STUDIES ON ESTRADIOL, GROWTH HORMONE AND SUPPRESSOR OF CYTOKINE SIGNALING-2 AND THE INFLUENCE IN LIVER METABOLISM

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Studies on Estradiol, Growth Hormone and Suppressor of Cytokine Signalling-2, and the influence in liver metabolism

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Thesis for Doctoral degree
(Ph.D.)

University of Las Palmas de Gran Canaria
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Hasta la más larga caminata empieza por un primer paso
(Confucio)

To my Family

ABSTRACT

Growth Hormone (GH) and 17β-Estradiol (E2) are critical regulators of body growth and composition, somatic development, metabolism and gender dimorphism in mammals. E2-GH interactions are physiologically and therapeutically relevant because they regulate endocrine, metabolic, and gender-differentiated functions in liver. A consensus exists that the response to sex-different GH patterns is the major cause of gender dimorphism in liver; however, it is likely that pituitary-independent mechanisms, such as the interaction with ERα, regulation of Suppressor of Cytokine Signaling (SOCS)-2 expression, and interaction with the GH-JAK2-STAT5 signaling pathway, may contribute to the pituitary-independent effects of estrogens.

In the general population, the endocrine and metabolic consequences of long-term exposition to estrogens or novel estrogen-related compounds and their influence on the GH axis are largely unknown. With the aim to explore the influence of E2 on GH-regulated endocrine, metabolic, and gender-differentiated functions in liver *in vivo*, we used two models with altered GH sensitivity. First, we carried out a comprehensive analysis on the molecular mechanisms of E2 effects, alone and in combination with GH, on liver functions in adult hypothyroid-orchidectomized rats. Transcriptomic and lipid analysis showed that E2 influenced genes involved in metabolism of lipids and endo-xenobiotics, and the GH-regulated endocrine, male-specific, metabolic, and immune responses. E2 induced a female-pattern of gene expression and inhibited GH-regulated STAT5b targeted genes. However, E2 did not prevent the inhibitory effects of GH on urea and amino acid metabolism-related genes. E2 decreased the hepatic content of saturated fatty acids and induced a transcriptional program that mimicked PPARα activation. In contrast, GH inhibited fatty acid oxidation. Second, we analysed the influence of SOCS2 on E2-regulated somatic growth and hepatic transcriptome in mice. In the absence of SOCS2 ($SOCS2^{-/-}$ mice), E2 did not prevent body weight gain and

longitudinal growth. The absence of SOCS2 influenced the effects of E2 on endocrine, metabolic, and immune systems responses in liver. GSEA showed that the gen sets regulated after 2 days of E2 treatment were similar in $SOCS2^{-/-}$ and wild-type littermates. However, some GO functions were significantly different. Particularly, E2 decreased hepatic immune response in the absence of SOCS2 whereas it was increased in wild-type mice. However, cell cycle and proliferation were stimulated by E2 in both genotypes. E2 increased expression of several GPCR that belong to the olfactory receptors family in $SOCS2^{-/-}$ mice. Notably, 625 and 678 genes were induced and repressed by E2, respectively, in $SOCS2^{-/-}$, but not in wild-type littermates.

In this Thesis, we also explored the influence of SOCS2 on metabolism by using two mice models with altered insulin sensitivity. First, we investigated the role of SOCS2 deletion in the development of high-fat-diet (HFD)-induced hepatic steatosis and insulin resistance. Our results showed that $SOCS2^{-/-}$ mice exhibited increased hepatic triacylglycerol secretion and were protected from HFD-induced steatosis compared to HFD-fed wild-type littermate. In contrast, HFD-triggered systemic insulin resistance and hepatic inflammatory response were more marked in $SOCS2^{-/-}$ mice. Immune and anti-inflammatory responses in $SOCS2^{-/-}$ mice were enhanced in muscle and adipose tissue. Macrophages analysis revealed increased phagocytic activity and cytokine production in the absence of SOCS2. Second, we investigated the impact of SOCS2 deletion on the development of type 1 Diabetes induced by multiple low dose streptozotocin (MLDSTZ), and the possible mechanisms involved. Untreated $SOCS2^{-/-}$ pancreas had higher β-cell mass than the control wild-type (WT) which explains the augmented serum insulin levels observed in $SOCS2^{-/-}$ mice. Pancreatic β-cells derived from $SOCS2^{-/-}$ mice showed increased GHR levels which suggest higher sensitivity to GHR-STAT5 signal in $SOCS2^{-/-}$ than in WT-derived β-cells. Upon MLDSTZ treatment, WT and $SOCS2^{-/-}$ mice developed diabetes. However, our results suggest that

SOCS2 ablation compensate β-cell destruction induced by MLDSTZ.

In summary, we provide *in vivo* evidence that 1) E2 exerts a significant impact on lipid content and transcriptome in liver and a marked influence on GH physiology; 2) SOCS2 is relevant to regulate E2 actions *in vivo*; 3) SOCS2, probably by controlling hepatic triglycerides secretion and inflammation, is an important regulator of hepatic homeostasis under conditions of HFD-induced stress; and 4) SOCS2 deletion, probably due to increased STAT5 activity, modulates susceptibility to MLDSTZ-induced diabetes in mice. Understanding E2, SOCS2 and GH interactions in physiological and pathological states could contribute to prevent health damage and improve clinical management of patients with growth, developmental and metabolic disorders.

RESUMEN

La hormona de crecimiento (GH) y el 17β-Estradiol (E2) son hormonas reguladoras del crecimiento corporal, el desarrollo, el metabolismo y el dimorfismo sexual en los mamíferos. Las interacciones que existen entre GH y E2 son fisiológica y terapéuticamente relevantes, debido a que regulan funciones endocrinas, metabólicas y de diferenciación sexual en el hígado. La respuesta a los patrones sexuales de liberación de la GH son la mayor causa de dimorfismo sexual en el hígado, si bien también existen diversos mecanismos independientes a la pituitaria como por ejemplo son la interacción con el receptor de estrógenos α (ERα), la regulación de la expresión del Supresor de la Señalización activada por Citoquinas 2 (SOCS2) y la interacción con la vía de señalización GH-JAK2-STAT5. Estos mecanismos contribuyen a los efectos independientes de la pituitaria que ejercen los estrógenos.

Actualmente, se desconocen las consecuencias de una exposición prolongada a estrógenos o compuestos derivados de estrógenos, así como su potencial influencia sobre la GH. Con el objetivo de explorar la influencia de E2 sobre las funciones reguladas por la GH en el hígado (endocrinas, metabólicas y diferenciación sexual) *in vivo,* en la presente Tesis Doctoral hemos usado dos modelos con sensibilidad alterada a la GH. Primeramente, llevamos a cabo un exhaustivo análisis de los mecanismos moleculares que producen los efectos de E2, solo y en combinación con la GH, en las funciones del hígado adulto de ratas hipotiroideas-orquidectomizadas. El análisis lipídico y transcriptomico realizado demostró que el E2 afecta a los genes involucrados en el metabolismo de lípidos y de endo-xenobioticos, y que la GH regula la respuesta inmune y metabólica. El E2 indujo un patrón de expresión genético femenino e inhibió los genes regulados por la GH a través de STAT5b. Sin embargo, el E2 no impidió los efectos inhibidores de la GH sobre los genes relacionados con el metabolismo de la urea y de los aminoácidos. El E2 disminuyó el contenido hepático de ácidos grasos

saturados e induce un programa transcripcional que imita la activación de PPAR. Por otro lado, la GH inhibe la oxidación de ácidos grasos. En segundo lugar, analizamos la influencia de SOCS2 sobre la regulación del crecimiento corporal y del transcriptoma hepático que produce el E2 en ratones. En ausencia de SOCS2 (ratones $SOCS2^{-/-}$), el E2 no afectó al peso corporal ni al crecimiento longitudinal. La ausencia de SOCS2 moduló los efectos del E2 *in vivo* sobre las funciones metabólicas y endocrinas en el hígado. El análisis GSEA mostro que los genes regulados tras dos días de tratamiento con E2 son similares en ambos genotipos. Sin embargo, algunas de las funciones GO son significativamente diferentes entre los genotipos. Particularmente, en ausencia de SOCS2, el E2 disminuye rápidamente la respuesta inmune hepática, la aumenta en los WT. Sin embargo, en ambos genotipos el E2 estimula el ciclo celular y la proliferación. En ratones $SOCS2^{-/-}$, el E2 aumenta la expresión de los GPCR pertenecientes a la familia de rectores olfatorios. De estos resultados cabe destacar la identificación de un elevado número de genes inducidos (625) o reprimidos (678) por E2, sólo en ausencia de SOCS2.

En esta Tesis Doctoral se exploró además la influencia de SOCS2 en el metabolismo del hígado *in vivo* usando dos modelos de ratones con la sensibilidad a la insulina alterada. Primeramente, investigamos el rol de SOCS2 en el desarrollo de la esteatosis hepática y la resistencia a la insulina inducidas por la administración de una dieta alta en grasa (HFD). Nuestros resultados muestran que los ratones $SOCS2^{-/-}$ presentaron un aumento en la secreción hepática de triacilglicerol y estaban más protegidos de la esteatosis hepática inducida por la HFD que los animales salvajes (WT) alimentados con la misma dieta. Por otro lado, la resistencia a la insulina sistémica y la respuesta hepática inflamatoria, desencadenadas por la HFD, fue más marcada en los ratones $SOCS2^{-/-}$. En el musculo y el tejido adiposo de los ratones $SOCS2^{-/-}$ la respuesta inmune y antiinflamatoria se vio aumentada. Un análisis de los macrófagos reveló la

existencia de un aumento de la actividad fagocitica y de la producción de citoquinas en ausencia de SOCS2. En segundo lugar, investigamos el impacto de la supresión de SOCS2 en el desarrollo de la Diabetes tipo I inducida por un tratamiento de múltiples exposiciones a bajas dosis Streptozotocin (MLDSTZ), así como el potencial mecanismo que se ve implicado. En ausencia de tratamiento, los ratones SOCS2$^{-/-}$ presentaban una mayor masa de células β que la cepa salvaje (WT) lo cual explica el nivel sérico de insulina aumentado en los ratones SOCS2$^{-/-}$ Las células β pancreáticas de los ratones SOCS2$^{-/-}$ mostraron un mayor nivel de los receptores de GH, lo cual sugiere una mayor sensibilidad a la señalización por GHR-STAT5 en las células β del páncreas en estos ratones en comparación con los WT. Tras el tratamiento con MLDSTZ, tanto los ratones SOCS2$^{-/-}$ como los WT desarrollaron diabetes. Sin embargo, nuestros resultados sugieren que la eliminación de SOCS2 podría compensar la destrucción de células β inducida por MLDSTZ.

En resumen, en la presente Tesis Doctoral proporcionamos evidencias *in vivo* que demuestran que 1) el E2 ejerce un impacto significante en el contenido lipídico y el transcriptoma del hígado, además de influenciar las acciones fisiológicas de la GH; 2) SOCS2 es un importante regulador de las acciones del E2 *in vivo*; 3) SOCS2, probablemente mediante el control de la secreción de triglicéridos hepáticos y la inflamación, es un importante regulador de la homeostasis hepática bajo condiciones de stress inducido por HFD; y 4) la supresión SOCS2 modula la susceptibilidad a la diabetes inducida por MLDSTZ en ratones, esto es probablemente debido a una mayor actividad de la vía GHR-STAT5 en las células β pancreáticas.

El comprender las interacciones de E2, SOCS2 y GH en los estados fisiológicos y patológicos podría contribuir a evitar daños de salud y mejorar el manejo clínico de los pacientes con trastornos del crecimiento, metabólicos y del desarrollo.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to by their Roman numbers in the text:

I. Fernández-Pérez L, Santana-Farré R, **Mirecki-Garrido M,** Garcia I, Guerra B, Mateos-Díaz C, Iglesias-Gato D, Díaz-Chico J.C, Flores-Morales A, Díaz M 2014. Lipidic and transcriptomic analysis reveals a functional interplay between estradiol and Growth Hormone in Liver. **PLOS ONE 9(5):e96305.**

II. **Mirecki-Garrido M**, Iglesias D, Borja Guerra, Mateos-Díaz C, Flores-Morales A, Fernández-Pérez L 2014. Then Suppressor of Cytokine Signaling-2 influences the hepatic effects of 17β-Estradiol on liver transcriptome (**manuscript in preparation**).

III. Zadjali F, Santana-Farré R, Vesterlund M, Carow B, **Mirecki-Garrido M**, Hernández-Hernández I, Flodström-Tullberg M, Parini P, Rottenberg M, Norstedt G, Fernández-Pérez L, Flores-Morales A 2012. SOCS2 inactivation protects against hepatic steatosis but worsens insulin resistance in high fat diet fed mice. **FASEB J 26 (8):3282- 3291.**

IV. **Mirecki-Garrido M***, Alkharusi A***,** Flores-Morales A, Fernández-Pérez L, Norstedt G 2014. Suppressor of Cytokine Signaling-2 gene deletion influences diabetes development induced by multiple low-dose streptozotocin (**manuscript in preparation**).

* M. Mirecki-Garrido and A. Alkharusi have contributed equally to this work

RELATED PUBLICATIONS

I. **Mirecki-Garrido M**, Guerra B, Mateos-Díaz C, Jiménez-Monzón R, Díaz-Chico N, Díaz-Chico JC, Fernández-Pérez L 2012. Influences of estrogens on biological and therapeutic actions of Growth Hormone in liver. **Pharmaceutical 5(7):758-778.**

II. Santana-Farré R*, **Mirecki-Garrido M***, Bocos C, Henríquez-Hernández LA, Kahlon N, Herrera E, Norstedt G, Parini P, Flores-Morales A, Fernández-Pérez L 2012. Influence of neonatal hypothyroidism on hepatic gene expression and lipid metabolism in adulthood. **PLoS ONE 7(5):7386.**

III. Zadjali F, Santana-Farre R, **Mirecki-Garrido M**, Ellis E, Norstedt G, Fernandez-Perez L**,** Flores-Morales A 2011. Liver X receptor agonist downregulates Growth Hormone signaling in the liver. **Horm Mol Biol Clin Investig 8:471-478**.

* M. Mirecki-Garrido and R Santana-Farré have contributed equally to this work

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LIST OF ABREVIATIONS

ALT	Alanine aminotransferase
AR	Androgen Receptor
CD	Control Diet
CHO	Cholesterol
CIS	Cytokine-inducible SH2 protein
Cyp	Cytochrome gene
DM	Diabetes Mellitus
E2	17β-estradiol
ER	Estrogen Receptor
FFA	Free Fatty Acids
GGT	Gamma glutamil transpeptidasa
GH	Growth Hormone
GHR	Growth Hormone Receptor
GR	Glucocorticoid Receptor
GHRH	GH releasing hormone.
HFD	High-Fat Diet
HOMA	Homeostatic model assessment
IGF-I	Insulin-like Growth Factor I
IGFBP	Insulin Growth Factor Binding Protein
ip	intraperitoneally
JAK	Janus kinase
KIR	Kinase Inhibitory Region
IL	Interleukin
LFD	Low-Fat Diet
LPS	Lipopolysaccharide
MLDSTZ	Multiple Low Dose of Streptozotocin
NAFLD	Non-Alcoholic Fatty Liver Disease
OX	Orchidectomy
PRL	Prolactin
sb	subcutaneously
SH2	Src homology 2 domain
SHP	SH2-Containing Protein Tyrosine Phosphatase
SOCS	Suppressor of Cytokine Signaling
$SOCS2^{-/-}$	SOCS2 knockout mouse
SS	Somatostatin.
SSI	STAT-Induced STAT Inhibitor
STAT	Signal Transducers and Activator of Transcription
TG	Triacylglycerols
TNF- α	Tumor Necrosis Factor α
WT	Wild type mice

1. INTRODUCTION

1.1. Growth Hormone

Growth Hormone (GH) is the main regulator of somatic growth, metabolism, and gender dimorphism in liver [1,2,3,4]. GH is predominantly linked to linear growth during childhood, but continues to have important metabolic actions throughout life. Its primary somatic effect is the promotion of longitudinal growth [5,6] but also induce diverse effects on cell growth, differentiation and metabolism. GH promotes the proportional growth of several organs in the body including liver, muscle and bone and exerts effects on cellular differentiation, metabolism and nutrient uptake [7,8,9,10,11]. GH is also involved in the regulation of immune cells and hematopoiesis [12,13] and can act on the brain to influence emotion, behaviour and other cognitive responses [14,15]. GH actions are pleiotropic and influenced by factors such as age, gender (sex hormones) and pituitary secretory pattern [16,17,18].

1.1.1. Growth Hormone Secretion. The somatotropic cells of the anterior pituitary gland are the primary producers of GH in the body. The regulation of pituitary GH secretion involves a complex neuroendocrine control system that includes the participation of several neurotransmitters and the feedback of hormonal and peripheral (metabolic) factors [19,20] (Fig.1). GH secretion from pituitary gland is regulated by two major hypothalamic peptides: the stimulatory GH releasing hormone (GHRH) and the inhibitory hormone somatostatin (SS). The balance of these stimulating and inhibiting peptides is in turn, indirectly, affected by many physiological stimulators (i.e, sex hormones, nutrients, sleep, and exercise,) and inhibitors (i.e., insulin growth factor I (IGF-I), and GH). In addition to hypothalamic (GHRS, SS) and endocrine (IGF-I, GH) factors, other peripheral (metabolic) factors influence pituitary GH release: free fatty acids (FFA), insulin, glucose, amino acids, leptin, neuropeptide Y, and ghrelin. These

factors are primarily related to or derived from the metabolic status of the organism, which is consistent with the role of GH in regulating substrate metabolism, adiposity, and growth, and appear to coordinate the metabolic status of the organism with GH secretion [21,22,23,24]. One GH is released into the bloodstream it travels to target organs where it binds to the Growth Hormone Receptor (GHR). GHR is ubiquitously present with the highest levels found in the cells of the liver. GHR signaling triggers the expression and release of hepatic IGF-I, which is a major mediator of somatic GH action [25,26]. In addition to the pituitary, GH is produced in extra-pituitary tissues, which indicates that GH has local paracrine-autocrine effects, distinct from its classic endocrine somatotropic effects [27].

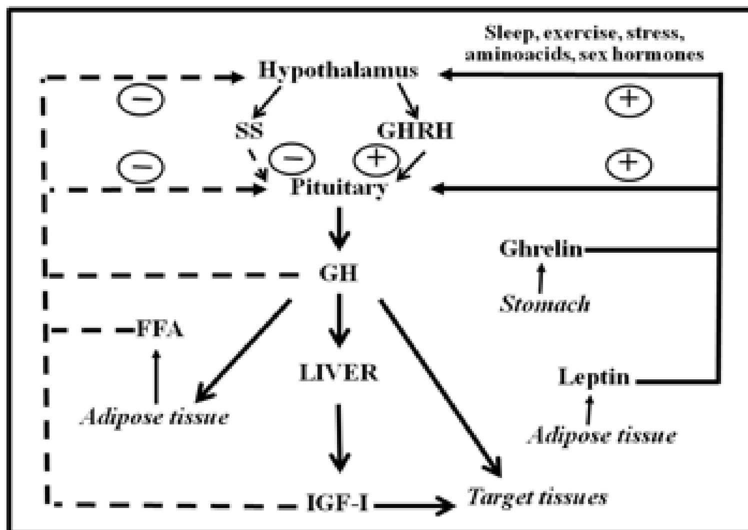

Figure 1. Regulation of pituitary GH secretion (Based on [28]).

- The Sexually Dimorphism of Pituitary Growth Hormone Secretion. The secretion of GH is controlled by neuroendocrine factors, which regulate pituitary GH secretion in a sexually dimorphic manner in many species, including rats, mice and humans [29,30,31,32]. Gender dimorphism in GH secretory patterns can, at

least partly, explain differences in growth and liver physiology between males and females. The sexually dimorphic pattern of GH secretion is also seen in humans, but not as marked as in the rat.

Sex steroids are physiological regulators of pituitary GH secretion and, indirectly, regulate sex-specific liver physiology [33]. From neonatal period of life, gonadal steroids play a critical role to maintain liver response to GH in adulthood. Sexual dimorphism in rodents seems to be regulated by estrogen secretion in adult females and by androgen secretion, neonatally and during adulthood, in males. Neonatal exposure to testosterone imprints the male program of neuroendocrine control of the pulsatile pituitary GH secretion that is first seen at puberty, when the adult pattern of GH secretion becomes evident, and continues throughout adulthood. The male characteristic metabolism in liver in adulthood is dependent on continuous androgen exposure. If such an androgen re-programming does not occur, the secretion pattern will remain as the feminine pattern (continuous GH secretion) [33]. In female rats, gonadectomy has little impact on hepatic steroid metabolism. However, estrogen treatment feminizes hepatic metabolism in male rats [34] (Fig.2).

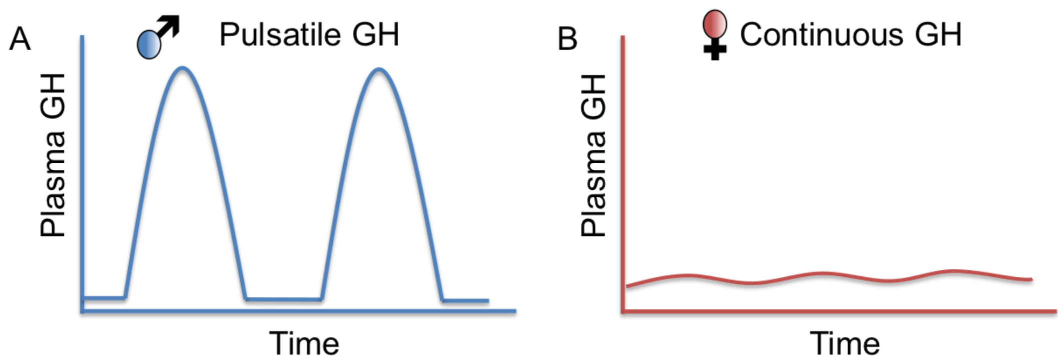

Figure 2. Plasma GH profile in adult male (A) and female (B) rats (Based on [35,36,37,38])

Figure 2A shows that the pulsatile plasma GH profile in adult male rats is characterized by peaks of plasma GH every 3-4 hours followed by interpulse interval where plasma GH levels are undetectable [33]. Consequently, intracellular activation of signal transducer and activator of transcription (STAT)-5b, main executor of GH signaling in liver, is also episodic and periods with low GH circulating levels are required to achieve maximal activation of STAT5. Adult female rats are characterized by more frequent pituitary GH release and a near continuous presence of GH in plasma (Fig. 2B). Female rats show reduced STAT5b activation compared with males [33]. These differences in STAT5b activation are responsible for several of the gender differences in hepatic gene expression. For example, the GH-free interval observed in the males (rats and mice) is required for the expression of male-specific liver genes, such as CYP2C11 [39], and most likely reflects the need to reset a GH-activated STAT5b signaling pathway. Genome-wide screens of gene expression have shown that GH- and sex-dependent regulation of hepatic gene expression is not confined to steroid or drug metabolism. Moreover, a number of other hepatic genes have been found to be up- and/or down-regulated by the different patterns of GH or sex-steroid exposure. GH- and sex-dependent hepatic transcripts encoding plasma proteins, enzymes, transcription factors and receptors involved in the metabolism of proteins, carbohydrates, lipids, or signaling regulation have been identified [40,41,42]. A consensus exists that the response to sex-different GH patterns is the major cause of gender dimorphism in liver; however, it is likely that factors other than the sexually dimorphic pattern of GH secretion are behind some sex differences in rat liver. Potential mechanisms that could contribute to “liver sexuality” are the pituitary-independent effects of estrogens through interaction with ERα or GH-JAK2-STAT5 signaling pathway in liver.

1.1.2. Growth Hormone Signaling. GH mediates its intracellular effects via the GHR which is ubiquitously expressed, especially in liver, fat, and muscle. GHR belongs to type I cytokine receptor, a family of receptors without intrinsic kinase activity [43]. GHR has an extracellular domain that is connected to a cytoplasmic domain via a flexible linker. The tyrosine kinase JAK2 is constitutively associated with a Box 1 region in the cytoplasmic domain of the GHR. In the inactive state, the JAK2 catalytic domain is masked by its pseudokinase domain. The GH molecule interacts with preformed dimmers of identical GHR pairs, which results in a conformational change in the receptors and associated JAK2 molecules [44]. This event unmasks the catalytic domain of JAK2 and results in activation of adjacent JAK2 molecules by transphosphorylation. Activated JAK2 phosphorylates the GHR cytoplasmic domain on tyrosine residues and subsequent JAK2-dependent and –independent intracellular signal transduction pathways evoke pleiotropic cell responses including changes in gene transcription, cell proliferation, glucose and lipid metabolism, or in cytoskeletal re-organization [8,40,45,46]. The main event in the GH signaling pathway is the recruitment of members of the STAT family of transcription factors to phosphorylated tyrosine residues in the GHR intracellular domain. Of the various STAT proteins (STAT 1 to 6), STAT5b has been widely associated with GH biological actions in liver [1,47]; although STAT1, 3, and 5a have also been shown to be recruited by the GHR. STAT5 phosphorylation by JAK2 results in their dissociation from the GHR, dimerization, and translocation to the nucleus where they modulate the transcription of target genes (e.g., IGF-I, ALS, SOCS2, SOCS3, CIS) [48,49]. The STATs represent one of the best-known pathways in GH-induced signaling; others include the MAPK and PI3K pathways (Fig. 3).

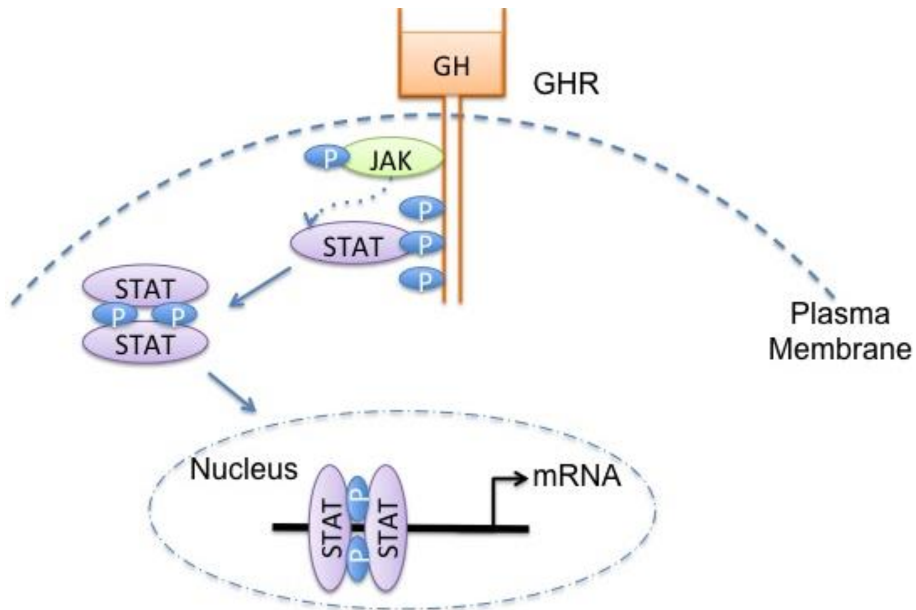

Figure 3. Schematic representation of GHR-JAK-STAT signaling activation (Based on [50,51]).

STAT5b is a key GH signaling intermediate for the regulation of target genes associated with several liver physiological processes, including modulation of body growth, cell cycle, and metabolism of lipids, bile acid, steroids, and drugs [1]. The phenotype of STAT5b$^{-/-}$ mice highlight its importance for GH signaling; postnatal growth is reduced in the males and the expression of female predominant genes in the liver is upregulated [52]. Disruption of STAT5b gene feminizes the male mice since it abolishes their ability to convert the male specific pulsatile GH secretion into pulsatile GHR signaling. Moreover, the signaling pattern became more continuous and similar to the secretion pattern seen in female [53]. In humans, inactivating mutation in the STAT5b gene cause a syndrome similar to Laron type dwarfism couple with autoimmunity likely due to low levels of T regulatory cells [54].

- Inactivation (cell desensitization) of Growth Hormone signaling. The conserved control of GHR-JAK2 activation kinetic in multiple cell models emphasizes the importance of mechanisms for desensitization of GH-dependent signaling pathway in GH physiology [55]. This is clearly illustrated in the case of hepatic GH actions where signal duration regulates gender differences in liver gene expression [47]. Studies on primary hepatocytes and several cell lines have shown that GH-induced JAK2-STAT5b activation is transient, with maximal activation achieved within the first 30 min of stimulation, followed by a period of inactivation. This period is characterized by an inability to achieve maximal JAK2-STAT5 activation by GH in the following 3-4 h, unless GH is withdrawn from the media [56]. Cell surface levels of GHR are the primary determinant of GH responsiveness. Transcriptional, translational and posttranslational level factors can influence GHR synthesis and, thereby, regulate cell sensitivity to GH actions. These factors include nutritional status, endocrine context, developmental stage, and estrogens [28]. GHR cell-surface translocation is also directly inhibited by IGF-I, likely contributing to a local feedback loop to hamper GH sensitivity [57]. Removal of cell surface GHRs by endocytosis is an early step in the termination of GH-dependent signaling. GHR ubiquitination is a key control mechanism in the down-regulation of GH signaling, modulating both GHR internalization and proteasomal degradation. In addition to GHR down-regulation, other mechanisms are needed to complete inactivation of GH signaling. Since activation of GH-dependent signaling pathways is critically based on protein phosphorylation on tyrosine, serine or threonine residues, the obvious mechanism for deactivation of this process is the action of protein phosphatases. First, several studies have resulted in the identification of phosphatases which are involved in the specific inactivation of GHR signaling. Second, Signal Regulatory Protein (SIRP)-α, which belongs to a family of ubiquitously expressed transmembrane glycoproteins, negatively regulates GH-activated signaling by inhibition of the phosphorylation of JAK2, STAT5b, STAT3, and ERK1-2 but the physiological

relevance of this mechanism is uncertain [58]. The ubiqutin ligase SOCS2 has been shown to be a key component of negative regulators of GHR-JAK2-STAT5 signaling pathway [59,60]. SOCS proteins have been shown to modify cytokine actions through a classic negative feedback loop. In general, SOCS protein levels are constitutively low, but their expression is rapidly induced by stimulation with different cytokines including GH. Evidence also indicates that growth factors (e.g., insulin), xenobiotics (e.g., dioxin, statins), and estrogens, can induce SOCS2 expression [59,61]. Consequently, regulation of SOCS2 protein expression provides a mechanism for cross-talk where multiple factors, including estrogens can regulate the activity of GH [62]. SOCS2 binds the GHR complex and promote its ubiquitination and subsequent proteasomal degradation [60].

1.1.3. Growth Hormone regulates Growth, Metabolism, Immunity and gender dimorphism of hepatic gene transcription

- Somatic growth. The important role of GH on longitudinal growth is evident from the phenotypes in animal models, $GHR^{-/-}$ mice are smaller than the wild type (wt) mice, with a 50% decrease in body weight [63]. The first hypothesis was that GH does not exert its growth effects directly on target tissues, but it works through an intermediate signaling substance [25] named as IGF-I [64]. Circulating IGF-I is mostly produced by the liver in response to GH. The important of IGF-I for longitudinal growth is evident from $IGF\text{-}I^{-/-}$ mice which have a 60% reduction in growth compared with their wt littermates [65]. However this hypothesis has been revised nowadays and it has became evident that not all the growth effects of GH are mediated by hepatic IGF-I. GH exert direct effects on muscle, bone and adipose tissue [20]. GH/IGF-I pathway is defective in patients suffering from Laron Syndrome, which is most commonly associated with mutations that inactivate or otherwise impair GHR function and block production of IGF-I, leading to profound growth retardation [66]. Laron patients have high levels of

circulating GH and low IGF-I levels, these patients are the equivalent to $GHR^{-/-}$, and these Laron mice lead a reduction in pro-aging signaling and protect against diabetes and cancer [67]. An IGF-I replacement therapy is not effective, the reason is believed to be because there is a reduced hepatic production of IGFBP which leads to a faster clearance of IGF-I. GH hyposecretion or deficiency at an early age results in dwarfism. GH hypersecretion occurring before or after puberty results in gigantism or acromegaly, respectively. GH has been approved for several conditions including: GH deficiency, Turner syndrome, chronic renal insufficiency, small for gestational age or intrauterine growth retardation, Prader Willi syndrome, and continued height deficit at puberty [68]. In contrast, transphenoidal pituitary resection surgery and administration of somatostatin analogous or GH receptor antagonists to patients with acromegaly lowers endogenous GH and IGF-1 levels [69,70].

- Metabolism. The effects of GH on metabolism include promoting lipolysis in adipose tissue, protein synthesis, and insulin resistance [71,72,73]. The key physiological function of GH is the promotion of protein synthesis and inhibition of protein degradation in muscle, bone and other large organs, inhibiting the catabolism of glucose and aminoacids by promoting the utilization of lipids as energy source. These systemic effects of GH are achieved through inhibition of insulin actions and the promotion of fatty acid (FFA) mobilization from adipose tissue and liver [4,8]. The mechanisms of GH actions on lipid metabolism are complex and involve both transcriptional and acute changes in catalytic enzyme activities [4]. It is well established that human GH is a lipolytic hormone in adipose tissue. Long-term administration of GH includes a decrease in fat deposition and an increase in fat mobilization, increasing circulating FFA and glycerol levels. GH reduces fat mass, particularly in individuals who have accumulated excess fat during periods of GH deficiency. Obesity is clinically evident in GH deficient (GHD) patients, a decline in GH levels correlates with

age-related adiposity [74] and lack of GH or GH signaling induces obesity earlier in mice [4]. GHD in adulthood causes a syndrome characterized by increased visceral adiposity, decreased muscle mass, metabolic disturbances, and increase mortality associated with cancer or vascular complications.

GH is considered to have diabetogenic properties [20,75]. GH anabolic actions in muscle and bone involve the stimulation of protein synthesis and promoting catabolism of fatty acids instead of glucose, that is why GH actions reach to hyperglycemia and decreased insulin sensitivity. In the adipose tissue GH promotes lipolysis and blocks the uptake of fatty acids through inhibition of lipoprotein lipase which leads to increased circulating level of FFA and glycerol [18,75,76]. This is very remarkable in the GH transgenic mice which develop insulin resistance but have a decreased body fat [77]. In liver, GH has a stimulatory effect on glucose production which may be a result of its antagonism of insulin action leading to hepatic/systemic insulin resistance [8]. GH increases glucose production by increasing glycogenolysis; however, it has either a stimulatory or no effect on gluconeogenesis. On the other hand, over-expressing the human GH gene in rat increases basal hepatic glucose uptake and glycogen content [78]. In contrast, GHD mice (Ames) and the $GHR^{-/-}$ mice have improved insulin sensitivity and up-regulated hepatic insulin signaling, suggesting that GH antagonizes insulin signaling locally in the liver [79]. GH-induced insulin resistance may be developed by the increased FFA mobilization from adipose tissue which can then affects liver insulin sensitivity, and lead to insulin resistance.

In the liver, GH promotes lipogenesis, inhibits lipolysis and increases lipid output by the liver [3]. Mice with liver deletion of GHR have decreased hepatic trygliceride secretion, insulin resistance and spontaneous hepatic steatosis [80]. In the case of glucose, GH in the liver promotes gluconeogenesis and glycogenolysis and reduces the uptake of glucose. In muscle, GH promotes amino acid uptake and

protein synthesis without increasing proteolysis [81]. Muscle specific deletion of GHR mice are protect from High-Fat Diet (HFD) induced insulin resistance, this could be attributable to the decreased diabetogenic GH action on the muscle [82]. In adipose tissue GH promotes lipolysis, targeted deletion of GHR in fat resulted in a doubling of the fat mass, but has no effect on glucose homeostasis [83]. GH also seems to play a role in insulin secretion from the β cells in the pancreas. Mice with GHR deletion in the β cells exhibit decreased glucose stimulated insulin release and decreased β cell hyperplasia in response to HFD [84].

- Immunity. Besides its actions on postnatal growth and metabolism GH also plays a role in the regulation of immune system. Several different immune cells have been shown to express both GHR, which suggest that GH signalling directly affects immune function, and GH, which implies that paracrine and autocrine hormone secretion might play role in immunity [85]. GH is able to directly affect cytokine production by immune cells, but the mechanism and effects are unclear. GH have been shown to promote the production of inflammatory cytokines such as Interleukin (IL) -1α, IL-6 and Tumor Necrosis Factor α (TNF- α) in immune cells both *in vitro* [86] and *in vivo* [87]. In a Finnish study, GH treatment of critically ill patients was found to increase both morbidity and mortality, likely through modulation of immune function [88]. While it is clear that GH modulates immune functions, the distinct effects and underlying mechanisms remain to be elucidated.

- Gender Dimorphism of Hepatic Gene Transcription. Sex hormones imprint a sex-dependent pattern of pituitary GH hormone secretion which is a major player in establishing and maintaining the sexual dimorphism of hepatic gene transcription that emerges in rodents at puberty [33]. Genomic and bioinformatics analysis have contributed to solve molecular mechanisms involved in GH-regulated hepatic gene transcription [40,41,46,47,89]. Sex-dependent expression and GH regulation characterizes several families of hepatic genes involved in

endo- and xenobiotic metabolism as well as relevant metabolic functions (e.g., lipid metabolism); 20-30% of all hepatic genes have a sex-specific expression pattern in rodents. Most of these hepatic sex differences are explained by the female-specific secretion pattern of GH, through the induction of female-predominant transcripts and suppression of male-predominant. Estrogens cause opposite effects on lipid and glucose metabolism which represents a relevant point of regulatory interactions between estrogens and GH.

1.2. The Suppressors of Cytokine Signaling

SOCS protein family was identified in 1997 by three different groups [90,91,92]. At present, the SOCS family represents eight different members: cytokine-inducible SH2 protein (CIS) and SOCS-1 to SOCS-7. The SOCS proteins have been shown to modify cytokine actions through a classic negative feedback loop. The SOCS family utilizes a feedback loop to inhibit cytokine responses and block the activation of the JAK/STAT signaling pathway [93]. Although today their greatest importance is held to be in the regulation of the cytokine-induced JAK/STAT pathway, these proteins are both induced by and are negative regulators of more than just cytokines, suggesting a role as signal modulators much wider than implied by their names, evidence indicates that cytokines, growth factors and steroid hormones, and several xenobiotics can regulate SOCS expression [94]. As mentioned above, intracellular GH signaling is regulated by SOCS proteins that influence the action GH elicits.

1.2.1. SOCS Proteins Structure. All SOCS proteins display a three-part architecture [93]. A central Src homology 2 (SH2) domain is involved in substrate binding through recognition of cognate phosphotyrosine motifs. It is flanked by a variable N-terminal region and a conserved C-terminal domain known as the SOCS box. The N-terminal domain contains an extended SH2 subdomain (ESS)

that contributes to substrate interaction [95,96]. Additionally, SOCS1 and SOCS3 present an extra domain, which is named (KIR) and is located at the N-terminal region near the SH2 domain. Members of the SOCS family contain N-terminal regions of variable length. For example, CIS, SOCS1, SOCS2, and SOCS3 have relatively short (50-80 residue) N-terminal regions, whereas SOCS4, SOCS5, SOCS6, and SOCS7 have longer N-terminal regions of up to 2700 residues [97], suggesting these four proteins form a sub-group within the SOCS family. SOCS4 and SOCS5 share greater sequence homology with each other than with other members of the SOCS family [97], with conservation largely restricted to the SH2 domain (92% amino acid identity) and suggests that while the SH2 domains may have an overlapping binding specificity [97], the N-terminal regions will have unique protein targets (Fig.4). The SOCS box is a structural domain found at the C-terminus it is usually coupled to a protein interaction module such as an SH2 domain in case of SOCS proteins. The SOCS box participates in the formation of E3 ligase complexes, marking activated cytokine receptor complexes for proteasomal degradation. A similar mechanism was recently uncovered for controlling SOCS activity itself. The SOCS box can also add unique features to individual SOCS proteins [97,98].

Figure 4. Schematic representation of SOCS protein structure domains (Based on [93]).

1.2.2. Mechanism of action of SOCS proteins. Since the discovery of the first member of the SOCS family, significant progress has been made in understanding the function and importance of these proteins. All SOCS proteins make use of their SH2 domain to bind to phosphorylated tyrosine residues. There are four major ways by which SOCS inhibits cytokine signaling: (1) blocking STAT recruitment to the cytokine receptor; (2) targeting the receptor for degradation by the proteasome; (3) binding to JAKs and directly inhibiting their kinase activity; (4) targeting JAKs for degradation by the proteasome (Fig. 5). First, SOCS proteins can suppress signaling by competing with downstream signal transducers for binding to share phosphorylated motifs of the activated receptor [99]. Therefore, the SOCS box can add unique features to individual SOCS proteins: it can function as an adaptor domain as was demonstrated for SOCS3 or as a modulator of substrate binding in case of CIS [100]. Second, SOCS proteins can regulate signal transduction by linking their substrates to the ubiquitination machinery via the SOCS box. Ubiquitination of a substrate via SOCS proteins can lead to its proteasomal degradation. In line with this, signal transduction of several cytokines is prolonged in the presence of proteasome inhibitors [101,102]. The SOCS box participates in the formation of E3 ligase complexes, marking activated cytokine receptor complexes for proteasomal degradation. A similar mechanism was recently uncovered for controlling SOCS activity itself, since SOCS2 was found to enhance the turnover of other SOCS proteins. Third, a small kinase inhibitory region (KIR) found in the N-terminal domain of SOCS1 and SOCS3 inhibits the activity of JAKs by acting as a pseudo-substrate [95]. SOCS1 can directly bind to the phosphorylated activation loop of JAK2, whereas SOCS3 shows only weak affinity for JAK2 and is thought to bind to the receptor in close proximity of the kinase [103,104].

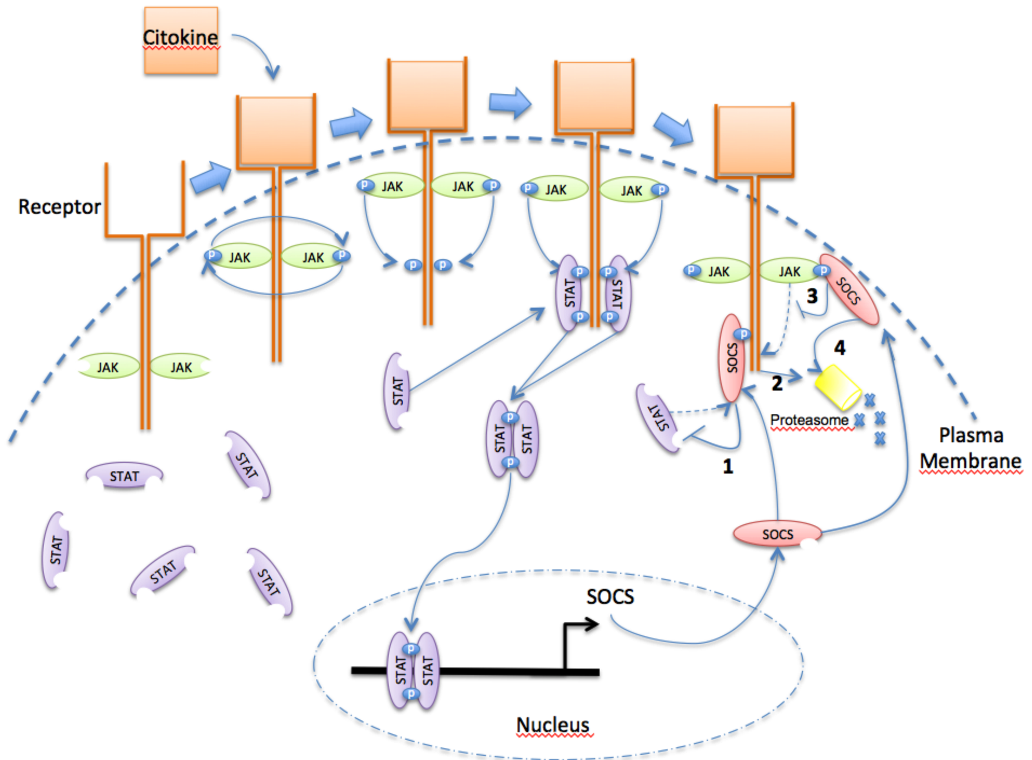

Figure 5. Schematic representation of SOCS-dependent negative regulation of GHR-JAK-STAT signaling pathway (Based on [93]).

Promiscuity and redundancy are notable features of the SOCS proteins system when tested *in vitro*. Any single SOCS can be induced by many cytokines *in vitro* and in turn act on several cytokine receptors, not necessarily the ones inducing their expression. GH induces the expression of CIS, SOCS1, -2, and -3; all of which have shown negative actions on the GHR when forcibly overexpressed in cell lines. Evidence also indicates that growth factors that do not belong to the 4-helical bundle cytokine family (*e.g.* insulin, chemokines), and even steroid hormones, can induce SOCS expression. Consequently, regulation of SOCS protein expression provides the mean for cross-talk where multiple factors can regulate the activity of specific cytokines [55] being regulated the SOCS family at the transcriptional, translational and post-translational levels.

Some emerging evidences suggest that SOCS molecules might also be degraded by others SOCS family members. SOCS2 appears to enhance the degradation of SOCS1 and SOCS3, and possibly CIS. SOCS6 and SOCS7 might also play a role in cross-modulation of other SOCS proteins. The SOCS box of SOCS2 appears to facilitate the targeting of others SOCS molecules for proteasomal degradation. [105].

1.2.3. Physiological actions of SOCS proteins. During the last few years considerable progress has been achieved regarding the role of specific SOCS proteins by the generation of knockout or transgenic mice [106]. *In vitro* various SOCS proteins can inhibit different cytokine receptors and in turn individual SOCS proteins can be induced by a bewildering variety of different stimuli. Thus, *in vitro* redundancy and pleiotropy characterize the SOCS proteins. However, *in vivo* data have shown that individual SOCS proteins show at least some degree of specificity for certain cytokines [107].

- Cytokine Inducible SH2-containing protein (CIS) was the first SOCS family member to be described and it is the most closely related to SOCS2 [100]. $CIS^{-/-}$ mice did not present a severe phenotype; however, it was recently suggested that hematopoietic growth factors (e.g. IL-3, erythropoietin) might be affected [108]. CIS also plays a role in limiting GM-CSF, IL-2, prolactin and growth hormone signaling by inhibition of STAT5 activation. CIS-transgenic mice resemble $Stat5b^{-/-}$ mice, with defects in growth and lactation due to reduced growth hormone and prolactin signaling. CIS-transgenic mice additionally display enhanced TCR signaling and impaired responses to IL-2 [109].

- Suppressor of Cytokine Signaling (SOCS)-1, -3, are the most investigated members of the SOCS family. They have been implicated in the regulation of cytokine signaling related to immunological function but also affects hormone

signaling and metabolism. SOCS1$^{-/-}$ mice are normal at birth, they exhibit stunted growth and die within 3 weeks of birth, with a syndrome characterized by severe lymphopenia, activation of peripheral T cells, fatty degeneration and necrosis of the liver, and macrophage infiltration of major organs [110]. SOCS3$^{-/-}$ mice die during the embryonic stage of development due to placental function defects. In other words, deletion of SOCS3 causes embryonic lethality; these embryos can be saved, however, by a tetraploid rescue approach. These observations demonstrate SOCS3's essential role in placental development and non-essential role in embryo development. Rescued *SOCS3*$^{-/-}$ mouse embryos exhibit prenatal lethality with cardiac hypertrophy, which suggests that SOCS3 is essential for regulating either LIF receptors or gp130 signaling [110]. SOCS3-deficient mice clearly show that although SOCS3 is essential for G-CSF, IL-6, LIF and leptin signaling, it is in fact, dispensable for regulation of IFNγ signaling [109].

- Suppressor of Cytokine Signaling (SOCS) -4, -5, -6 and -7. The latest additions to the SOCS family are also its largest member [97]. SOCS4$^{-/-}$ mice have not been reported and SOCS5 and SOCS6-deficient mice do not display an overt phenotype. Deletion of the SOCS7 gene highlights a critical role in regulation of insulin signaling [111,112,113]. SOCS7$^{-/-}$ mice are slightly smaller than the wild-tyoe littermates and they suffer from hydrocephalus and have enhanced insulin signaling, likely due to an increase in β-cell mass [111,114].

- Suppressor of Cytokine Signaling (SOCS)-2, The phenotype of SOCS2 null mice identifies SOCS2 as the key physiological player in the negative regulation of GH-dependent somatic growth [60,115,116]. As mentioned above, SOCS2 acts as an ubiquitin-ligase for the GHR, inhibiting GH signaling [60]. Consequently, SOCS2$^{-/-}$ mice are characterized by marked gigantism with increased bone length and proportional enlargement in skeletal muscles due to increased GH sensitivity [117,118]. SOCS2$^{-/-}$ male mice are characterized by a 40% increase in body

weight, SOCS2$^{-/-}$ mice are giants but not obese [117]. SOCS2$^{-/-}$ female mice are a 20% heavier than wt littermates. A similar phenotype is also observed in the *high-growth* (hg) mutation, which is caused by a deletion of the socs2 locus [115]. Dual-Knockout of SOCS2 and STAT5b and crossing of SOCS2$^{-/-}$ mice with GHRH signaling deficient mice abolished the original phenotype and confirmed that the increased growth observed in SOCS2$^{-/-}$ mice is due to increased sensitivity to GH but with normal systemic insulin-like growth factor-1 (IGF-I) levels [116,119]. Because SOCS2$^{-/-}$ mice do not have increased circulating IGF-1 levels, it is likely that the increased bone growth and observed structural differences within SOCS2$^{-/-}$ growth plates are a direct consequence of altered SOCS2-mediated GH/IGF-1 signaling local to the growth plate [120,121,122,123]. Our own studies revealed that SOCS2$^{-/-}$ mice are protected from high-fat-diet (HFD)-induced steatosis. Paradoxically, the HFD-fed SOCS2$^{-/-}$ mice showed worsening of glucose tolerance and exacerbated inflammatory response that was manifested by enhanced production of inflammatory cytokines in liver and fat tissues [62]. Recently, we have identified SOCS2 as an important regulator of hepatic homeostasis (lipid and glucose metabolism and inflammation) under conditions of high-fat dietary stress [124]. In addition previous work has shown that SOCS2 deficiency induces changes in hepatic gene expression that only partially overlap with known GH induced effects, suggesting that not all the effects attributed to SOCS2 are GH dependent [118]. We have also identified SOCS2 as an important regulator of pancreas physiology (manuscript in preparation). Other studies have demonstrated that SOCS2 is essential for the regulation of GH actions not directly related to somatic growth. For example, SOCS2 can block GH-dependent inhibition of neural stem cell differentiation. Consequently SOCS2$^{-/-}$ mice have fewer neurons in the developing cortex, whereas SOCS2 over-expression results in increased neural differentiation [109,122]. It has also been demonstrated that SOCS2 inhibits intestinal epithelial proliferation [125]. A role of SOCS-2 in the differentiation process of mesenchymal precursors has also been shown in mice

myoblasts [126]. Stable transfection of SOCS2 into C2C12 cells resulted in the acceleration of proliferation and survival and inhibition of spontaneous myotube formation. In addition, SOCS2 potentiated bone morphogenic protein (BMP)-induced trans-differentiation of C2C12 cells into osteoblast phenotypes [126]. Further process affected by SOCS2 include mammary gland development [127], bone mineral density [128], and allergic response [129]. Besides its actions on GH signaling SOCS2 has been implemented in the regulation of other cytokines and immune cell regulation [94,130,131,132]. SOCS2$^{-/-}$ mice are more susceptible to infection and exhibit increased levels of pro-inflammatory cytokines in response to certain pathogens. Also, expression of SOCS2 is induced by lipoxins and has been demonstrated to mediate some of their anti-inflammatory actions [133].

1.3. Estrogens

Estrogens are a group of steroid hormones produced by enzymatic modification of cholesterol. Estardiol (E2) is the major natural estrogen in mammals and has physiological actions not only limited to reproductive organs both in females and males [134]. The role of E2 in the development and regulation of female reproduction is well established. However, recent studies demonstrate that E2 has a multiple effects on metabolism (e.g., anti-diabetic effects) [135]. Relevant to this Thesis, the liver is a direct target of estrogens because it expresses Estrogen Receptor alpha (ERα) which is connected, among others, with lipid and glucose homeostasis [136,137,138] and somatic growth [139].

1.3.1. Estrogen signaling. Estrogen signaling can be mediated by multiple receptors [140]. Most of the known estrogenic effects are mediated via direct interaction of estrogen with the DNA-binding transcription factors, ERα and ERβ. Classical estrogen signaling occurs through a direct binding of ER dimers to estrogen responsive elements in the regulatory regions of estrogen target genes

followed by activation of the transcriptional machinery at the transcription start site. In addition, estrogen can modulate gene expression by a second mechanism in which ERs interact with other transcription factors, like STAT5, through a process referred to as transcription factor cross-talk. Estrogen may also elicit effects through non-genomic mechanisms, which involve the activation of downstream kinase pathways like PKA, PKC, and MAPK via membrane-localized ERs. An orphan G protein-coupled receptor (GPR)-30 in the cell membrane mediates non-genomic and rapid estrogen signaling. Finally, E2 has a similar affinity for ERα and ERβ, these receptors are activated by a wide range of ligands including Selective Estrogen Receptor Modulators (e.g., raloxifen) as well as many other compounds. ERβ is expressed in the ovary, prostate, lung, gastrointestinal tract, bladder, and hematopoietic and the central nervous systems, while ERα is mainly expressed in reproductive tissues, kidney, bone, white adipose tissue, and liver. The liver expresses ERα but almost undetectable levels of ERβ which indicates that specific actions of estrogens in liver can be mimicked by using selective ERα agonists such as propyl-pyrazole-triol [141]. Collectively, the above mentioned data indicate that the mechanisms involved in ER signaling are influenced by cell phenotype, the target gene, and the activity or crosstalk with other signaling networks. The liver represents a site where physiologically and therapeutically relevant interactions between estrogens and GH can be developed. Particularly relevant is the interaction of estrogens with GHR-JAK2-STAT5 signaling pathway in the regulation of somatic growth, lipid and glucose metabolism, and “liver sexuality”.

1.3.2. Estrogens regulate somatic growth, metabolism and gender dimorphism of hepatic gene transcription.

- Somatic growth. It is well known that sex steroids and GH interact closely to regulate pubertal growth [34]. Interestingly, loss of ERα (ERKO), but not ERβ, mediates important effects of estrogen in the skeleton of male mice during growth

and maturation [139]. A phenotype like to ERαKO mice can be found for aromatase-deficiency in mice or human, which are deficient in estrogens [142]. In addition, gender-related differences in body composition are in part mediated by sex steroids modulating the GH-IGF-I axis [143,144,145]. This is supported by the observation of gender differences in body composition emerge at the time of pubertal growth. Furthermore, the efficiency of GH activity is also modulated by estrogens in adulthood. This is exemplified by women being less responsive than men to GH treatment [146]; GH treatment induces a greater increase in lean mass and decrease in fat mass, or a greater increase in indices of bone turnover and in bone mass, in GH-deficient male compared to female patients. Relevant to GH physiology is the alteration of IGF-I bioavailability by oral administration of pharmacological doses of estrogens [reviewed in [62]]. IGF-I tissue availability and activity are regulated by IGF binding proteins (IGFBPs) [3,20,147]. IGF-I circulates almost entirely as a ternary complex bound to IGFBP-3 and the acid labile subunit (ALS) both of them are strongly GH-regulated in liver. The inhibition of GHR-JAK2-STAT5 signaling pathway in liver, most likely contributes to the effects of estrogens on IGF-I, ALS, and IGFBP-1. Thus, estrogens exert profound effects on liver-derived IGFPBs when administered by the oral route which most likely modify the biological actions of IGF-I. In addition, oral administration of pharmacological doses of estrogen can inhibit GH-regulated metabolic effects (e.g., lipid oxidation, protein synthesis) [148]. These effects on metabolism and body composition are attenuated by transdermal administration, suggesting that liver is the major site of regulatory control by estrogen.

Estrogens can modulate GH actions on liver by modulating GH responsiveness, which includes changes in hepatic GHR expression and crosstalk with GH-activated JAK2-STAT5 signaling pathway [62] (Fig. 6). Particularly, E2 can induce SOCS2 and SOCS3 expression which in turn negatively regulates GHR-JAK2-STAT5 signaling pathway leading to reduction in transcriptional activity in

liver. Therefore, beside E2 regulation of sex dimorphic pattern of pituitary GH secretion, induction of SOCS expression and inhibition of JAK2-STAT5 signaling is a very relevant mechanism that, in part, could explain how estrogens directly inhibit the effects of GH in several STAT5-regulated actions (i.e., somatic growth, body composition, metabolism, and gender-related hepatic functions). We have observed that long-term administration of physiological doses of E2 to GH-deficient male rats (hypothyroid) regulates several members of SOCS family by a complex interplay with GH and thyroid hormones [61]. Hypothetically, other members of the negative regulators of STAT family may also contribute to estrogen interaction with GH signaling in liver. This is explained by ERα stimulation of PIAS3 expression which binds to STAT3 and blocks STAT3 DNA-binding activity. Interestingly, E2 activation of ER followed by direct interaction of ER with STAT5 may also inhibit STAT5-dependent transcriptional activity [149,150]. On the other hand, it has been shown that E2-activation of ERα or ERβ, via non-genomic mechanisms, induces STAT5 (and STAT3)-dependent transcriptional program in endothelial cells [151]. Overall, these studies have shown a direct interaction between ER and STAT5 signaling and also demonstrate that functional consequence of this cross-talk depends on the precise milieu of the intracellular environment.

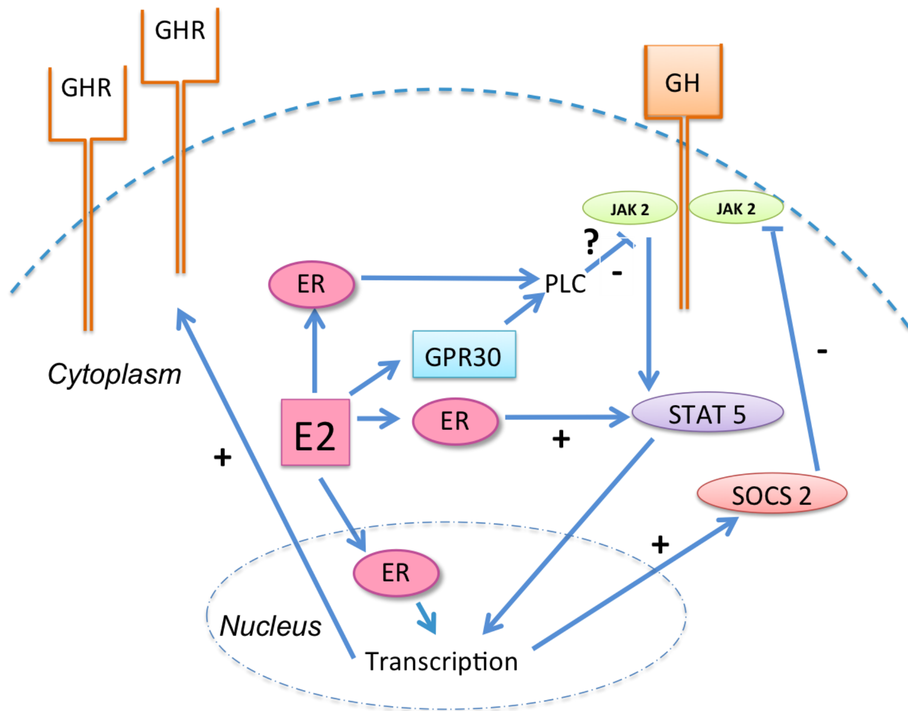

Figure 6. Schematic representation of signaling pathway activated by E2 and its crosstalk with GH-JAK2-STAT5 signaling (Based on [28]).

- Lipid and glucose metabolism. Several studies have suggested that E2-mediated signaling can have an important role in the control of lipid and glucose metabolism [136,137]. Studies in both human and rodents suggest that altered levels of E2 or its receptors can lead to a metabolic syndrome-like phenotype (i.e, insulin resistance, adiposity, dyslipidemia). For example, postmenopausal women are three times more likely to develop metabolic syndrome associated abnormalities than premenopausal women [152]. Furthermore, estrogen/progestin based hormone replacement therapy in postmenopausal women has been shown to lower visceral adipose, fasting serum glucose and insulin levels [153]. Clinical observations in ERα deficient male or with decreased levels of aromatase noted the development of increased body weight, insulin resistance, and hyperinsulinemia [154,155]. Similarly, the $ER\alpha^{-/-}$ and the aromatase deficient mice develop insulin resistance, intra-abdominal adiposity, steatosis and impaired lipid oxidation in

liver, which can be reverted by E2 treatment [155,156,157]. The beneficial influence of E2 in relation to normalizing lipid and glucose homeostasis is also evidenced in ob/ob and high-fat diet fed mice, models of obesity and type 2 diabetes. In both models, E2 treatment improves glucose tolerance and insulin sensitivity, and reduces weight in HFD-fed mice [158,159]. Studies in ERα$^{-/-}$ mice have shown that ERα mainly mediates beneficial metabolic effects of estrogens such as anti-lipogenesis, improvement of insulin sensitivity and glucose tolerance, and reduction of adiposity [136,137]. In addition to the observations from ERα$^{-/-}$, selective ablations of ERα in the hypothalamic brain region or the hematopoietic/myeloid cells have both been reported to give rise to an increase in body weight and reduced glucose tolerance [160,161]. Insulin resistance in ERα$^{-/-}$ mice is largely localized to the liver, including increased lipid content and hepatic glucose production. Surprisingly, the liver-selective ablation of ERα (LERKO) did not recapitulate the observed ERα$^{-/-}$ mice phenotype [162]. LERKO mice did not increase body weight nor developed glucose intolerance or insulin resistance, even when challenged with a HFD. The authors suggest the presence of unidentified compensatory mechanism/s or that hepatic insulin resistance occurs as a secondary effect upon ablation of E2 signaling in other cell types. Furthermore, treatment of ob/ob mice with the ERα selective agonist propyl-pyrazole-triol can improve glucose tolerance and insulin sensitivity which supports the critical role of ERα signaling in the control of glucose homeostasis.

1.4. Growth hormone and estrogens interaction in liver

E2 and GH signaling play a critical role in liver physiology and pathology in both female and male. A main tissue target is liver, which can respond in a sex specific manner to GH and sex hormones. The absence of E2 or GH signaling causes a metabolic syndrome-like phenotype (i.e., adiposity, fatty liver, metabolic disturbances) which can be ameliorated by E2 [136,155,156,157] or GH [3,163]

replacement, respectively. These findings suggest that E2 and GH signaling regulate overlapping cellular networks related with physiological control of liver metabolism. E2 – GH interactions are physiologically and therapeutically relevant in the liver-regulation of endocrine (e.g., IGF-I), metabolic (e.g., lipid and glucose metabolism), and sex-differentiated (e.g., endo- and xenobiotic metabolism) functions. In the general population, the endocrine and metabolic consequences of long-term exposition to estrogens or novel estrogen-related compounds and their influence on the GH axis are largely unknown. The widespread exposition of estrogen or estrogen-related compounds in human makes the interplay of E2 with GH in liver clinically relevant [164]. Thus, a better understanding of this complex interaction in physiological and pathological states could contribute to prevent health damage and improve clinical management of patients with growth, developmental and metabolic disorders.

Estrogens can modulate GH actions in liver by acting centrally, regulating pituitary GH secretion, and, peripherally, modulating GH signaling [47] (Fig.7). Most previous studies have been focused on the influence of estrogens on pituitary GH secretion [34] and the gender-specific GH secretion release from pituitary has been shown to have a great impact on hepatic transcriptional regulation [33]. However, there is also strong evidence that estrogens modulate GH action at the level of GHR expression and signaling. Particularly, E2 has been shown to induce SOCS2 which in turn negatively regulates GHR-JAK2-STAT5 signaling pathway [61,62]. This interplay could be clinically relevant because the GHR-JAK2-STAT5 signaling is of particular importance in the regulation of endocrine, metabolic, and sex-differentiated actions of GH in liver. Importantly, disruption of GHR-JAK2-STAT5 signaling is associated with hepatic metabolic changes that include fatty liver, fibrosis, and hepatocellular carcinoma [1].

Many aspects of hepatic function are perturbed by pharmacological concentrations of estrogen [165] and when estrogens are orally administrated, impair the GH-regulated endocrine and metabolic function of the liver in humans [166]. It is well established the role of the estrogens in the GH–IGF-I axis, in fact, oral estrogen administration to women reduces serum levels of IGF-I, despite elevating GH levels [166] and suppresses GH stimulation of lipid oxidation [164,167], this effects occur in normal and GH-defficient women and are avoided by transdermal administration of physiological dose of estrogen. Oral estrogen thus results in a significant loss of lean body mass and an increase in fat mass. Moreover, E2 is known to play an essential role in the pubertal growth, E2 rescues pubertal growth during GH resistance through novel mechanism of GHR-independet stimulation of hepatic IGF-I production [168]. Since the liver makes most of the circulating IGF-I and much of the body´s lipid oxidation, elevated estrogen levels in the hepatic portal vain would be detrimental to these GH stimulated process [169]. This explains why oral E2 administration is more deleterious than transdermal administration. Moreover estrogen down-regulation of GH signaling could be attributed to the well known ability of high-dose estrogen to inhibit growth. As suggested by Leung et al. upregulation of SOCS2 could contribute to inhibition by estrogen of prolactin induced lactation, the sexual dimorphism in red blood cell mass, and the modulatory effects of estrogen on immune function, all of them involve the JAK-STAT signaling.

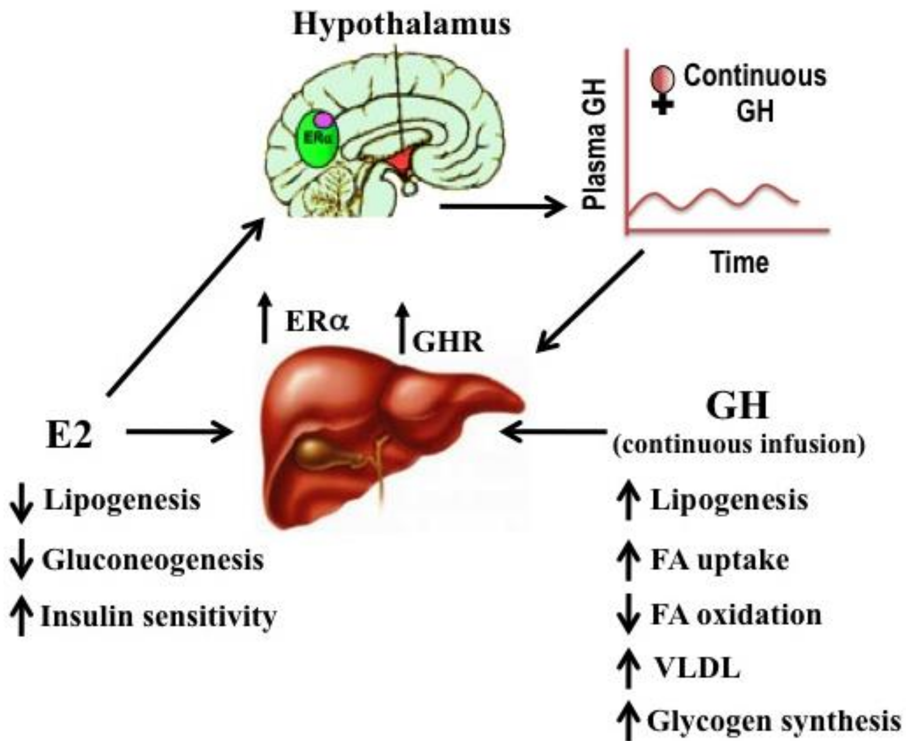

Figure 7. Phyisiological effects of E2 and GH (female pattern) on lipid and glucose metabolism (Based on [28]).

1.5. Relationship between the Metabolic Syndrome and GH, E2 and SOCS.

The Metabolic Syndrome is a complex disease that is increasing at epidemic rates in Western countries. Insulin resistance, obesity, diabetes and NAFLD (Non Alcoholic Fatty Liver Disease) are the components of the metabolic syndrome. Fatty liver development is a multifactorial process and certain metabolic disorders are associated with it, such as obesity, hyperlipidemia and insulin resistance. Fatty liver o steatosis (i.e, accumulation of triglycerides in the liver) is pathological. The prolonged lipid storage can result in an activation of inflammatory reactions and loss of metabolic competency. Insulin resistance is considered as the factor that most contributes to the development of NAFLD (as insulin controls energy

balance by regulating the metabolism of carbohydrate and lipid; in relation to the lipid content of the liver, insulin modulates the synthesis and secretion and β-oxidation of fatty acids). However, the molecular basis for liver steatosis formation is poorly understood. There are extensive clinical and experimental evidences suggesting that metabolic alterations associated with NAFLD are regulated, among others, by E2, GH, and SOCS2.

E2 may interfere with endocrine, metabolic, and gender-differentiated functions in liver in both females and males. As mentioned above, estrogen, through its interaction with the ERα, exerts direct effects on liver [62,170]. Indirect mechanisms also play a crucial role because of the E2 influence on the pituitary GH secretion and the GHR-JAK2-STAT5 signaling pathway in the target tissues inducing the expression of SOCS2, which is a negative regulator of the GHR-JAK2-STAT5 signaling pathway [59]. The GHR-JAK2-STAT5 signaling pathway is of particular importance in the regulation of endocrine, metabolic, and sex-differentiated actions of GH in liver. The ability of GHR-JAK2-STAT5 signaling pathway to regulate hepatic lipid metabolism has been highlighted in recent mouse genetic studies showing that hepatic inactivation of GHR [80], it is associated kinase, JAK2 [170] or its downstream signaling intermediary, STAT5b [171] leads to fatty liver [1]. Hepatic steatosis is known to be associated with decreased insulin sensitivity and SOCS2 was shown to be a potent regulator of proinsulin processing and insulin secretion in β cells. We have now identified SOCS2 as an important regulator of hepatic homeostasis under conditions of High-Fat dietary stress [62].

An insufficient number of insulin-producing β cells is a hallmark of both type 1 and type 2 diabetes. SOCS proteins are powerful inhibitors of pathways involved in survival and function of pancreatic β cells, such as those induced by insulin and GH [116,172]. Lactogen signaling is critical for β cell proliferation and β cell

function during pregnancy. The most important mediator of lactogen signaling is the JAK2/STAT5 pathway [173] and CIS and SOCS2 are induced during pregnancy [174]. Constitutive production of SOCS2 in pancreatic β cells leads to hyperglycaemia and glucose intolerance [175]. In a Japanese cohort, several SNPs in the SOCS2 gene promoter region were found to be associated with increased risk of type 2 Diabetes Mellitus [176]. Elimination of SOCS1 increases interferon response and potentiates β cell death [177] while SOCS3 is a potent protector against type 1 Diabetes Mellitus through suppressing IL-β and TNFα in pancreatic β cells [178,179]. SOCS3 knock–down β cells also show resistance to apoptotic cell death [180]. Therefore, one therapeutic strategy to increase functional β cell mass in order to overcome insulin deficiency could be SOCS regulation.

1.6. Relevance of Pharmacogenomic to explore molecular mechanisms of GH, E2 and SOCS2.

In the field of Endocrinology different hormones affects the function of each other and their actions also depend largely on nutritional status and other parameters. The fact that most hormones have tissue specific effects, make such studies even more challenging. Nevertheless, finding out how different hormones affect the trasncriptome, proteome and metabolic content of cells is great importance for the understanding of hormone action and disturbances in endocrine system. The first papers describing microarray analysis *in vivo* gene expression in the endocrine field were published in 2001-2002 [40,181,182], since then the technology and the fields of investigation have extensively growth. The studies of gene expression used in this Thesis are particularly attractive to the Endocrine Pharmacology field, as regulation of gene expression is a key mechanism whereby hormones exert their actions. Some papers have been published in the field of the gene expression profiling in molecular studies of hormone actions in our group, [40,182,183,184]. In Rico-Bautista (2005) [118], were identify genes and metabolic parameters that

might contribute to the $SOCS2^{-/-}$ phenotype by cDNA microarray, demonstrating that although SOCS2 deficiency induces significant changes in hepatic gene expression, only a fraction of these overlap with known GH-induced effects in the liver, suggesting that SOCS2 might be an important regulator of other growth factors and cytokines acting on the liver. Studying changes in gene expression after short-term hormonal treatment could help to elucidate direct mechanism of hormone action, while the effects of long-term treatment may help explain the physiological effects of hormones. E2 relationship in GH resistance models were study in some papers, Long-term administration of physiological doses of E2 to GHD male rats (hypothyroid) regulates several members of SOCS family by a complex interplay with GH and thyroid hormones [61] also a global expression analysis of GH actions in liver using microarrays clearly indicates that most of the known physiological effects of GH can be explained through its effects on the transcription of specific genes [40,47,48,184,185].

Pharmacogenomic has the potential to clarify tissue specific actions of hormones and explain observed physiological effects previously lacking molecular explanation. Pharmacogenomic is also a tool to personalize medicine combining bioinformatics with proteomic, metabolomics, and others new technologies, to explore pathophysiology and to characterize more precisely an individual´s risk for disease, as well as response to treatment. In this Thesis, gene expression profiling and system biology network studies have been used to construct hypotheses concerning the molecular mechanisms and metabolic networks underlying actions of E2, GH and SOCS2. By collecting and comparing transcription profiles concerning hormone actions in situations like varying hormonal status, diets, or gene deletions, it might be possible to elucidate the role of hormones in different states of disease.

2. AIMS

The main aim of the work presented in this Thesis was to characterize the influence of E2 and GH interplay and SOCS2 on liver metabolism. Particularly, we focused on the following aims:

- Characterize E2 and GH interplay on liver metabolism (Paper I).
- Characterize the role of SOCS2 in E2-regulated somatic growth and liver metabolism (Paper II).
- Characterize the role of SOCS2 in HFD-induced fatty liver (Paper III).
- Characterize the role of SOCS2 in MLDSTZ-induced Diabetes (Paper IV).

3. MATERIALS AND METHODS

3.1. Animals. All the studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the University of Las Palmas de Gran Canaria and conducted in accordance with European and Spanish laws and regulations. All Animals were kept under standard conditions, constant 12-h/12-h light/dark cycles, and in a controlled temperature (21-23 ºC) environment, and free access to autoclaved standard chow (A04 SAFE Panlab, Barcelona, Spain) and tap water throughout the experiments. SOCS2 deleted (SOCS2$^{-/-}$) and wild-type (WT) littermates (C57BL/6J) male mice (Paper II and III) have been previously reported [124]

- **To investigate the molecular mechanisms of E2 and GH interplay in male liver (Paper I).** Adult (2-3 months old) male Sprague-Dawley rats (n=6 per group) were used throughout these experiments. Generation of hypothyroid animals (TX) was performed as previously described [186,187] (see Paper I). Two weeks after starting MMI administration, male rats were orchidectomized (OX) or sham–operated to make TXOX rats or testis-intact controls (TX), respectively (Fig 1). Six rats were not treated with MMI and were subjected to sham-surgery to provide euthyroid testis-intact controls (INTACT). Four days after OX, we began HRT with E2 benzoate (Sigma) (50 µg/kg/day; sc; 5 days per week) (TXOXE2) or vehicle (0.2 ml corn oil; sc; 5 days per week) (TXOX) to TXOX rats for 20 days [188,189] before hormonal replacement for 7 days with E2 plus GH (TXOXE2GH) or vehicle plus GH (TXOXGH). GH (0.3 mg/kg/day) was administered as two daily sc injections at 12-h intervals (08:00h and 20:00h) to mimic the male-specific GH secretion [39,190]. TX and TXOX control animals received equivalent amounts of the vehicle alone. Hypothyroidism status was corroborated by monitoring the body weight gain at 7-day intervals and the serum levels of T4 and T3. Twenty-four hours (in the case of E2) or twelve hours (in the

case of GH) after the last injection, the animals were killed by exsanguinations. On PND94, blood samples were collected and serum stored at -80ºC until analysis. Portions of the liver were snap frozen in liquid nitrogen and stored at -80ºC until processed for mRNA analysis.

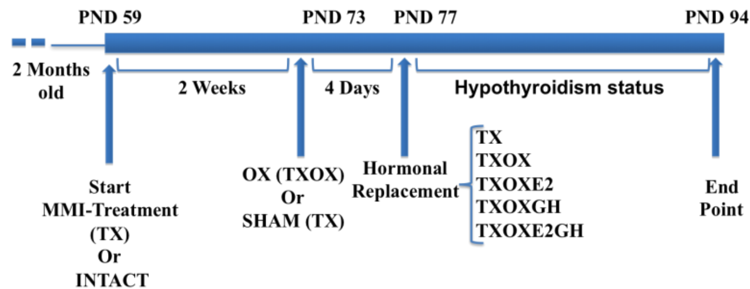

Figure 1. Schematic representation for hormonal replacement in hypothyroid-orchidectomized rats.

- **To investigate the role of SOCS2 on the effects of E2 on somatic growth and liver transcriptome (Paper II).** We carried out two different studies in male mice. First, we investigated the influence of SOCS2 on E2-regulated somatic growth in intact (non-orchidectomized) male mice. For this purpose SOCS2$^{-/-}$ and WT littermates (C57BL/6) mice [124] (2 months old) (n=6 per group) were treated with E2 benzoate (Sigma, St. Louis, MO) (50µg/kg/48h; sc) or vehicle (corn oil:ethanol; 90:10; v/v; 50 µl/48h; sc) during 15 weeks [191] (see Fig.2). Second, we investigated the influence of SOCS2 on E2-regulate liver transcriptome in orchidectomized (OX) mice. For this purpose, SOCS2$^{-/-}$ and WT (n=6 per group) (4 months old) were OX or sham operated [192]. After one week of recovery period, they were injected with E2 benzoate (100 µg/kg; sc) or vehicle (corn oil:ethanol; 90:10; v/v) for 1 day, 2 days or 21 days (5 days/week) [193] (see Fig. 3).

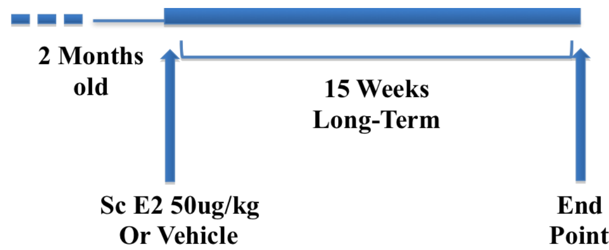

Figure 2. Schematic representation for long-term E2 treatment in SOCS2-/- and WT male mice (non-orchidectomized) that was used to investigate the role of SOCS2 on E2-regulated somatic growth.

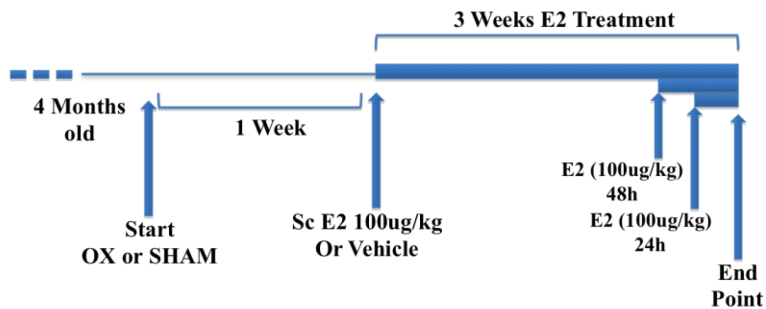

Figure 3. Schematic representation for E2 treatment in SOCS2-/- and WT male mice (orchidectomized) that was used to investigate the role of SOCS2 on E2-regulated liver transcriptome.

The diet was removed from the cages, twelve hours before the mice were sacrificed, to minimize the effect of food. Serum samples were collected and stored at -80ºC until analysis. Portions of the liver were washed with cold PBS, snap frozen in liquid nitrogen, and stored at -80ºC until being processed for mRNA or biochemical analysis.

- **To investigate the role of SOCS2 in High-Fat Diet (HFD)-induced fatty liver and insulin resistance (Paper III).** SOCS2$^{-/-}$ and WT littermates (8–10 weeks old) were fed with a standard control diet (CD) (SAFE-diet A04, Panlab SLU, Barcelona, Spain) or HFD (OpenSource Diets -D12492), New Brunswick, NJ, USA) (see Paper III). The mice were sacrificed after an ip injection of 0.75 U/kg human recombinant insulin (Actrapid, Novo Nordisk, Denmark) or saline as

a control. Tissues were stored at −80°C until analysis. Pieces of pancreas and liver were excised for histological examination. Blood samples were collected through cardiac bleeding, and plasma was used to measure various analytes.

- **To investigate the role of SOCS2 on Diabetes Mellitus induced by multiple low-dose streptozotocin (MLDSTZ) (Paper IV).** We used the MLDSTZ model to induce type 1 Diabetes [194] in SOCS2$^{-/-}$ and wild type littermates (Fig. 4). Six months-old male mice received 5 consecutive daily ip injections of STZ (50 mg/kg b.w.). Then, STZ administration was stopped and blood glucose, body weight, food and water intake, glucose tolerance, and insulin sensitivity were recorded. Mice were considered diabetics if the blood glucose levels were greater than 300 mg/dl. Sixty days after STZ treatment was stopped, mice were sacrificed and tissues were collected for further studies.

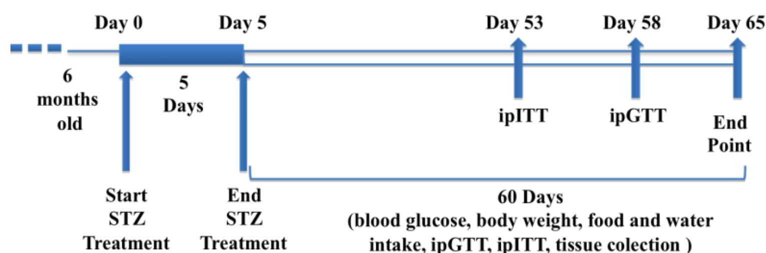

Figure 4. Schematic representation for the multiple low dose streptozotocin (STZ) treatment in SOCS2-/- and WT male mice that was used as a model of autoimmune diabetes and β-cell destruction *in vivo*.

3.2. Somatic growth and food intake analysis. Body weights, tail lengths, and food intake were measured once a week for all animals. The measurement of tail length was used for monitoring growth [195,196,197]. The percentages of weight rate (WR) or tail rate (TR) were calculated by the following formulas: WR=[(W (g) (new) – W (g) (old)/W (g) (old)]*100 or TR = [(T (cm) (new) – T (cm) (old)/T (cm) (old)]*100, respectively. Food consumption was estimated by subtracting the amount of food left on the grid from initial food weight. Food spilled on the floor

of the cage was not weighed, but spillage was minimal because the diet was supplied as pellets. The weekly caloric intake was calculated on the basis of food consumption x caloric value of the diet (2900 kcal/g). Feed efficiency (FE), which denotes the body weight increase per gram of food consumed or the ability to transform calories consumed into body weight [198], was calculated by the following formula: mean body weight gain (g)/total caloric intake.

3.3. Intraperitoneal glucose (ipGTT) and insulin (ipITT) tolerance tests. For ipGTT, mice were fasted 16h overnight followed by an intraperitoneally (ip) injection of D (+)-glucose (20% in 0.9% NaCl) at a dose of 2g/kg b.w. For ipITT, human insulin was ip injected into 4h fasted mice at a dose of 0.75 U/kg b.w. Blood glucose levels were measured using a glucometer (Roche Diagnostics, Switzerland). Insulin was measured in blood samples collected at fasting time point 0 during ipGTT. Homeostatic model assessment (HOMA)-IR (insulin resistance) index was calculated as follows: fasting insulin (ng/ml)×fasting glucose (mM). HOMA-β (β-cell function) index was calculated as follows: 20×fasting insulin (μU/ml)/fasting glucose (mM) – 3.5 [199].

3.4. Serum biochemistry. Cholesterol (CHO), triacylglycerols (TG), gamma glutamil transpeptidase (GGT), and creatinine were measured were measured by a CLIMA MC-15 clinical chemistry photometer (RAL, Spain). (Papers II and IV). CHO and TG were also assayed using colorimetric enzymatic kits (Roche/Hitachi Diagnostic GmbH, Mannheim, Germany) (see Paper III). Plasma alanine aminotrasferase (ALT) activity was measured using commercial assay kit (Cayman Chemical Co., MI, USA) (Paper III). Serum levels of insulin (Crystal Chem Inc.), leptin and IGF-I (Quantikine, R&D systems), and GH (Millipore, MA, USA) (Papers II, III and IV) were determined by using mouse/rat immunoassays (ELISA) according to manufacturer recommendations. The ELISA included quality controls provided by the manufacturers, and the standard curves of the

assays were performed in accordance with the manufacturer's provided samples. All the samples were assayed together and each sample was assayed in duplicate.

3.5. RNA isolation. Total RNA was isolated by the homogenization of frozen liver as previously described [156]. All samples were treated with RNase-free DNase (Promega, Madison, WI) and further purified by RNAse MiniElute Cleanup Kit (Quiagen). The yields and purity of RNA were measured by a ND-1000 Spectrophotometer NanoDrop (NanoDrop Technologies), and the quality of total RNA was analyzed with an Experion automated electrophoresis system (Biorad).

3.6. DNA microarray platforms. 2-color microarray-based platforms were used to perform genome expression studies. In Paper I, a microarray containing 27000 rat 70-mer oligo probe sets (KTH Microarray Center; www.biotech.kth.se) was used to evaluate the effects of hypothyroidism and hormonal replacement on liver gene expression (Fig.5). In Paper II, microarrays containing 44000 mouse 60-mer oligo probe sets (Agilent) were used to evaluate the changes in liver transcriptome, in the presence (WT) or in the absence of SOCS2 ($SOCS2^{-/-}$), caused by E2. Briefly, 5µg (Paper I) or 0.1µg (Paper II) of high-quality total RNA were reversed-transcribed, labelled, and hybridized following the manufacturer's protocol Pronto™ Plus System (Promega) (Paper I) or Agilent (Life Technologies) (Paper II), respectively. After 16 h of hybridization, the slides were scanned using GenePix (Axon Instruments, CA) (Paper I) or G2565CA (Agilent) (Paper II) microarray scanners. Four independent hybridizations were performed comparing individual animals from the different experimental groups for a total of 4 analyses (Fig.5 and 6).

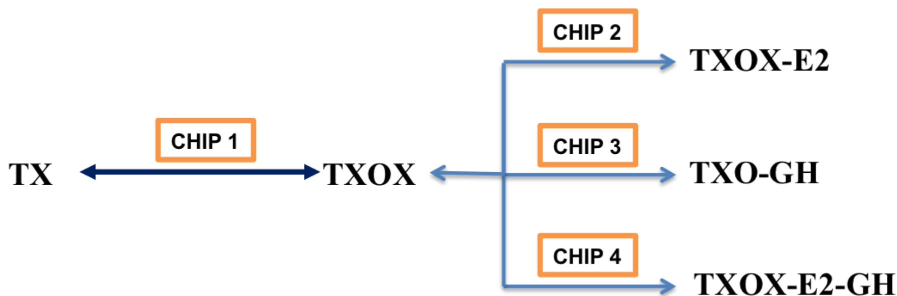

Figure 5. Schematic representation of comparison between groups (↔) that was used to evaluate the effects of hormonal replacement on liver gene expression in hypothyroid-orchidectomized (TXOX) rats (Paper I).

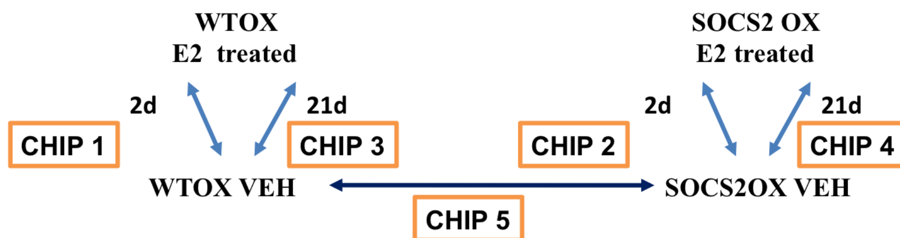

Figure 6. Schematic representation of comparison between groups (↔) that was used to evaluate the effects of E2 treatment on liver transcriptome in wild type (WT) and SOCS2$^{-/-}$ orchidectomized (SOCS2OX) mice (Paper II).

3.7. Microarray data processing and analysis. Image acquisition and analysis were performed by using GenePix Pro 6 (Paper I) or Feature Extraction (Agilent) (Paper II) software. The LOWESS (Locally Weighted Scatter Plot Smoother) method was used to normalized raw intensity data [200]. If the measured probe sets were not present in at least 3 of the 4 chips, they were considered as no information and therefore were eliminated to reduce data complexity. Identification of differentially expressed genes was performed using the SAM (Significance Analysis for Microarrays) statistical technique [201]. Functional and system biological network analysis were performed on the basis of the Gene Ontology (GO) enrichment of differentially-expressed genes using DAVID [202] and the results depicted using Cytoscape [203]. For the graphical representation,

significance cut-off was set on *p* value < 0.05 and corrected *q* value (Benjamini) < 0,1. GO graphs interpretation: node (inner circle) size corresponds to the number of genes up-regulated by GH or E2; node border (outer circle) size corresponds to the number of genes down-regulated by GH or E2; color of the node and border corresponds to the significance of the gene set for up or down regulated genes, respectively (dark red=significantly enriched, light red=enriched no significantly; grey=absent); edge size corresponds to the number of genes that overlap between the two connected gene sets. Green edges correspond to shared up-regulated genes and blue edges correspond to shared down-regulated genes.

3.8. Analysis of gene expression by real time quantitative-PCR (qPCR). The mRNA expression levels of genes were measured using qPCR. Briefly, 2 µg of total RNA was treated with RNase-free DNase I (Promega) and reverse transcribed using iScript (Bio-Rad) according to manufacture instructions. Two µl of cDNA served as a template in a 20 µl qPCR reaction mix containing the primers and SYBR Green PCR Master Mix (Diagenode, Belgium). Quantification of gene expression was performed according to the manufacturer's protocol using ABI PRISM® 7000 SD RT-PCR (see description in papers). Data were extracted and amplification plots generated with ABI SDS software. All amplifications were done in duplicate and C_t scores were averaged for subsequent calculations of relative expression values. The level of individual mRNA measured by qPCR was normalized to the level of the housekeeping genes cyclophiline, 36B4, and ribosomal 28S by using Pfaffl method [204]. Exon-specific primers were designed by the Primer 3 program (see Tables of primers in papers) [205].

3.9. Histochemistry (Paper III and IV). In paper III, pancreas was fixed over-night in 4% paraformaldehyde at 4 °C and by paraffin embedded. In paper IV, frozen livers were cryosectioned and subjected to insulin and glucagon immunostaining (manuscript in preparation). Primary antibodies against insulin

and glucagon were obtained from Dako-Cytomation (Stockholm, Sweden). In paper III, primary antibodies were detected with a biotinylated secondary antibody (anti-guinea pig IgG) in conjunction with Vectastatin ABC peroxidase kit (Vector Laboratories, Carpenteria, CA) and chromogen diaminobenzidine (Sigma-Adrich, St. Louis, MO). Images were captured by bright-field microscope under the same magnification and light illumination In paper IV, secondary antibody (anti-guinea pig IgG) in conjunction with Vectastatin ABC peroxidase kit (Vector Laboratories, Carpenteria, CA) and chromogen diaminobenzidine (Sigma-Adrich, St. Louis, MO) and images were captured by immunofluorescence. Slides were counterstained in standard hematoxylin/eosin. Frozen sections were also stained with Oil Red O to visualize fat deposits in the liver as previously described [206] (Paper III).

3.10. Pancreas insulin analysis. In Paper III and IV, one third of the pancreas mass, from the tail, was digested and homogenized in 1 mL acidic ethanol (70% ethanol and 37% HCl) for insulin quantification per unit weight pancreas. Insulin was determined using an ultrasensitive rat insulin ELISA kit (Crystal Chem, IL, USA).

3.11. Hepatic lipid analysis. In Paper I, frozen liver aliquots were used for lipid extraction. Aliquots of lipid extracts were quantified after separation by one-dimensional TLC and image analysis using the G5-700 Bioimage TLC scanner (Bio-Rad, CA). The spots were quantified as integrated optical density against an internal standard of cholesteryl formate and against calibration curves of the different lipid standards [207].

3.12. General statistical analysis. The significance of differences between the groups was tested by either a two-tailed Student's t test or a one-way ANOVA, which was followed by *post hoc* comparisons of the group means according to the

GraphPad Prism 5 program (GraphPad Software, San Diego, CA). Factor scores were analyzed by two-way ANOVA to evaluate the combined effects of hormonal treatments, as well as their interactions. Multivariate analyses were performed using the SPSS package (version 15.0, SPSS Inc, Chicago, IL, USA). Student's *t*-test was used to test for the significant differences between two groups. Statistical significance was reported if $P<0.05$ was achieved.

4. RESULTS AND DISCUSSION

4.1. Functional interplay between E2 and GH in liver (Paper I; doi:10.1371/journal.pone.0096305). In this study, we combined lipidic and transcriptomic analysis to obtain comprehensive information on the molecular mechanisms of E2 effects, alone and in combination with GH, to regulate liver functions in adult hypothyroid-castrated male rats. This is a model with reduced GH activity that was used to minimize the influence of internal hormones on hormonal replacements (E2 and GH) and to explore E2 influence in male-differentiated functions. We show that E2 and GH replacements in hypothyroid-castrated male rats have a significant impact on lipid content and transcriptome in the liver and that E2 exerts a marked influence on GH-regulated endocrine, metabolic, immune, and gender specific responses in the liver.

Hypothyroidism impaired body weight gain and decreased circulating levels of IGF-I and biological markers of GH-STAT5b signaling activity in the liver (i.e., mRNA levels of IGF-I, ALS, SOCS2, CIS, and CYP2C11) [208]. These changes were totally or partially restored by intermittent GH administration to TXOX rats. However, the effects of GH were largely prevented by E2 which is in line with the negative effects of estrogens on continuously GH administration in hypophysectomized female rats [209]. The positive effects of E2 on hepatic SOCS2, CIS, and FGF21 transcripts, suggest that E2 might prevent the activation of GH-STAT5b signaling in liver through induction of these negative regulators of GH signaling [62,210]. Similarly, estrogen administration in humans can prevent the GH-induced increase in IGF-I, IGFBP-3, lipid oxidation, and protein synthesis [164,211]. The effects of hypothyroidism on growth are associated, in part, with an increased hepatic amino acid catabolism and urea synthesis [212]. Biological network analysis showed that intermittent GH administration to TXOX rats causes a positive regulation of cellular catabolism, whereas the genes involved in the

metabolism of amino acids and urea (i.e., OTC, ASS1, aminotransferases, and methyltransferases) are significantly down-regulated. This is in line with the positive effects of GH on nitrogen balance, which have been previously studied in hypophysectomized rats [8,40,213].

GH serves as an anabolic hormone that promotes lipolysis and prevents lipogenesis in adipose tissue, which increases the availability of fatty acids (FFA) for energy expenditure [8]. E2 is also able to interfere with this process by preventing the induction of some genes related to fat utilization, such as ApoC2, which activates the enzyme LPL that hydrolyzes TG. Therefore, E2 actions in liver can impact the peripheral metabolic actions of GH.

Lipogenesis is often increased in situations of reduced energy expenditure such as hypothyroidism, GH deficiency, E2 deficiency, or aging [214]. Accordingly, our analysis of the hepatic lipid content revealed that TXOX rats contained significantly increased levels of total saturated fatty acids (SFA) compared to INTACT control rats. E2 replacement did not modify the mRNA expression levels of key regulators of hepatic lipogenesis [i.e., sterol regulatory element binding protein (SREBP)1c, acetyl-Co A carboxylase alpha (ACC), fatty-acid synthase (FAS)] [215], whereas it activated a PPARα transcriptional program that promotes fatty acid catabolism in liver [216,217]. This was evidenced by the E2 increased expression of the PPARα gene itself and the PPARα target genes involved in the β/ω-oxidation of fatty acids (i.e., CTE-I, CPT-2, Fasd6, Fasd1, Fasd2, Scd1, ACOX1, ECH1, BAAT, FGF21, CYP4A1, CYP4A3) (Table S2). Accordingly, E2 replacement caused a significant reduction in SFAs. Overall these findings are indicative of a positive crosstalk between E2 and PPARα that is supported by multiple independent studies [136,159,218,219]. Interestingly, despite the increased expression of genes involved in β-oxidation, we detected a significant increase in hepatic TG content in E2 treated TXOX rats, which is likely explained

by effects on lipid transport. The first step of long chain fatty acids uptake is its translocation across the plasma membrane. Notably, E2 increased the transcription of several known PPARα activated genes encoding proteins that have been implicated in fatty acids uptake and activation such as CD36, ACSL4 and SLC27A5 (FATP5) [220,221]. We have previously demonstrated that the fatty acid transporter CD36 is predominantly expressed in female rat livers and proposed that this sexual dimorphism depends on the GH secretion pattern, which can be influenced by E2 treatment. E2 also increased transcripts of the SLC27A5 gene which encodes FATP5, an fatty acid transporter that is an acyl-CoA synthetase (bile acid ligase) that catalyzes the conjugation of bile acids with amino acids before excretion into bile canaliculi [222]. Following fatty acids uptake, the first step for the intracellular use of long chain fatty acids is its esterification with CoA. This reaction is catalyzed by acyl-CoA synthetases such as ACSL4 which was also induced by E2 in TXOX rat liver. The produced acyl-CoAs are substrates for ß-oxidation but also can prime the synthesis of TG, phospholipids, CE, and ceramides and therefore are also a primary source of signaling molecules [223]. The notion that E2 may regulate the formation of lipid signaling intermediaries is supported by the stimulation of fatty acids elongase-5 (Elovl5). Elovl functions with fatty acid desaturases to generate many of the long-chain PUFAs assimilated into cellular lipids (i.e., 20:4n-6 and 22:6n-3). However, it is worth mentioning that E2 administration did not alter VLCPUFA metabolism because the levels of 20:4n-6, 20:5n-3 and 22:6n-3 remained similar to values in the TXOX group. It has been reported that E2 might play a critical role in lipogenesis and Scd1 transcription [136], a gene that encodes a rate-limiting enzyme to generate MUFAs such as 18:1 n−9 and 16:1 n−7. Previous studies have reported that the absence of E2 or ERα in rats provoked a profound increase in lipogenesis and Scd1 transcription [137], which suggests that E2 inhibits Scd1 transcription. Interestingly, the antilipogenic effect of E2 therapy, while maintaining efficient TG export and reduced phospholipid transfer protein, has been reported to depend

on hepatic ERα [219,224]. Our study, however, shows that E2 increased the Scd1 gene expression and that this effect was paralleled by reduced hepatic content of 18:0 and increased of 18:1 n−9 (the main product of SCD reaction) contents, in total and, especially, in neutral lipids compared with TXOX animals, which indicates that E2 modulates SCD1 activity in TXOX liver. Surprisingly, E2 also downregulated Scd2 gene expression in TXOX rat livers. The significance of this opposed transcriptional regulation of Scd genes is unknown, but given that transcript levels of Scd1 are about 1800 times higher than that of Scd2 in the rat liver [225], changes in 18:1n-9 and 18:0 must be entirely attributed to variations in Scd1 gene expression. Overall, the changes in the lipid composition and gene expression profile seen in E2-treated TXOX rats support the finding that E2-PPARα functional interactions play a physiological role in the regulation of hepatic lipid metabolism.

E2 has the ability to reduce circulating CHO in women and in animal models fed on a high-fat diet [226]. However, E2 was unable to efficiently reverse hypercholesterolemia or hypotriglyceridemia in TXOX rats. This result may be due to the fact that E2 reduced expression levels of several transporters of CHO (and CE), including ApoB and ABCA1 in TXOX rats, which most likely contributed to maintaining an increased hepatic level CE. E2 may also induce intracellular CHO mobilization by modulating enzymes involved in CE and CHO synthesis and/or turnover [214,227]. Distinct enzymes can catalyze the CHO to CE conversion in liver: lecithin:cholesterol acyltransferase (LCAT), which uses phosphatidylcholine (PC) as a source of acyl changes and ACAT, which uses acyl-CoA. Because the levels of lysophosphatidylcholine (LPC) were undetectable in all groups, our initial conclusion was that E2 stimulated the ACAT2 reaction to increase CE. However, we did not detect changes in the expression level of the ACAT gene, which did not discard posttranslational modification of enzymes in the CE cycle in the liver from E2-treated TXOX rats.

An increased level of hepatic CE, together with the increased TG and decreased FFA hepatic contents in GH-treated TXOX rats, resemble the effects of E2 on hepatic lipid composition and suggest that some effects of E2 might be GH mediated. A striking consequence of the combined replacement with E2 and GH is the complete restoration of MUFA levels from total and neutral lipids, an effect attributable to the increase in 18:1n9, likely through alteration of Δ9 desaturase expression. Moreover, GH and E2 increased hepatic CE and the combined effect of the two hormones were additive with regard to CE because its levels doubled those found in INTACT animals and were approximately 30% higher than in the E2 or GH groups which indicates a more efficient hepatic CHO metabolism. Accordingly, in the presence of E2, GH reduced hepatic CHO content compared not only to the TXOX group but also in relation to the E2- or GH-treated TXOX groups. The hepatic content of TG was, however, significantly increased by GH in E2-pretreated TXOX rats, which suggests that combined treatment by E2 and GH dramatically enhances lipogenesis. It is known that in contrast with its lipolytic effects in adipose tissue, GH exerts lipogenic actions in liver through stimulation of SREBP1, which is usually accompanied by increased hepatic TG (VLDL) secretion [8]. Indeed, our lipid profiling analysis suggested that intermittent GH administration to TXOX rats increased lipogenesis in the liver. However, in contrast to the effects of a continuous infusion of GH in hypophisectomized rats [40], intermittent GH administration to TXOX rats did not increase SERBP1, whereas several genes involved in fatty acids transport (e.g., FABP) and the biosynthesis of unsaturated fatty acids from 18:2n-6 and 18:3n-3 (e.g., fatty acid desaturases 4, 5 and 6) were induced. Interestingly, intermittent GH administration to TXOX rats down-regulated the expression of the lipin gene, an SREBP1c target gene, which is critical in the regulation of cellular levels of DG and TG and a key regulator of fatty acid oxidation in adipose tissue, skeletal muscle, and liver tissue [228]. These findings support the hypothesis that the female pattern of GH

administration is a more efficient stimulus to induce lipogenic effects in the liver than the male pattern [184,229]. Another mechanism whereby GH might promote lipogenesis in the liver is through the down-regulation of lipid oxidation. We have previously shown that continuous GH administration to hypophysectomized [40] and to old-intact [46] male rats inhibited PPARα. Accordingly, our lipidomic and genomic analysis showed that intermittent GH administration to TXOX rats also leads to down-regulation of the PPARα signaling pathway. In particular, GH represses the expression of PPARα itself, ACOX-1, CPT-1, FGF21, and several members of the CYP4A family, which are involved in fatty acid oxidation.

In summary, our study adds novel data that highlight the impact of subcutaneous E2 administration on liver physiology and its interplay with GH. These results highlight the role of E2 as a critical regulator of liver metabolism in mammals and add further weight to the hypothesis that E2 acts as an important regulator of GH actions in the liver. The E2-GH interplay in the liver is relevant because of the physiological roles that these hormones have in mammals and the widespread use of estrogen and estrogen-related compounds in human. Notably, this is the first study to demonstrate that hepatic lipid profiles are endowed with singular fingerprints that may be used to segregate different groups with altered hormone status. This includes different hormonal replacements (E2 or GH) that induced overlapping changes in gene expression. Therefore, liver lipid profiling can serve to identify cryptic hormone deficiencies or exposure to hormones or hormone-like substances.

4.2. The Suppressor of Cytokine Signaling-2 influences the effects of E2 on somatotropic-liver axis and liver transcriptome (Paper II; manuscript in preparation). In Paper I, we added novel data that highlight the impact of subcutaneous E2 administration on liver physiology and its interplay with GH. The E2-GH interplay in the liver is relevant because of the physiological roles that these hormones have in mammals and the widespread use of estrogen and estrogen-related compounds in human. In this study (Paper II), we have carried out a transcriptomic analysis to obtain comprehensive information on the molecular mechanisms of E2 effects *in vivo* in the presence (WT) or in the absence ($SOCS2^{-/-}$) of SOCS2. The influence of SOCS2 in E2-regulated somatic growth and liver transcriptome seen in this study could contribute to better understand the molecular mechanisms involved in the endocrine and metabolic consequences of exposition to estrogens or novel estrogen-related compounds and their influence on the GH-liver axis.

As first approach to assess the influence of SOCS2 on the effects of E2 in liver we studied changes in somatotropic-liver axis in the absence or in the presence of SOCS2. After 30 days, E2 reduced body weight gain in the two genotypes. However, reduction of body weight by E2 was more prolonged and increased in the WT group (data not shown). As expected [118], when E2 treatment was started (time 0), $SOCS2^{-/-}$ mice had higher body weight than WT. Therefore, body weight gain measurements were normalized by body weight at time 0 this shows that E2, in comparison with vehicle-treated mice, caused higher reduction of body weight in WT (Fig.1A) than in $SOCS2^{-/-}$ (Fig.1B) mice. At end point, significant differences, in comparison with vehicle-treated mice, in body (Fig.1C) and femur (Fig.1D) lengths remained in E2-treated WT but not in E2-treated $SOCS2^{-/-}$ mice. Notably, E2 increased hepatic IGF-I mRNA levels (Fig. 1E) in both genotypes without changes in circulating IGF-I (Fig. 1F).

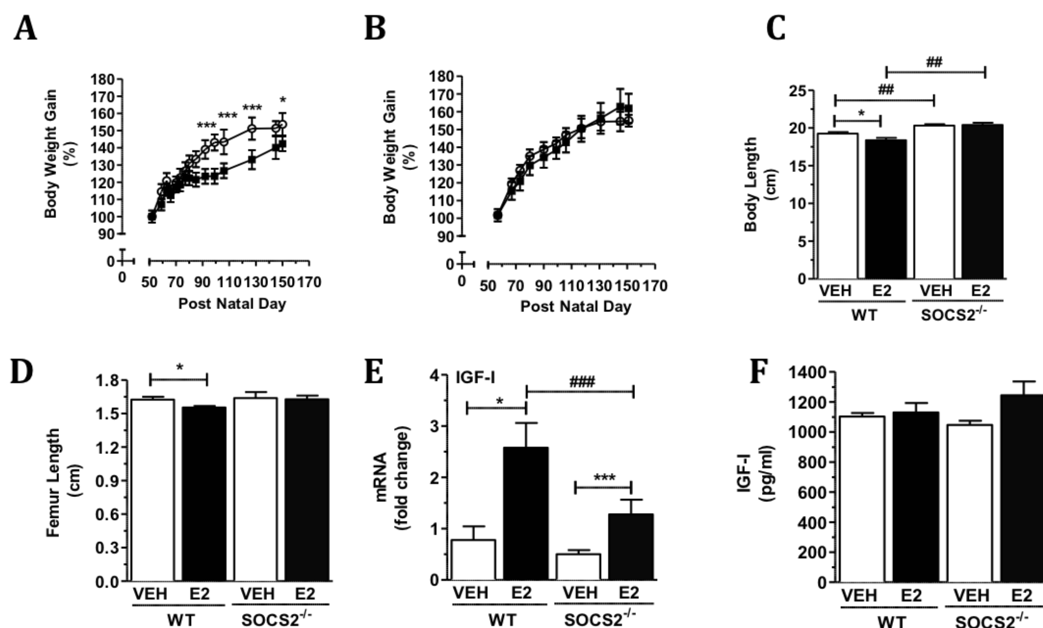

Figure 1. Influence of SOCS2 on E2-regulated somatotropic-liver axis. Wild-type ($SOCS2^{+/+}$) and $SOCS2^{-/-}$ male mice were treated with E2 benzoate (E2B) (50μg/kg/48h) or vehicle (VEH) during 90 days as described in Material and Methods. Normalized body weights on postnatal day 50 (time 0 of E2 or vehicle treatment) in WT (A) and SOCS2-/- (B) mice were monitored at 7 days intervals in the absence (white square) or in the presence of E2 (black square). The lengths of body (C) and femur (D), the hepatic IGF-I mRNA levels (E), and circulating IGF-I (F) were measurements at end point. Results are expressed as mean ± SEM from six individual animals in each group. The mean mRNA expression level of IGF-I in the VEH $SOCS2^{+/+}$ group is defined as 1, with all other expression values reported relative to this level. *P<0.05; **P<0.01; ***P<0.001 for comparison with vehicle treated groups; #P<0.05; ##P<0.01; ###P<0.001 for comparison between genotypes.

E2 has been shown to induce SOCS2 in liver, which in turn negatively regulates STAT5-mediated transcriptional activity [61,62,193]. In contrast, the molecular consequence of SOCS2 gene deletion is a prolonged activation of STAT5 [230]. Therefore, we next carried out mRNA quantitative analysis of SOCS2, CIS, SOCS1 and SOCS3, which are negative regulators of GHR-STAT5 signaling [48,210]. E2 increased the hepatic levels of SOCS2 mRNA (Fig. 2A) in WT whereas it was undetectable in SOCS2-/- mice. In WT mice, E2 induced hepatic mRNA expression levels of CIS (Fig.2B) and SOCS1 (Fig.2C) whereas SOCS3

(Fig.2D) was unchanged. Notably, in vehicle-treated SOCS2$^{-/-}$ mice, the mRNA expression levels of CIS (Fig.2B) and SOCS3 (Fig.3D were significantly reduced whereas SOCS1 (Fig.2C) stayed unchanged, in comparison with WT mice. In contrast, E2 was not capable of increasing the mRNA expression levels of CIS, SOCS1 or SOCS3 in SOCS2$^{-/-}$ mice. These findings suggest that SOCS2 can modulate the effects of E2 on negative modulators of somatotropic-liver axis.

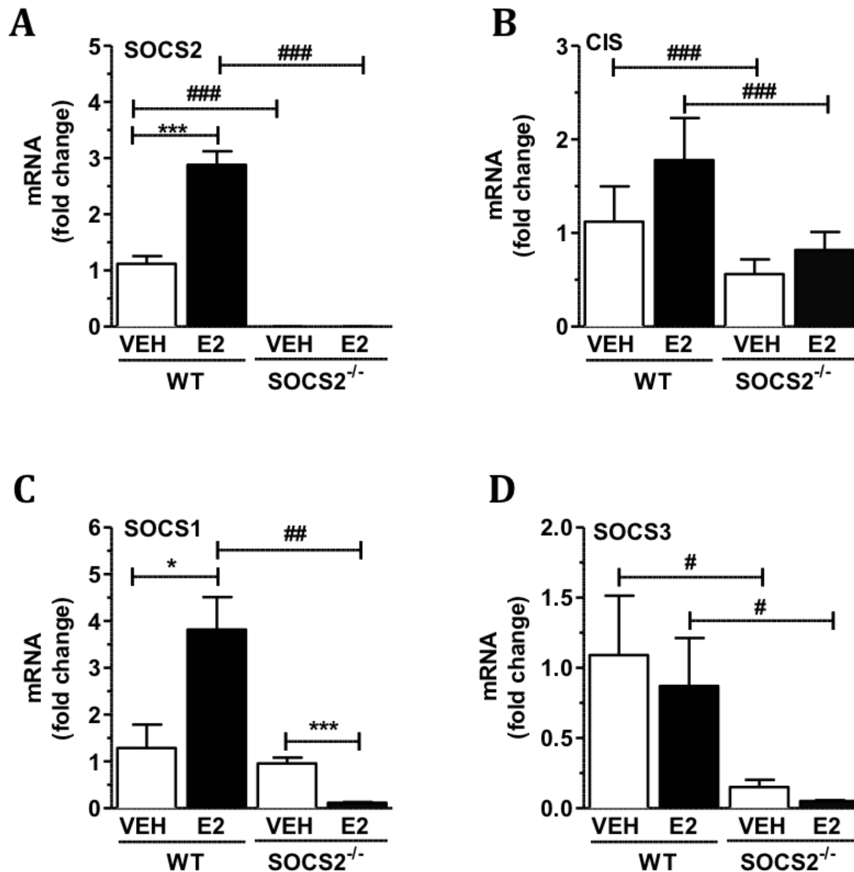

Figure 2. Influence of SOCS2 on E2-regulated Suppressors of Cytokine Signaling genes in liver. Wild-type (SOCS2$^{+/+}$) and SOCS2$^{-/-}$ male mice were treated with E2 benzoate (E2B) (50 µg/kg/48h) or vehicle (VEH) during 90 days as described in Material and Methods. The hepatic mRNA levels of SOCS2 (A), CIS (B), SOCS1 (C), and SOCS3 (D) were measured by qPCR after mice were sacrificed on postnatal 90 day as described in Material and Methods. Results are expressed as mean ± SEM (n=6). The mean mRNA expression level of each gene in the VEH SOCS2$^{+/+}$ group is defined as 1, with all other expression values reported relative to this level. *P<0.05; **P<0.01; ***P<0.001 for

comparison with vehicle treated groups; #, P<0.05 ##, P<0.01, ###P<0.001 for comparison between genotypes.

Most previous studies have been focused on the influence of estrogens on pituitary GH secretion [34] and the gender-specific GH secretion release from pituitary has been shown to have a great impact on hepatic transcriptional regulation [33]. Thus, we next measured the influence of E2 on GH-regulated gene markers of hepatic gender dimorphism [33] in WT and SOCS2$^{-/-}$ mice. In the absence of E2 treatment, mRNA levels of MUP, a male-specific gene, decreased (Fig.3A), whereas Cyp2B9 and A1bg (Fig.3B), two female-specific genes, were increased. These findings suggest that absence of SOCS2 is enough by itself to feminize male liver and influence the effects of E2 on gender dimorphism. In both genotypes, E2 decreased MUP (Fig.3A) whereas Cyp2b9 (Fig.3B) and A1bg (Fig.3C) were highly increased which show that E2 feminizes the adult liver. Overall, these findings support our hypothesis that SOCS2 can influence the effects of E2 on somatotropic-liver axis.

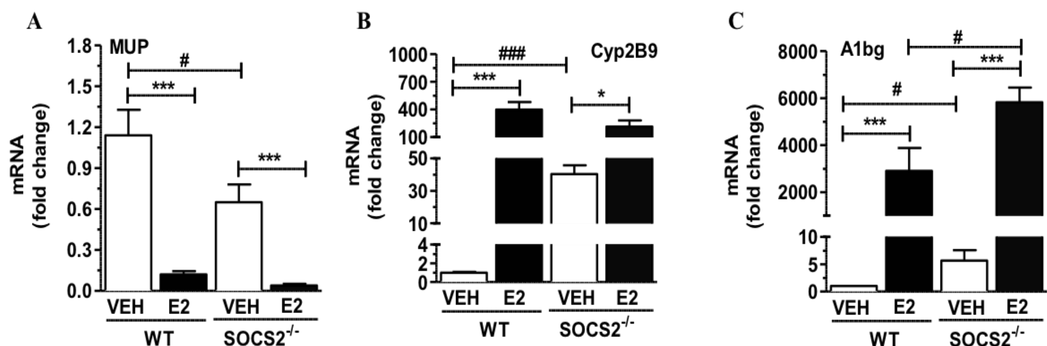

Figure 3. SOCS2 influence on E2-regulated gender dimorphism in liver. Wild-type (SOCS2$^{+/+}$) and SOCS2$^{-/-}$ male mice were treated with E2 benzoate (EB) (50 µg/kg/48h) or vehicle (VEH) during 90 days as described in Material and Methods. The hepatic mRNA levels of MUP (A), Cyp2b9 (B), and A1bg (C) were measured by qPCR after mice were sacrificed on postnatal 90 day as described in Material and Methods. Results are expressed as mean ± SEM (n=6). The mean mRNA expression level of each gene in the VEH SOCS2$^{+/+}$ group is defined as 1, with all other expression values reported relative to this level*P<0.05; ** P<0.01; ***P<0.001 for comparison with vehicle treated groups; #, P<0.05; ##, P<0.01; ###, P<0.001 for comparison between genotypes.

Several studies suggest that E2-mediated signaling can have an important role in the control of lipid and glucose metabolism [136,137]. Studies in both human and rodents suggest that altered levels of E2 or its receptors can lead to a metabolic syndrome-like phenotype (i.e, insulin resistance, adiposity, dyslipidemia). As mentioned in this Thesis (Introduction), the beneficial influence of E2 in relation to normalizing lipid and glucose homeostasis has been evidenced in ob/ob and high-fat diet fed mice, two models of obesity and type 2 diabetes, respectively [158,159]. Furthermore, treatment of ob/ob mice with the ERα selective agonist propyl-pyrazole-triol can improve glucose tolerance and insulin sensitivity which supports the critical role of ERα in the control of glucose homeostasis. In the absence of E2 treatment, $SOCS2^{-/-}$ mice had lower levels of TG (Fig.4B), leptin (Fig.4C), and perirenal fat (Fig.4D), in comparison with WT mice. In agreement with previously reported data [124], these findings show that $SOCS2^{-/-}$ male mice have reduced adiposity. Interestingly, E2 further reduced circulating TG (Fig.4b) and leptin (Fig.4C) in WT mice but not in $SOCS2^{-/-}$. In contrast, circulating CHO (Fig. 4A) was reduced in $SOCS2^{-/-}$ but not in WT mice.

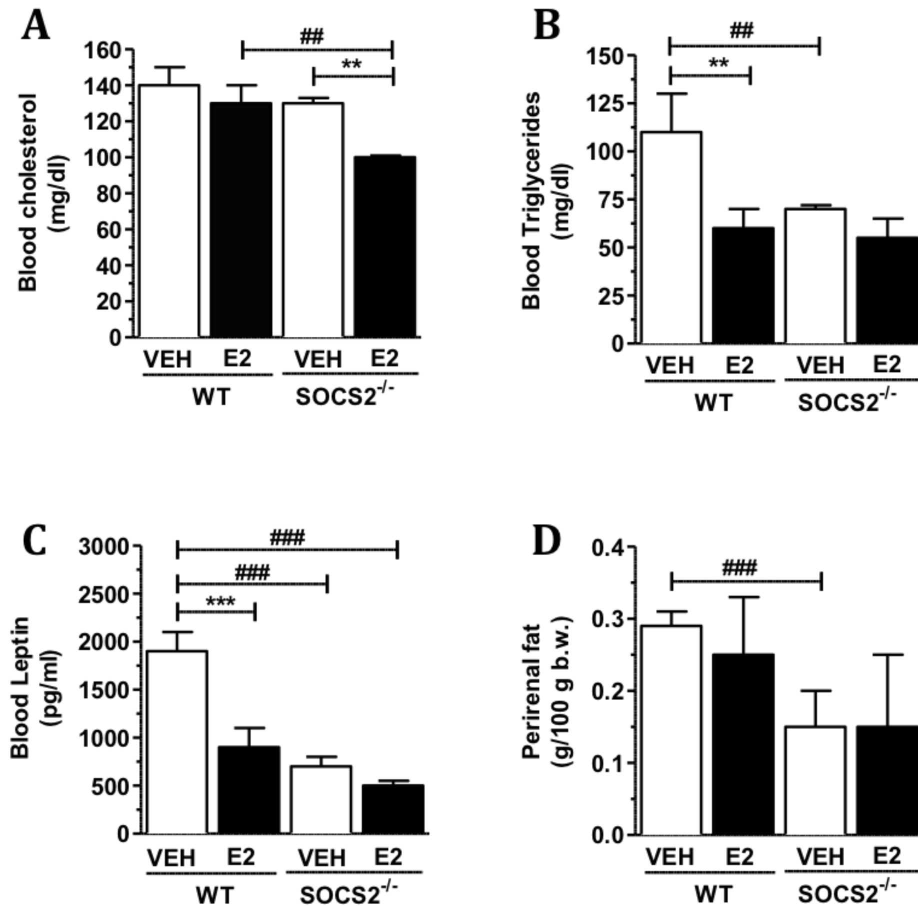

Figure 4. SOCS2 influences the effects of E2 on circulating lipids and adiposity index. Wild-type ($SOCS2^{+/+}$) and $SOCS2^{-/-}$ male mice were treated with E2 benzoate (EB) (50 μg/kg/48h) or vehicle (VEH) during 90 days as described in Material and Methods. Then, the circulating levels of cholesterol (A), triglycerides (B), leptin (C), and perirenal fat (D) were measured as described in Material and Methods. Results are expressed as mean ± SEM (n=6). *P<0.05; ** P<0.01; ***P<0.001 for comparison with vehicle treated groups; #, P<0.05; ##, P<0.01; ###, P<0.001 for comparison between genotypes.

Independently of genotype, E2 induced the hepatic mRNA levels of FAT/CD36 (Fig.5A), a receptor involved in long-chain fatty acids uptake and expressed, predominantly, in female rat liver [231]. Lipid receptors that play a critical role in lipid metabolism [232], such as LXR (Fig.5B) and PPARα (Fig.5C), were down-regulated by E2 in both genotypes. However, L-FABP (Fig.5D) and FAS (Fig.5E)

were differently regulated by E2 in WT, in comparison with SOCS2$^{-/-}$ mice, whereas SREBP1c (Fig.5F), a relevant gene in the control of lipogenesis [233], remained unchanged. Overall, in this Thesis we add novel findings suggesting that SOCS2 can influence the effects of E2 on lipid metabolism in liver.

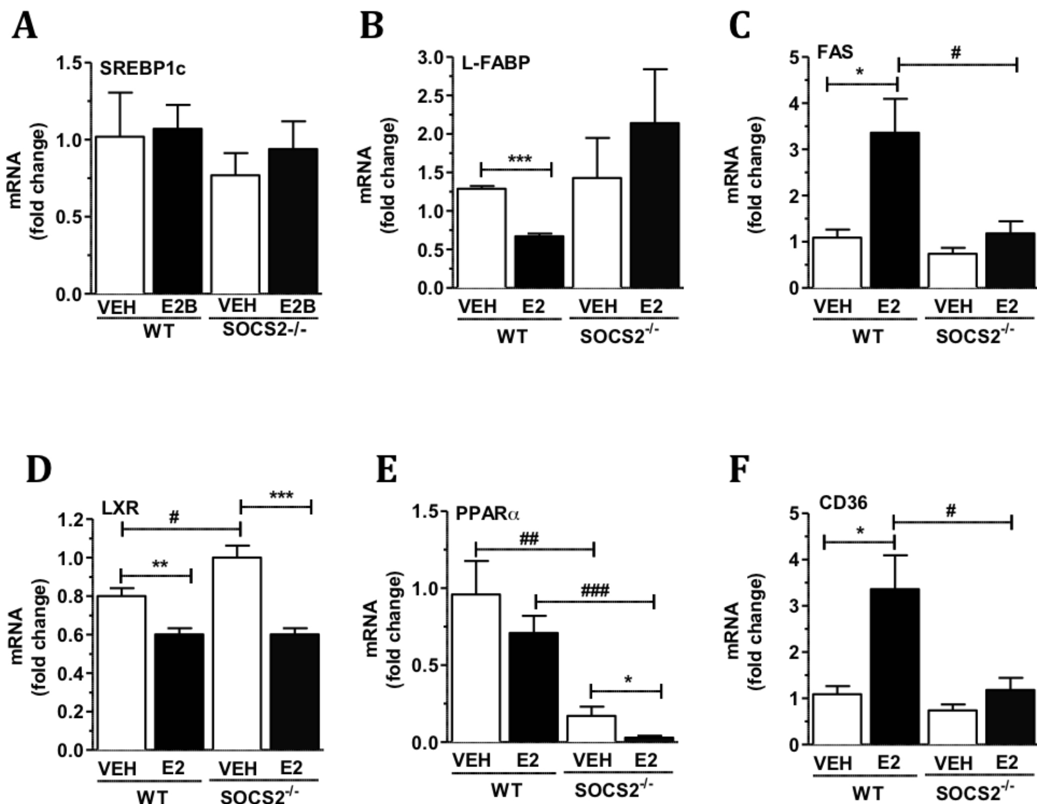

Figure 5. SOCS2 influences the effects of E2 on hepatic lipid genes. Wild-type (SOCS2$^{+/+}$) and SOCS2$^{-/-}$ male mice were treated with E2 benzoate (EB) (50µg/kg/48h) or vehicle (VEH) during 90 days as described in Material and Methods. Then, the hepatic mRNA levels of CD36 (A), LXR (B), PPARα (C), L-FABP (D), FAS (E), and SREBP1c (F) were measured. Results are expressed as mean ± SEM (n=6). *P<0.05; ** P<0.01; ***P<0.001 for comparison with vehicle treated groups; #, P<0.05; ##, P<0.01; ###, P<0.001 for comparison between genotypes.

The role of SOCS2 on the effects of E2 in glucose homeostasis was also explored in this paper. As previously described in this Thesis [124], SOCS2$^{-/-}$ mice, in comparison with WT, showed higher fasting (basal) glucose levels (Fig.6A). Interestingly, maximum blood glucose peak was detected 20 min earlier in

SOCS2$^{-/-}$ mice (Fig.6C) than in WT, and it remained higher at 30 min (Fig.6D) after glucose administration. Differences in AUC between genotypes were also detected (Fig.6E). Notably, independently of genotype, E2 did not improve glucose tolerance.

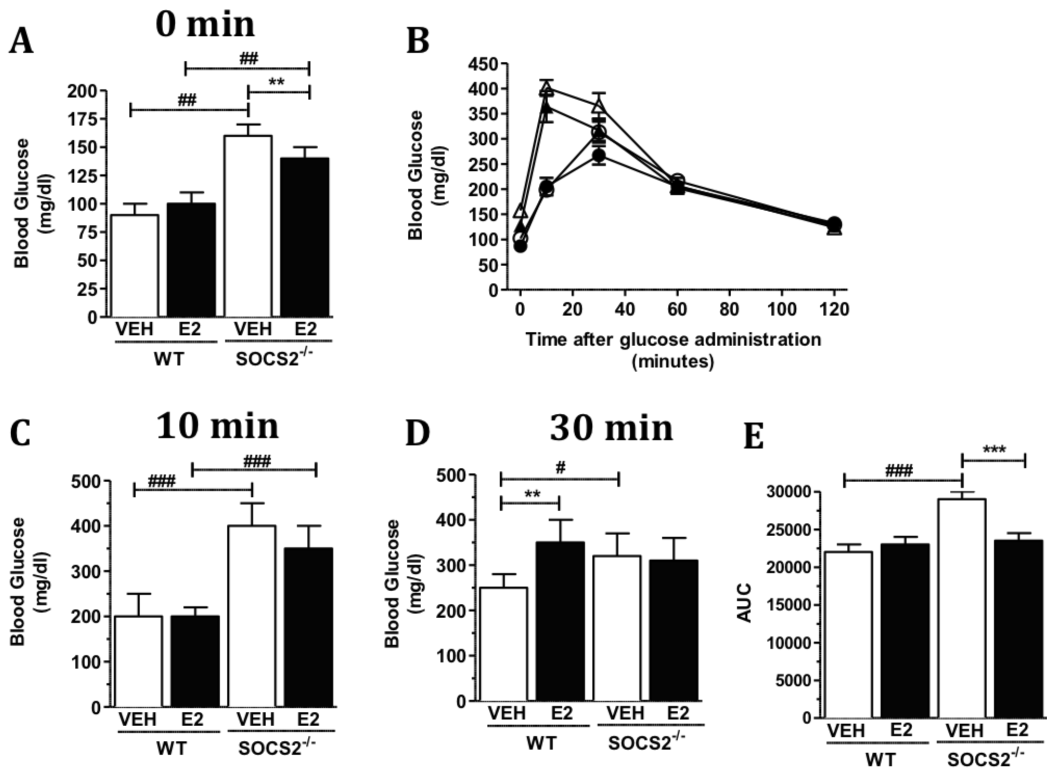

Figure 6. SOCS2 influence on E2-regulated glucose homeostasis. Wild-type (SOCS2$^{+/+}$) (circle) and SOCS2$^{-/-}$ (triangle) male mice were treated with vehicle (VEH) (white symbol) or E2 benzoate (EB) (50 µg/kg/48h) (black symbol) during 90 days as described in Material and Methods. Basal glucose (A), ipGTT (B), blood glucose at 10 min (C), blood glucose at 30 min (D) and AUC (E) were analyzed. Two-way ANOVA was performed to test for significant differences among groups and genotypes. Student´s t test was used to test for the significant differences between two groups. Data are shown as mean ± SEM. *P<0.05; ** P<0.01; ***P<0.001 for comparison with vehicle treated groups; #, P<0.05; ##, P<0.01; ###, P<0.001 for comparison between genotypes.

Next, we studied the role of SOCS2 on the effects of E2 in insulin sensitivity. The ipITT showed that both genotypes had similar sensitivity to insulin (Fig.7A).

Indeed, the absence of differences in AUC (Fig.7B) suggest that both genotypes responds similarly to insulin and E2. However, SOCS2$^{-/-}$ mice maintained higher blood glucose (2-fold) at 120 min (Fig.7C) which suggest that they are more resistant to exogenous insulin-induced hypoglycaemia than WT.

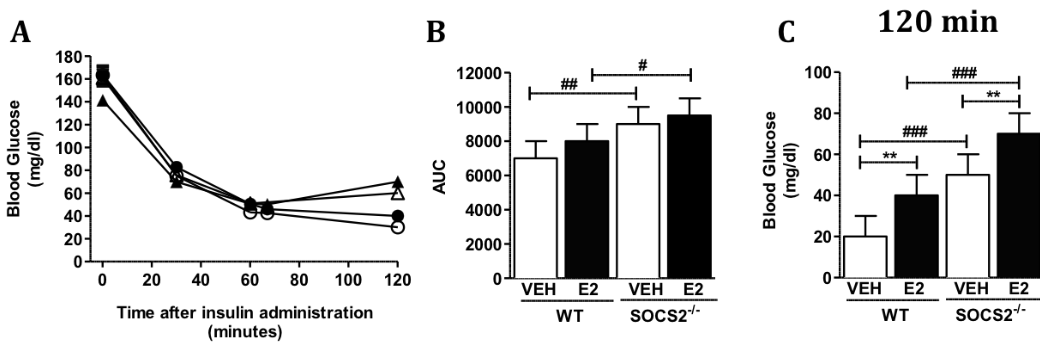

Figure 7. SOCS2 influence on E2-regulated insulin sensitivity. Wild-type (SOCS2$^{+/+}$) and SOCS2$^{-/-}$ male mice were treated with E2 benzoate (EB) (50 µg/kg/48h) or vehicle (VEH) during 90 days as described in Material and Methods. ipITT (A), AUC (B) and blood glucose at 120 min (C) were analyzed. Two-way ANOVA was performed to test for significant differences among groups and genotypes. Student´s t test was used to test for the significant differences between two groups. Data are shown as mean ± SEM. *P<0.05; ***P<0.001 for comparison with vehicle treated groups; #, P<0.05; ##, P<0.01; ###, P<0.001 for comparison between genotypes.

Finally, we performed a genome wide gene expression analysis to better understand the influence of SOCS2 on E2-regulated liver physiology. This paper identified 4631 genes (3356 up-regulated; 1275 down-regulated) that were differentially regulated in WT mice after 2 days of E2 treatment . However, E2 regulated lower than 50% (2330 total; 1425 up-regulated; 905 down-regulated) in SOCS2-/- mice than in WT. Next, we identified the active biological processes from expression profiles by GO enrichment analysis (GSEA) [202] and system biological network [203] analysis (Fig. 8-9). GSEA showed that the gen sets regulated after 2 days of E2 treatment were similar in SOCS2$^{-/-}$ and wild-type littermates. However, some GO functions were significantly different. Particularly, E2 decreased hepatic immune response in the absence of SOCS2 whereas it was

increased in wild-type mice. However, cell cycle and proliferation were stimulated by E2 in both genotypes. E2 increased expression of several GPCR that belong to the olfactory receptors family in SOCS2$^{-/-}$ mice.

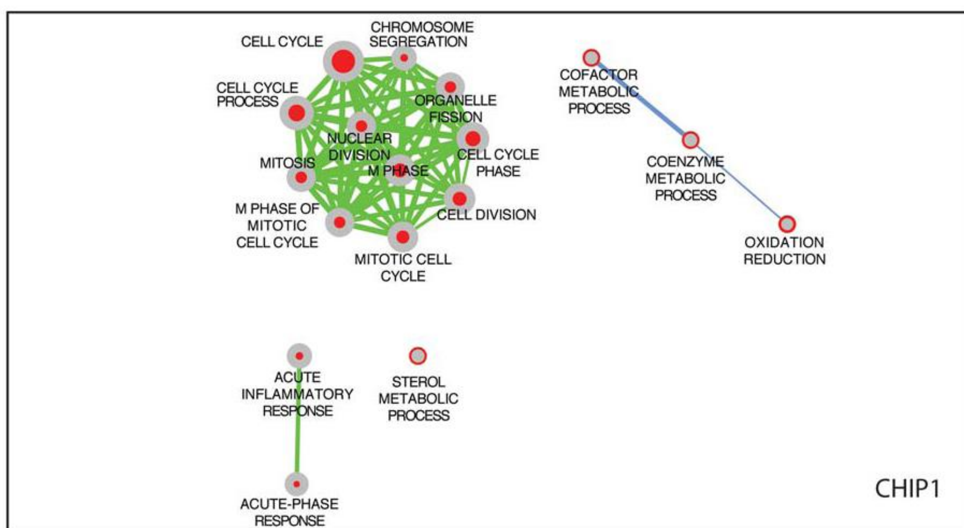

Figure 8. System biological network analyses of the effects of 2 days of E2 treatment on liver transcriptome in wild-type mice. The genes differentially-expressed in the livers were identified by DNA microarrays as described under Material and Methods. Then, functional and system biological network analysis were performed on the basis of the GO enrichment of differentially-expressed genes in liver using DAVID, and the results depicted using Cytoscape. Node (inner circle) size corresponds to the number of genes up-regulated by E2; node border (outer circle) size corresponds to the number of genes down-regulated by E2; color of the node and border corresponds to the significance of the gene set for up or down regulated genes, respectively (dark red = significantly enriched, light red = enriched no significantly; grey = absent); edge size corresponds to the number of genes that overlap between the two connected gene sets. Green edges correspond to shared up-regulated genes and blue edges correspond to shared down-regulated genes.

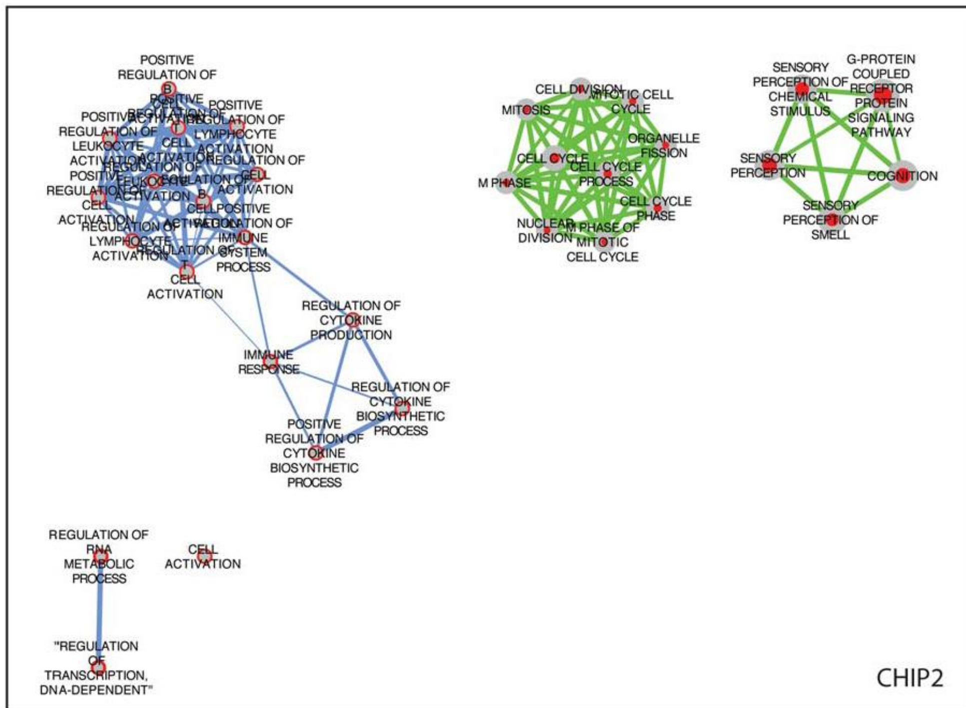

Figure 9. System biological network analyses of the effects of 2 days of E2 treatment on liver transcriptome in SOCS2$^{-/-}$ mice. The genes differentially-expressed in the livers were identified by DNA microarrays as described under Material and Methods. Then, functional and system biological network analysis were performed on the basis of the GO enrichment of differentially-expressed genes in liver using DAVID, and the results depicted using Cytoscape. Node (inner circle) size corresponds to the number of genes up-regulated by E2; node border (outer circle) size corresponds to the number of genes down-regulated by E2; color of the node and border corresponds to the significance of the gene set for up or down regulated genes, respectively (dark red = significantly enriched, light red = enriched no significantly; grey = absent); edge size corresponds to the number of genes that overlap between the two connected gene sets. Green edges correspond to shared up-regulated genes and blue edges correspond to shared down-regulated genes.

Notably, 625 and 678 genes were induced and repressed by E2, respectively, in SOCS2$^{-/-}$, but not in wild-type littermates (see Table 1-2 for small representation). Overall, these results reveal an extensive re-programming of liver physiology by short-term E2 treatment and the influence of SOCS2$^{-/-}$ on E2's effects *in vivo*.

ID Probe	Unigene/Refseq	Symbol	Gene description	R		q(%)
				mean ± SD		
A_55_P1995537	NM_010824	Mpo	Myeloperoxidase, mitochondrial protein	3,41	0,85	0,00
A_52_P15388	NM_008522	Ltf	Lactotransferrin	2,13	0,61	0,67
A_55_P1983921	NM_021352	Crybb3	Crystallin, beta B3, transcript variant 1	1,95	0,93	1,49
A_51_P167292	NM_009892	Chi3l3	Chitinase 3-like 3	1,93	0,55	0,67
A_55_P2163098	NM_134066	Akr1c18	Aldo-keto reductase family 1, member C18	1,90	0,36	0,00
A_51_P461067	ENSMUST00000103420	G1m	Immunoglobulin heavy constant gamma 1	1,85	0,64	0,93
A_55_P1953387	NM_001272097	Fabp5	Fatty acid binding protein 5, epidermal	1,74	1,31	4,03
A_51_P199168	NM_007702	Cidea	Cell death-inducing DNA fragmentation factor, alpha subunit-like effector A	1,73	0,72	0,99
A_52_P213889	NM_172476	Tmc7	Transmembrane channel-like gene family 7	1,66	1,08	3,07
A_51_P488196	NM_028472	Bmper	BMP-binding endothelial regulator	1,65	0,28	0,00
A_55_P2024155	NM_001033324	Zbtb16	Zinc finger and BTB domain containing 16	1,65	1,16	3,38
A_51_P520650	NM_177639	Dlgap1	Discs, large (Drosophila) homolog-associated protein 1	1,62	1,26	4,03
A_55_P2002975	NM_205795	Mrgprb4	MAS-related GPR, member B4	1,55	0,96	2,80
A_55_P2054913	NM_001011863	Olfr406	Olfactory receptor 406 (Olfr406)	1,54	0,56	0,99
A_55_P2149921	TC1780716	Q80XJ7	Aldo-keto reductase family 1, member A4	1,52	0,91	2,80
A_55_P2185900	NM_032002	Nrg4	Neuregulin 4	1,49	0,87	2,80
A_51_P295896	NM_028934	4930452B06Rik	RIKEN cDNA 4930452B06 gene	1,47	0,56	0,99
A_55_P2290388	NM_001043354	Rorb	RAR-related orphan receptor beta (Rorb),	1,46	0,53	0,99
A_55_P2001553	NM_020043	Igdcc4	Immunoglobulin superfamily, DCC subclass, member 4	1,45	0,16	0,00

Table 1. Representative list of hepatic genes that were upregulated in SOCS2-/- mice instead of WT mice after 2 days of E2 administration. Two days of E2 administration to OXSOCS2-/- and OXWT mice was performed as described in Material and Methods. Then, differently expressed genes in the liver trancriptome were identified by DNA microarrays. The analysis is based on the SAM statistical technique and differentially-expressed genes were discovered using a FDR less than 5% and a mean ratio of log2 > |0.58|. The table shows probe ID, Unigene/Refseq, gene symbol, gene description, R (log2 E2-treated OXSOCS2-/- / vehicle-treated SOCS2-/-), and q (%).

ID Probe	Unigene/Refseq	Symbol	Gene description	R		q(%)
				mean ± SD		
A_55_P2408588	NM_007489	Arntl	Aryl hydrocarbon receptor nuclear translocator-like	-2,29	1,22	1,93
A_52_P480044	XR_105914	BC023105	PREDICTED: cDNA sequence BC023105	-2,21	0,72	0,94
A_55_P2213968	NR_045840	4933416M07Rik	RIKEN cDNA 4933416M07 gene, non-coding RNA	-2,14	0,88	1,17
A_66_P120728	NM_001011791	Olfr193	Olfactory receptor 193	-2,10	0,90	1,49
A_51_P107315	NM_145423	Slc5a8	Solute carrier family 5 (iodide transporter), member 8	-2,02	0,52	0,94
A_65_P11137	NM_019541	Cts8	Cathepsin 8 (Cts8)	-1,96	0,22	0,00
A_55_P2056714	NM_001042612	Nlrp9c	NLR family, pyrin domain containing 9C	-1,95	0,90	1,57
A_55_P2151591	ENSMUST00000032909	Pde3b	Phosphodiesterase 3B, cGMP-inhibited	-1,94	0,73	1,09
A_51_P210031	NM_001252679	Smr2	Submaxillary gland androgen regulated protein 2 (Smr2)	-1,90	0,94	1,93
A_55_P2121042	NM_001082531	Pla2g2a	Phospholipase A2, group IIA (platelets, synovial fluid)	-1,85	0,53	0,94
A_52_P122649	NM_175647	Dmrta1	Doublesex and mab-3 related transcription factor like family A1	-1,81	0,67	1,09
A_52_P669922	NM_032541	Hamp	Hepcidin antimicrobial peptide	-1,80	0,28	0,00
A_55_P2095859	NR_037604	Rdh18-ps	Retinol dehydrogenase 18, pseudogene, non-coding RNA	-1,78	0,49	0,94
A_52_P186751	NM_198677	BC061237	cDNA sequence	-1,77	0,58	1,09
A_51_P267969	NM_011259	Reg3a	Regenerating islet-derived 3 alpha	-1,77	0,60	1,09
A_55_P2073024	NM_001034859	Gm4841	Predicted gene 4841	-1,76	0,46	0,94
A_51_P322473	NM_172417	2310042D19Rik	RIKEN cDNA 2310042D19 gene	-1,74	0,07	0,00
A_55_P2051082	XR_168616	Gm11634	PREDICTED: predicted gene 11634	-1,72	0,25	0,00

Table 2. Representative list of hepatic genes that were downregulated in SOCS2-/- mice instead of WT mice after 2 days of E2 administration. Two days of E2 administration to OXSOCS2-/- and OXWT mice was performed as described in Material and Methods. Then, differently expressed genes in the liver trancriptome were identified by DNA microarrays. The analysis is based on the SAM statistical technique and differentially-expressed genes were discovered using a FDR less than 5% and a mean ratio of log2 > |0.58|. The table shows probe ID, Unigene/Refseq, gene symbol, gene description, R (log2 E2-treated OXSOCS2-/- / vehicle-treated SOCS2-/-), and q(%).

4.3. Relationship between SOCS2, hepatic steatosis and insulin resistance in high fat diet fed mice (Paper II; FASEB J 2012, 26:3282- 3291). In this study, we analysed the metabolic response of the SOCS2$^{-/-}$ mice to a hypercaloric, fat-rich diet. The SOCS2$^{-/-}$ mice exhibited enhanced hepatic TG secretion and were protected from HFD-induced liver steatosis. However, they displayed severe systemic insulin resistance associated with hyperinsulinemia and worsened insulin sensitivity in the liver compared with the WT mice on a similar diet. The HFD-fed SOCS2$^{-/-}$ mice also exhibited enhanced expression of inflammatory cytokines in liver demonstrating a novel role of SOCS2 as a negative regulator of macrophage activation under conditions of high-fat dietary stress. Importantly, the SOCS2$^{-/-}$ mice phenotype is clearly different from the liver-specific SOCS3 and SOCS1 knockout mice phenotypes [234,235], which show enhanced liver steatosis, highlighting the functional differences between members of the SOCS family in the regulation of hepatic metabolism.

In previous studies, we showed that SOCS2 is a negative regulator of hepatic GH receptor signaling [116]. The reduced steatosis observed in the HFD-fed SOCS2$^{-/-}$ mice compared with the HFD-fed WT mice is in agreement with the well-known actions of GH in promoting hepatic lipid mobilization [236] and strongly suggests that enhanced GH signaling in the liver is in part responsible for these effects. Indeed, we found mRNA levels of the known GH-regulated genes *ApoB, FABP1* and *DGAT2*, which are involved in TG assembly and secretion to be increased in the SOCS2$^{-/-}$ mice. Detailed analysis of mice with a hepatic specific deletion of JAK2 has unveiled another mechanism whereby GH controls liver fat content [170]. These mice, denoted JAK2L, have elevated levels of circulation GH, which in turns leads to increased adipose tissue lipolysis. This increases FFA supply to the liver leading to steatosis [170]. The SOCS2$^{-/-}$ mice have reduced levels of circulating GH and in opposition to JAK2L exhibit increased fat mass, suggesting that reduced adipose tissue lipolysis in SOCS2$^{-/-}$ mice may also contribute to

reduced hepatic TG accumulation. Noticeably, we did not detect significant changes in circulating FFA or hepatic expression of CD36 in SOCS2$^{-/-}$ mice compared to what has been reported in the JAK2L mice. Further experiments are needed to analyse the influence of GH and adipose tissue lipolysis in the SOCS2$^{-/-}$ mice liver.

Surprisingly, the SOCS2$^{-/-}$ mice showed an exacerbated response to HFD feeding leading to worsened insulin sensitivity, whereas the SOCS2$^{-/-}$ and WT mice showed few differences in insulin signaling when maintained on a normal control diet. This suggests that hyperactivity of the hepatic GH receptor signaling alone, as it is observed in SOCS2$^{-/-}$ mice, is unlikely to account for the diet-dependent deterioration in glucose control observed in this study. A more likely explanation is that the anti-insulinic actions of GH are exacerbated by diet related mechanisms under SOCS2 control. We provide evidence for the existence of three of such mechanisms: hyperinsulinemia, peripheral adiposity and excessive production of inflammatory cytokines. Toll-like receptor driven macrophage activation in liver and adipose tissues by high-fat feeding leads to the production of inflammatory cytokines, a process that is required for diet-induced hepatic insulin resistance [237]. The loss of SOCS2 leads to an altered response to HFD in mice, resulting in increased expression of inflammatory cytokines and enhanced NF□B activation. This seems to be mediated by direct actions of SOCS2 on macrophage activation, as demonstrated in *ex vivo* experiments showing that BMDM from the SOCS2$^{-/-}$ mice exhibit increased phagocytic activity *in vitro* and are hyper-responsive to LPS stimulation leading to expression of *IL-6, iNOS, IL-1β* and *INF-γ* .A previous study described an anti-inflammatory role of SOCS2, mediating the activity of aspirin-triggered lipoxins [133]. Our data suggest that the anti-inflammatory actions of SOCS2 may be mediated to some extent through inhibition of the LPS response in macrophages leading to NFκB activation. Transgenic mice with enhanced NFκB activity in hepatocytes exhibit insulin resistance driven by

inflammation in the absence of TG accumulation [238], which resembles the HFD fed SOCS2-/- mice.

Measurements of hepatic cytokine production provide a strong indication that liver non-parenchimal cells participate in the hepatic response to HFD in SOCS2$^{-/-}$ mice. However, whether this altered reactivity is related to GH or other signals remains unknown. In relation to GH, we previously showed that GH treatment of SOCS2$^{-/-}$ mice results in enhanced hepatic expression of cytokine-regulated genes [116,118] and GH is known to exacerbate the inflammatory response in LPS-treated rodents and worsen the conditions of critically ill patients [88]. Further work is needed to clarify the role of GH signaling in different tissues and cell types in the HF-fed SOCS2$^{-/-}$ mice.

Studies in both human and rodents suggest that E2 protects from HFD-induced diabetes, steatosis, and adiposity [226,239,240,241]. Now, data from our lab also suggest a gender dimorphism in the HFD-fed SOCS2$^{-/-}$ model (manuscript in preparation). Interestingly, when SOCS2$^{-/-}$ mice were fed with standard diet (CD), there were no differences in glucose tolerance (ipGTT) between females and males. However, HFD diet caused more marked glucose intolerance in female than in male SOCS2$^{-/-}$ mice (Fig. 1).

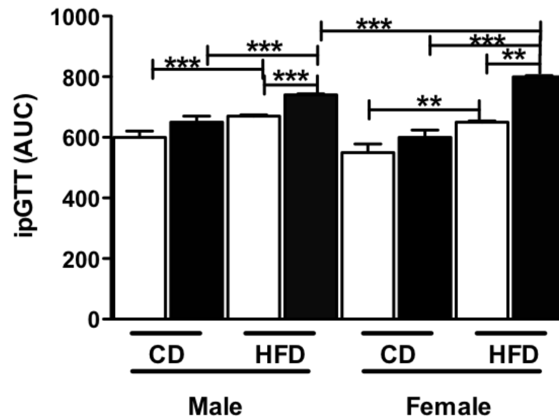

Figure 1. Dimorphism glucose tolerance in HFD-fed SOCS2$^{-/-}$ mice. WT (white columns) and SOCS2$^{-/-}$ (black columns) mice were fed with chow diet (CD) or high-fat diet (HFD) **as** described in Material and Methods. Two weeks before mice were sacrificed, the glucose tolerance tests (ipGTT) were performed in males and females. Results are expressed as mean ± SEM (n=6). *P<0.05, **P<0.001, ***P<0.0001

The test for insulin sensitivity (ipITT) showed that, under CD treatment, both SOCS2$^{-/-}$ and wild type littermates, independently of gender, behave similarly. As described above, both HFD-fed SOCS2$^{-/-}$ groups (females and males) showed lower hypoglycemic response to exogenous insulin than wild type mice. Surprisingly, the HFD-fed SOCS2$^{-/-}$ female group showed better insulin sensitivity compared to HFD-fed SOCS2$^{-/-}$ males (Fig. 2).

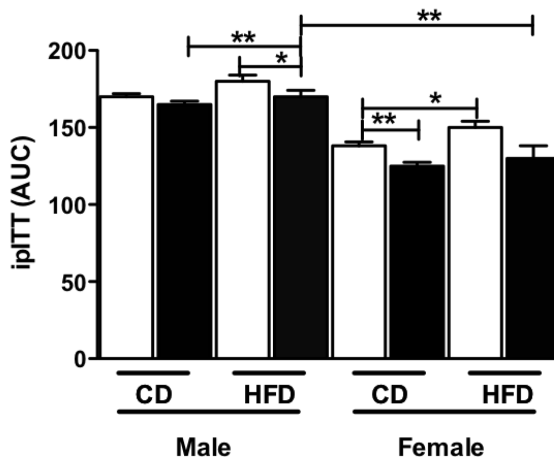

Figure 2. Dimorphism insulin sensitivity in HFD-fed SOCS2$^{-/-}$ mice. WT (white columns) and SOCS2$^{-/-}$ (black columns) mice were fed with chow diet (CD) or high-fat diet (HFD) as described in Material and Methods. A week before mice were sacrificed, the insulin tolerance tests (ipITT) were performed in males and females. AUC = area under curve of blood glucose levels. Results are expressed as mean ± SEM (n=6). *P<0.05, **P<0.001, ***P<0.0001

Furthermore, HFD diet caused less grade of steatosis in female than in male SOCS2$^{-/-}$ mice with a lower level of hepatic TG content. Finally, HFD-fed SOCS2$^{-/-}$ females, in comparison with HFD-fed SOCS2-/- male, showed 2,5-fold higher levels of circulating leptin (Fig. 3 A) and up to 9-fold lower plasma levels of inflammatory markers (i.e., IL6, INFγ, RANTES, TNFα, and IL1-β) (Fig. 3 B-F). Our data suggest that E2 protects from HFD-induced steatosis and insulin resistance in the absence of SOCS2.

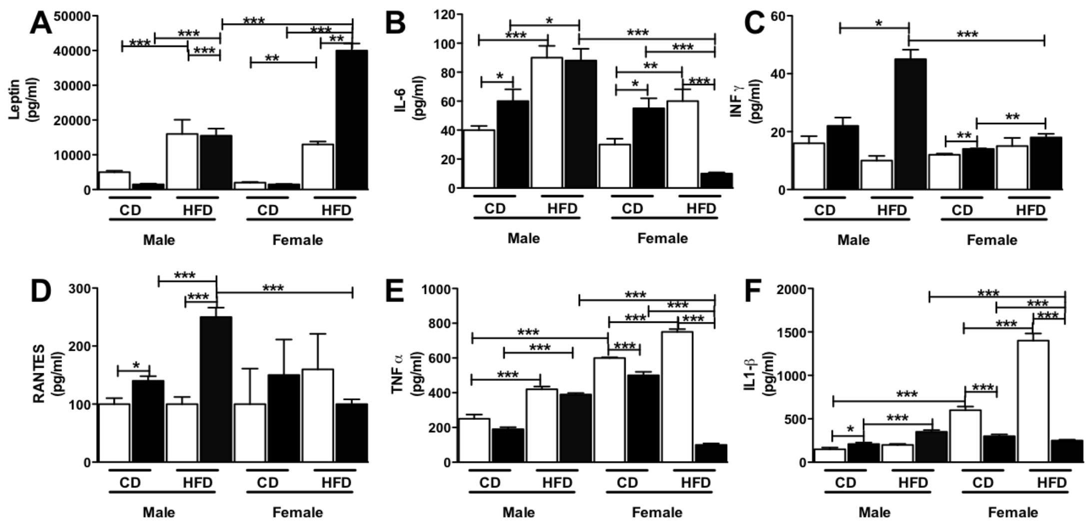

Figure 3. Dimorphism in inflammatory response in HFD-fed SOCS2$^{-/-}$ mice. WT (white columns) and SOCS2$^{-/-}$ (black columns) mice were fed with chow diet (CD) or high-fat diet (HFD) as described in Material and Methods. Then, the circulating levels of leptin (A), IL-6 (B), INFγ (C), RANTES (D),TNFα (E), and IL1β (F) were analysed in males and females. Results are expressed as mean ± SEM (n=6). *P<0.05, **P<0.001, ***P<0.0001

At physiological levels, the SOCS2$^{-/-}$ mice constitute a novel model system for the study of the metabolic syndrome with unique features that are relevant to the human disease. SOCS2$^{-/-}$ display no clear metabolic phenotype, these mice do not show obvious alteration in insulin sensitivity [118], in contrast GH transgenic mice develop marked insulin resistance [59,77,242]. HFD-fed SOCS2$^{-/-}$ mice displayed exacerbated insulin resistance compared to HFD fed WT. Fasting plasma insulin levels were increased in SOCS2$^{-/-}$ mice on HFD but we did not observe any differences in pancreatic levels of insulin or islet morphology, suggesting that the impaired insulin sensitivity is not cause by altered β-cell function. Moreover HFD induced a marked upregulation of the hepatic gene expression of proinflammatory cytokines in the SOCS2$^{-/-}$ mice. We conclude that SOCS2 has a protective role in the development of insulin resistance, probably by suppressing the release of proinflammatory cytokines.

In conclusion, our results demonstrated SOCS2 plays an important role in regulating the response to high-fat dietary stress. The SOCS2$^{-/-}$ mice provide a novel model to understand the complex relationship between inflammation, GH actions and nutrition in the control of hepatic glucose and lipid homeostasis. Future use of this model may help to outline the contribution of different mechanisms in the development of fat-induced hepatic insulin resistance and components related to lipid overload in comparison with components related to inflammatory stress.

4.4. Suppressor of Cytokine Signaling (SOCS)-2 gene deletion influences diabetes development induced by multiple low-dose streptozotocin (Paper IV; manuscript in preparation). It has been reported that experimental elimination of different SOCS has relevant physiological functions. For example, some studies have shown that SOCS are potent regulators of insulin signaling and beta cell functioning. Therefore, elimination of SOCS1 increases interferon response and potentiates islet cell death [177] while SOCS3 is a potent protector against type 1 Diabetes Mellitus (DM) through suppressing IL-B and TNF in pancreatic beta cells [178,179]. SOCS3$^{-/-}$ beta cells also show resistance to apoptotic cell death [180]. On the other hand, in the context of diabetes, SOCS2 has not been extensively studied, because it has mainly been implicated in GH and prolactin (PRL) signaling. However, it has been reported that SOCS2 is a potent regulator of pro-insulin processing and insulin secretion in β-cells [243]. Moreover, constitutive production of SOCS2 in β-cells leads to hyperglycaemia and glucose intolerance [175]. We have previously identified that SOCS2 negatively regulates GH signaling and SOCS2$^{-/-}$ mice grow larger than controls without elevation of GH [118,119]. The absence of SOCS2 makes mice highly sensitive to GH because SOCS2 deficiency leads to a reduced GH receptors (GHR) breakdown and, subsequently, more GHR. The elevated sensitivity of the SOCS2$^{-/-}$ mice to GH, and probably also to prolactin (PRL), is the rationale to investigate, in this study

IV, how SOCS2 ablation may influence the development of diabetes in a mice model of autoimmune diabetes and β-cell destruction (multiple low-dose of streptozotocin (MLDSTZ)) [194], fed with standard diet.

To analyze SOCS2 glucose homeostatsis and metabolism, the MLDSTZ was used as a model of autoimmune diabetes and B-cell destruction *in vivo* [194]. All diabetes development parameters were monitored, and both MLDSTZ-treated $SOCS2^{-/-}$ and WT mice developed sever diabetes after day 9 from injecting first dose of STZ, whereas vehicle-treated mice remained normoglycemic (Fig. 1). However, Figure 1A, shows that $SOCS2^{-/-}$ mice were more resistant to develop diabetes.

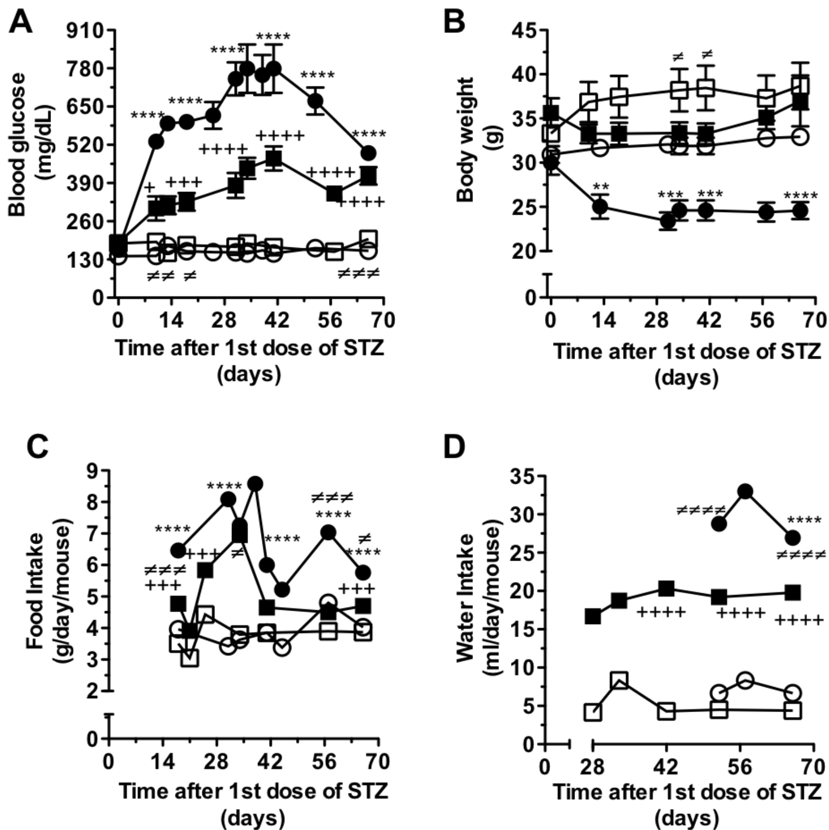

Figure 1. MLDSTZ-induced diabetes in mice. WT (circle) and $SOCS2^{-/-}$ (square) adult male mice received 5 consecutive daily ip injections of vehicle (open symbols) or STZ (50 mg/kg b.w.) (black symbols) as described in Material and Methods. Then, STZ was stopped and blood glucose (A), body weight (B), food intake (C), and water intake (D) were recorded. Results are expressed as mean ± SEM from six individual animals in each group. WT-Veh vs WT-STZ: *P<0.05, **P<0.001, ***P<0.0001; SOCS2-/- Veh vs SOCS2-/- STZ: +P<0.05, ++P<0.001, +++P<0.0001; WT-Veh vs SOCS2-/- Veh: ≠P<0.05, ≠≠P<0.001, ≠≠≠P<0.0001.

In the glucose tolerance (ipGTT), the area under the glucose curve (AUC blood glucose) was lower in MLDSTZ-treated $SOCS2^{-/-}$ mice than in MLDSTZ- WT mice (Fig. 2 A-B).

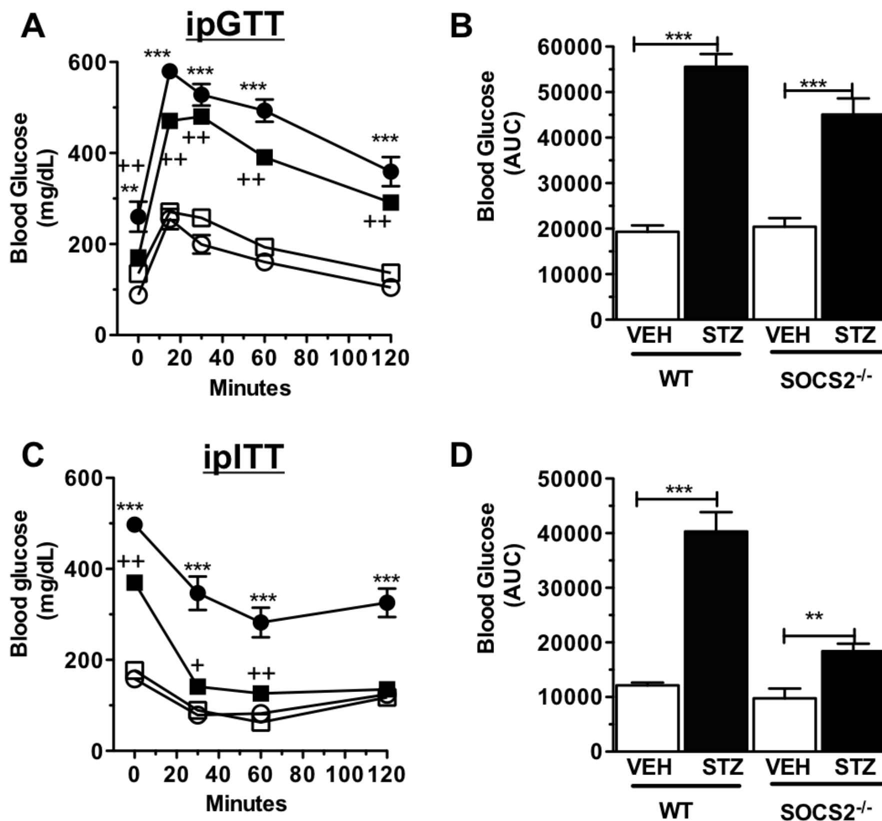

Figure 2. MLDSTZ-induced diabetes in wild type and SOCS2$^{-/-}$ mice. Adult male mice received 5 consecutive daily ip injections of vehicle (open symbols) or STZ (50 mg/kg b.w.) (black symbols) as described in Material and Methods. The glucose tolerance [ipGTT (A) and AUC (B)] and insulin sensitivity [ipITT (C) and AUC (D)] were performed on day 53-58 after the first dosis of STZ. Results are expressed as mean ± SEM from six individual animals in each group. WT-Veh vs WT-STZ: *P<0.05, **P<0.001, ***P<0.0001; SOCS2-/-Veh vs SOCS2-/-STZ: +P<0.05, ++P<0.001, +++P<0.0001; WT-Veh vs SOCS2-/-Veh: ≠P<0.05, ≠≠P<0.001, ≠≠≠P<0.0001.

Since the overall glucose tolerance has been changed in response to the deletion of SOCS2, next we performed a test to assess the insulin sensitivity (ipITT). These results suggest higher degree of insulin sensitivity with SOCS2 ablation (Fig. 2 C y D). Moreover, we further observed higher fasting plasma insulin (Fig.3B) and HOMA-IR index in the MLDSTZ treated SOCS2$^{-/-}$ mice group (Fig. 3A). The higher HOMA-IR observed in MLDSTZ- treated SOCS2$^{-/-}$ mice was likely due to the increased insulin levels observed in these animals compared to WT. Taken

together, these results suggest that SOCS2 ablation seems to compensate β-cell destruction induced by MLDSTZ.

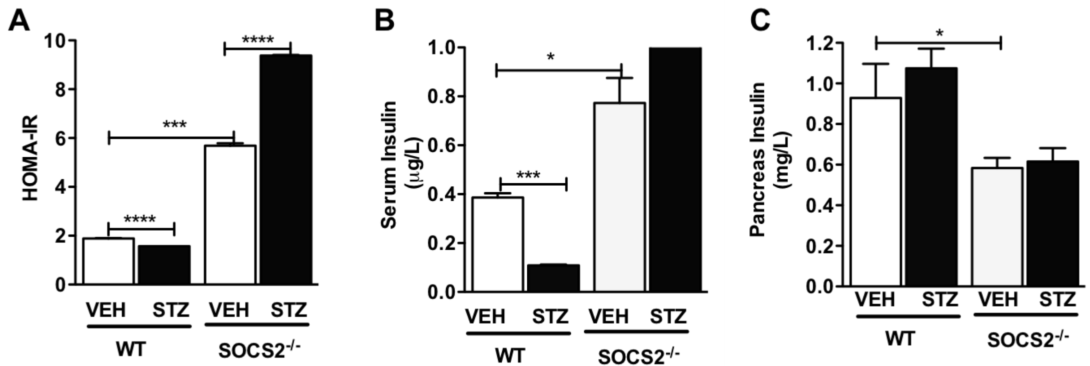

Figure 3. MLDSTZ-induced diabetes wild type and SOCS2$^{-/-}$ mice. Adult male mice received 5 consecutive daily ip injections of vehicle (open bars) or STZ (50 mg/kg b.w.) (black bars) as described in Material and Methods. The HOMA-IR index (A) and serum insulin (B) were measured after overnight food withdrawal on day 58 after first dose of STZ. Finally, mice were sacrificed and the insulin content per pancreatic tissue weight were determined (C). Results are expressed as mean ± SEM from six individual animals in each group. *P<0.05, **P<0.001, ***P<0.0001.

An increase in β-cell mass is the case during normal pregnancies [244] and this phenomena is likely caused by a stimulation of GH/PRL receptors [245]. Some studies have shown that SOCS2$^{-/-}$ mice have significant increase in pancreatic tissue weight compared to WT mice. A previous study has reported that both WT and SOCS2$^{-/-}$ have normal pancreatic islet architecture[246], similar insulin content and HOMA-B index of the islet function [124], but SOCS2$^{-/-}$ mice have higher β-cell mass compared to WT. In order to study the potential influence of SOCS2 ablation on beta cell mass and pancreatic islets size, we performed insulin immunostaining assays in non-treated (control) SOCS2$^{-/-}$ and WT pancreas. Our results showed that SOCS2$^{-/-}$ pancreas have higher β-cell mass and bigger islets size than the control WT pancreas (Fig. 4). This result seems to explain the augmented serum insulin levels observed in SOCS2$^{-/-}$ compared to WT (Fig. 3B).

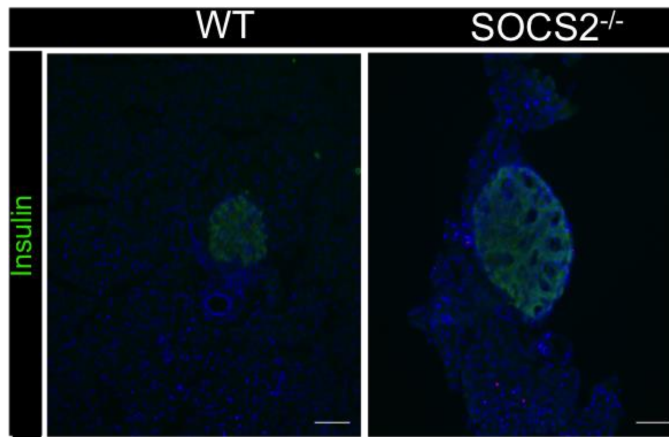

Figure 4. Immunohistochemistry analysis of insulin in pancreas from wild-type (WT) and SOCS2-deleted (SOCS2$^{-/-}$) mice.

Constitutive production of SOCS2 in β-cell leads to hyperglycaemia and glucose intolerance [175]. As it is mentioned above, SOCS2$^{-/-}$ mice are highly sensitive to GH (and probably also to PRL) because SOCS2 deficiency leads to higher GHR content (and probably also to higher PRLR). In order to confirm that SOCS2$^{-/-}$ animals are more GH/PRL sensitive than WT [59], we performed GHR and PRLR immunostaining assays in pancreatic sections obtained from both experimental groups. Our results demonstrated that GHR and PRLR protein content are augmented in SOCS2$^{-/-}$ pancreas compared to WT (Fig. 5). Higher pancreatic GHR content in absence of SOCS2 leads to an enhanced GH-induced JAK2/STAT5b signaling. Previous reports have shown that pancreatic β-cell growth, survival and insulin production are stimulated by GH through GHR/JAK2/STAT5b activation [174,247,248,249,250]. Thus, our findings of augmented pancreatic GHR/PRLR levels in SOCS2$^{-/-}$ may explain the higher β-cell mass and insulin production observed in SOCS2$^{-/-}$ pancreas compared to WT.

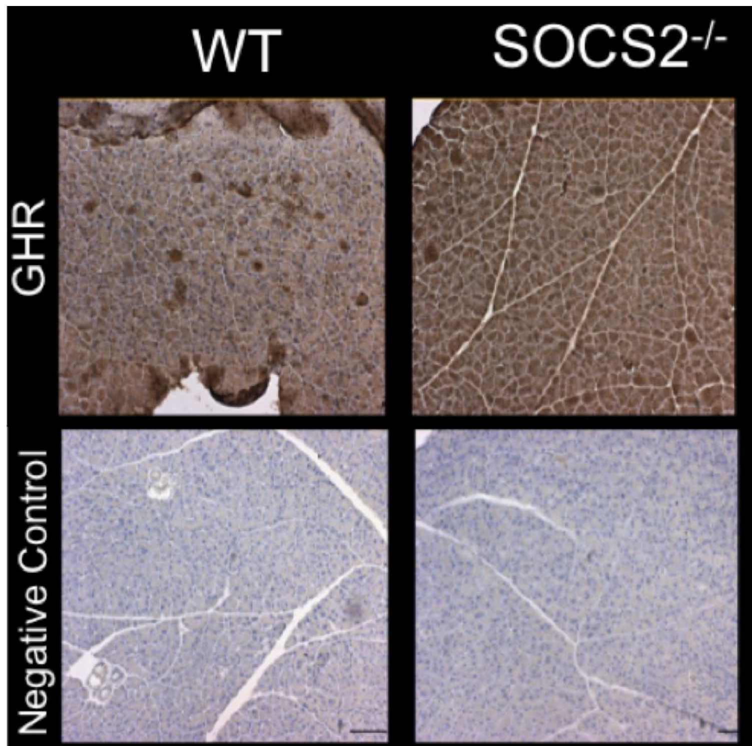

Figure 5. Immunohistochemistry analysis of GHR in pancreas from wild-type (WT) and SOCS2-deleted (SOCS2$^{-/-}$) mice.

In summary, this study identified SOCS2 as an important regulator of insulin homeostasis *in vivo* and suggests that inhibition of SOCS2 may be used as therapeutic target to ameliorate diabetes development [251].

5. CONCLUSSIONS AND GENERAL DISCUSSION

Our studies add novel data that highlight the impact of subcutaneous E2 administration on liver physiology and its interplay with GH. These results highlight the role of E2 as a critical regulator of liver metabolism in mammals and add further weight to the hypothesis that E2 acts as an important regulator of GH actions in the liver. The E2-GH interplay in the liver is relevant because of the physiological roles that these hormones have in mammals and the widespread use of estrogen and estrogen-related compounds in human. Our findings show that E2 induces a female pattern in adult hypothyroid-orchidectomized (TXOX) rat liver. First, we observed that E2 administration to TXOX rats increased pituitary GH synthesis and secretion, which simulate a “female pattern”, and leads to up-regulation of female genes. Second, we found that E2 on GH actions in TXOX rat liver can also be attributed to the E2 interplay with GH-JAK2-STAT5 signaling pathway through induction of negative regulators of GH-JAK2-STAT5 signaling pathway such as SOCS2, CIS and FGF21 (Paper I). Ours results clearly show that E2, directly or indirectly, can impact the effects of GH on liver physiology.

Interestingly, the effects of E2 produced a disturbance in the expression of hepatic genes related to body growth and lipid metabolism. This effects result in a reduced body length in WT group treated with E2, whereas these effects were not significant in SOCS2$^{-/-}$ mice. Moreover, E2 has the ability to control energy in balance, food intake and body fat distribution. Reduced E2 levels are associated with changes in body weight and fat distribution in humans, which parallel the findings in animals. In our results WT mice treated with E2, resulted in a reduced body weight gain while no effects were seen in the SOCS2$^{-/-}$ E2 treated mice.

Studies in both human and rodents suggest that reduced levels of E2 or its receptors can lead to a metabolic syndrome-like phenotype. E2 protects from

High-Fat-Diet (HFD)-induced diabetes, steatosis, and adiposity. These effects show a gender dimorphism being female more protected than male mice from HFD-induced damage.

The results presented in this Thesis suggest that SOCS2, a physiological inhibitor of cytokine signaling, might play a physiological role in the regulation of hepatic steatosis in HFD-fed male mice. In addition, SOCS2 can mediate the inhibitory effects of E2 on GH-JAK2-STAT5 signaling pathway, which controls the hepatic lipid metabolism, the body size in mammals and it is an important regulator of glucose metabolism. In fact, GH has diabetogenic action inducing insulin resistance. SOCS2 Knockout mice show a GH dependent gigantism, but do not show alterations in insulin sensitivity. In contrast, GH transgenic mice are marked insulin resistant. Thus, $SOCS2^{-/-}$ mice provide a novel model to understand the complex relationship between inflammation, GH actions and nutrition in the control of hepatic glucose and lipid homeostasis. $SOCS2^{-/-}$ mice exhibit enhance liver TG secretion and are protected from HFD induced liver steatosis, being the females more protected than males. However, $SOCS2^{-/-}$ HFD mice display severe systemic insulin resistance, associated to hyper-insulinemia and worse insulin sensitivity in liver compared to WT mice fed similar diet. $SOCS2^{-/-}$ mice fed with a HFD also exhibit enhanced expression of inflammatory cytokines in liver and fat tissue, demonstrating a novel role of SOCS2 as negative regulator of macrofague activation in situations of dietary stress.

Finally, experimental elimination of SOCS2 identifies this protein as an important regulator of insulin homeostasis *in vivo*. Our results demonstrate that MLDSTZ-induced diabetic $SOCS2^{-/-}$ mice are more resistant to develop diabetes. Moreover, we also found that SOCS2 ablation is related to better conserved insulin sensitivity. We also observed that $SOCS2^{-/-}$ mice have higher beta cell , bigger beta islets size in pancreas, and higher serum insulin concentrations and HOMA-IR compared to

WT. SOCS2$^{-/-}$ mice are more sensitive to GH (and probably to PRL) and exhibit higher pancreatic GHR and PRLR levels compared to WT. GH stimulates pancreatic β-cell growth, survival and insulin production through GHR-JAK2-STAT5b signaling pathway. Thus, SOCS2 ablation may be related to improved insulin sensitivity and lower insulin resistance through a stronger and non-negatively regulated action of GH on pancreas. These results suggest that SOCS2 inhibition may be used as therapeutic target to ameliorate diabetes development.

6. FUTURE PERSPECTIVES

The findings in this thesis increase our knowledge about the SOCS2, E2 and GH interactions. Understanding E2, SOCS2 and GH interactions in physiological and pathological states may contribute to prevent health damage and improve clinical management of patients with growth, developmental and metabolic disorders.

- In the general population, the endocrine and metabolic consequences of long-term exposition to estrogens or novel estrogen-related compounds and their influence on the GH axis are largely unknown; key importance knowledge in the understanding of the relationship of E2 and GH is SOCS2 and its regulation. Disturbances of SOCS2 would require further investigations of the hormonal and environmental factors in the control of its expression.
- Future experiments in SOCS2$^{-/-}$ mice treated with E2 should elucidate the molecular mechanism that lead SOCS2 to effects E2 actions (metabolic, somatic growth and sexual-dimorphism in liver) *in vivo.*
- Further work is needed to clarify the role of GH signaling in different tissues and cell types in SOCS2$^{-/-}$ mice in response to HFD.
- Future use of SOCS2$^{-/-}$-HFD model may help to outline the contribution of different mechanisms in the development of fat-induced hepatic insulin resistance and components related to lipid overload in comparison with components related to inflammatory stress.
- Further experiments should be done to understand the complex interplay between SOCS2 and DM.

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RESUME OF THE MAIN ASPECT OF THE THESIS IN SPANISH

Estudios sobre el Estradiol, la Hormona de Crecimiento y el Supresor de la Señalización de Citoquínas-2, y la influencia en el metabolismo hepático

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Tesis Doctoral

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1. OBJETIVOS

El objetivo principal del trabajo presentado en esta Tesis es caracterizar la influencia del E2 y la GH y su relación con SOCS2 en el metabolismo hepático. En particular, nos hemos centrado en los siguientes objetivos específicos:

- Caracterizar la relación que existe entre el E2 y la GH en el metabolismo hepático (Articulo I).
- Caracterizar el rol de SOCS2 en el crecimiento somático y en el metabolismo hepático regulado por E2 (Articulo II).
- Caracterizar el papel de SOCS2 en el desarrollo de hígado graso inducido por HFD (Articulo III).
- Caracterizar el papel de SOCS2 en el desarrollo de la Diabetes Tipo I inducida por MLDSTZ (Articulo IV).

2. PLANTEAMIENTO

Actualmente, se desconocen las consecuencias de una exposición prolongada a estrógenos o compuestos derivados de estrógenos, así como su potencial influencia sobre la GH. Con el objetivo de explorar la influencia de E2 sobre las funciones reguladas por la GH en el hígado (endocrinas, metabólicas y diferenciación sexual) *in vivo,* en la presente Tesis Doctoral hemos usado dos modelos con sensibilidad alterada a la GH. Primeramente llevamos a cabo un exhaustivo análisis de los mecanismos moleculares que producen los efectos de E2, solo y en combinación con la GH, en las funciones del hígado adulto de ratas hipotiroideas-orquidectomizadas. En segundo lugar, analizamos la influencia de SOCS2 sobre la regulación del crecimiento corporal y del transcriptoma hepático que produce el E2 en ratones. En esta Tesis Doctoral se exploró además la influencia de SOCS2 en el metabolismo del hígado *in vivo* usando dos modelos de ratones con la sensibilidad a la insulina alterada. Primeramente, investigamos el rol de SOCS2 en el desarrollo de la esteatosis hepática y la resistencia a la insulina inducidas por la administración de una dieta alta en grasa (HFD). En segundo lugar, investigamos el impacto de la supresión de SOCS2 en el desarrollo de la Diabetes tipo I inducida por un tratamiento de múltiples exposiciones a bajas dosis Streptozotocin (MLDSTZ), así como el potencial mecanismo que se ve implicado.

3. METODOLOGÍA

A. Diseño de la experimentación animal

Todos los experimentos se realizaron de acuerdo a las recomendaciones de la Guía de Cuidado y Uso de los animales de Laboratorio de la Universidad de Las Palmas de G.C. y con las leyes reguladoras españolas y Europeas. El comité Ético de Experimentación Animal de la Universidad de Las Palmas de G.C aprobó los protocolos y se hizo todo los posible para minimizar el sufrimiento de los animales.

- **Para investigar el mecanismo molecular mediante el cual E2 y GH afectan a la fisiología del hígado en los machos (Articulo I),** utilizamos ratas machos adultas (2 meses de edad) *Sprague-Dawley*. Mediante una técnica previamente descrita [186,187] se generó hipotiroidismo (TX) en estos animales (Articulo I). Dos semanas después del inicio de la administración de MMI, las ratas fueron castradas (OX) u operadas simulando el procedimiento (SHAM) lo que dio lugar a dos grupos TXOX y TX, siendo estas últimas, ratas con los testículos intactos (Fig. 1). Seis ratas no fueron tratadas con MMI pero con ellas se simuló la misma operación. Estas ratas constituyeron el grupo INTACT, que corresponde a animales eutiroideos con los testículos intactos. Cuatro días después de la operación se inicio el tratamiento de reemplazo hormonal (HRT) con E2 benzoato (Sigma) (50 µg/kg/día; sc; 5 días a la semana) (TXOXE2) o vehículo (0.2 ml aceite de maíz; sc; 5 días a la semana) (TXOX) durante 20 días [188,189], antes del reemplazo hormonal con hormonal bien con E2 o GH durante 7 días, lo que generó TXOXE2GH, o con vehículo y GH, lo que generó el grupo TXOXGH. La GH (0.3 mg/kg/día) se administró diariamente dos veces al día mediante inyecciones subcutáneas (SC) en intervalos de 12 horas (08:00h y a las 20:00h), con el objetivo de simular el patrón masculino de secreción de GH [39,190].

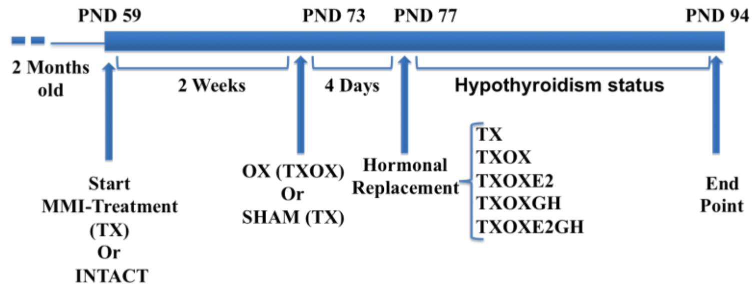

Figure 1. Hipotiroidismo y HRT para investigar el mecanismo molecular de interacción de la E2 y GH en el hígado de las ratas macho.

- **Para investigar el rol que juega SOCS2 sobre los efectos de E2 en el crecimiento somático y el metabolismo (Articulo II),** se usaron ratones de 4 meses de la cepa C57BL/6 (WT) y deficientes en SOCS2 ($SOCS2^{-/-}$) los cuales fueron orquiedectomizados (OX) u operados SHAM [192]. Después de una semana de recuperación se administró por vía SC 100 µg/kg E2 benzoato (Sigma, St. Louis, MO) o vehículo (aceite de maiz:etanol; 90:10; v/v) durante 24h, 48h o durante 3 semanas (5 días/semana E2) o vehículo ante del sacrificio [193] (Fig. 2). Doce horas antes del sacrificio, se retiró la comida de las jaulas de los animales para minimizar el efecto de la comida. Las muestras de suero se guardaron a -80ºC hasta su análisis. Una porción del hígado se congeló en nitrógeno liquido y se guardo a -80ºC para la extracción posterior del ARN mensajero (mRNA) (análisis RT-PCR y Microarray). Los Páncreas y los hígados de los animales fueron fijados para un posterior análisis histológico.

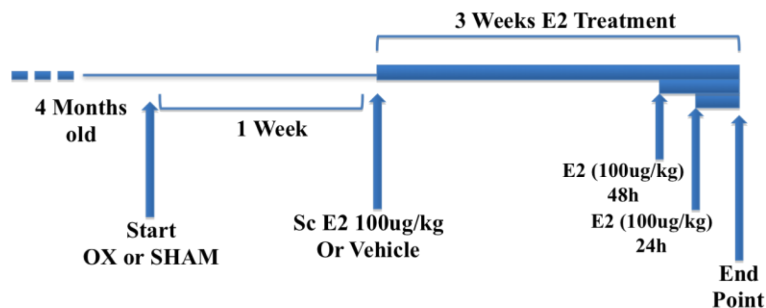

Figure 2. Tratamiento con E2 para investigar el rol de SOCS2 sobre los efectos que ejerce E2 en el crecimiento somático y el metabolismo.

- ***Tratamiento a largo plazo con E2.*** Ratones de dos meses de edad fueron tratados durante 15 semanas con E2 benzoato (Sigma, St. Louis, MO) (50µg/kg; sc; cada 48 h) o vehículo (aceite de maiz: etanol; 90:10; v/v) [191] (Fig. 3).

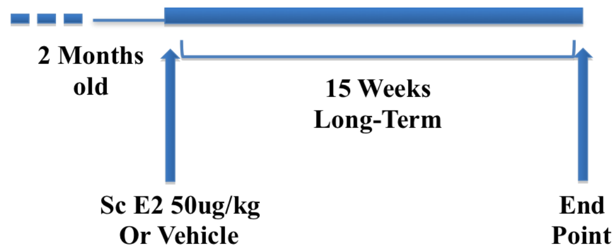

Figure 3. Tratamiento a largo plazo con E2 con el objetivo de investigar el papel de SOCS2 en la regulación del crecimiento somático por E2.

- **Para investigar el papel de SOCS2 en el desarrollo del hígado graso y de la resistencia a la insulina inducidos por la HFD (Articulo III)** ratones SOCS$^{-/-}$ y WT (C57BL/6) fueron alimentados con dieta HFD (OpenSource Diets (D12492), New Brunswick, NJ, USA) y dieta estándar (SAFE-diet A04, Panlab SLU, Barcelona, Spain) (Articulo III). Posteriormente, los animales fueron sacrificados tras una inyección intraperitoneal (IP) de 0.75U/kg de insulina recombinante humana (Actrapid, Novo Nordisk, Denmark) o suero salino como control. Los tejidos fueron almacenados a -80ºC hasta su posterior análisis. Páncreas e hígado fueron extraídos para un examen histológico. Las muestras de sangre se recolectaron por exanguinacion cardiaca y el plasma se utilizo para la medida de diferentes analitos.

- **Para investigar el papel de SOCS2 en el desarrollo de Diabetes Mellitus inducidad por MLDSTZ (Articulo IV)** se utilizó el modelo de inducción de Diabetes tipo I mediante MLDSTZ [194] en ratones SOCS2$^{-/-}$ y WT(Fig. 4). Para estos experimentos se usaron ratones de 6 meses que recibieron

cinco inyecciones consecutivas por vía IP de STZ (50 mg/kg b.w.). A continuación, se monitorizaron los niveles de glucosa, el peso corporal, y la ingesta de agua y comida. Para este estudio, se consideró que los ratones eran diabéticos cuando sus niveles de glucosa en sangre eran mayores a 300mg/dl. Los animales fueron sacrificados, recolectándose sus tejidos para posterior análisis, 60 días después del tratamiento con STZ.

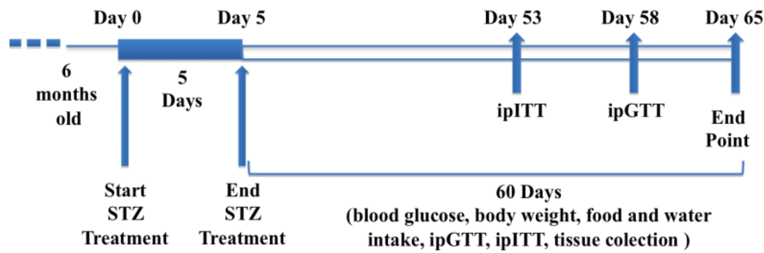

Figure 4. Representación esquematica del modelo de diabetes autoimmune y destrucción de celulas-β inducido por exposicion multiple a bajas dosis de STZ ratones SOCS2-/- y WT.

4. APORTACIONES ORIGINALES

4.1. Functional interplay between E2 and GH in liver (Paper I; doi:10.1371/journal.pone.0096305). En este estudio combinamos un análisis lipídico y transcripcional para obtener una exhaustiva información del mecanismo molecular por el cual E2, solo y en combinación con GH, regula las funciones del hígado de ratas macho castradas hipotiroideas. Este es un modelo con reducida actividad de la GH por lo que minimiza la influencia de las hormonas internas en la terapia de reemplazo hormonal (E2 y GH) y permite explorar la influencia de E2 en las funciones de diferenciación masculina. En este estudio, demostramos que el tratamiento con E2 y GH en ratas machos hipotiroideas castradas ejerce un efecto significativo en el contenido lipídico y en el transcriptoma del hígado, y que el E2 influye determinantemente en las funciones reguladas por la GH en el hígado (endocrinas, metabólicas, inmunes y de diferenciación sexual).

El hipotiroidismo afecta a la ganancia de peso y disminuye los niveles circulantes de IGF-I además de los marcadores biológicos de la actividad de la vía de señalización GH-STAT5b en el hígado (niveles de mRNA de IGF-I, ALS, SOCS2, CIS y CYP2C11) [208]. Estos cambios son parcial o totalmente restablecidos mediante la administración de GH en las ratas TXOX. Sin embargo, los efectos de la GH son en gran parte impedidos por el E2, lo cual esta en consonancia con los efectos negativos de los estrógenos en la administración continua de GH descrita en ratas hembras hipofisectomizadas [209]. El efecto positivo del E2 sobre la transcripción hepática de SOCS2, CIS y FGF21, sugiere que el E2 podría evitar la activación de la señalización GH-STAT5b en este tejido mediante la inducción de estos reguladores negativos de las señalización activada por la GH [62,210]. Del mismo modo, la administración de estrógenos en humanos (a través de la GH) evita la inducción de IGF-I, IGFBP-3, de la oxidación lipídica y de la síntesis de proteínas mediada por GH [164,211]. Los efectos del hipotiroidismo sobre el

crecimiento se asocian en parte con un aumento de le catabolismo de aminoácidos y la síntesis de urea en el hígado [212]. Diversos análisis biológicos multidisciplinares muestran que una administración intermitente de GH a ratas TXOX produce una regulación positiva del catabolismo celular mientras los genes involucrados en el metabolismo de aminoácidos y urea (i.e., OTC, ASS1, aminotransferasas, and methyltransferasas) ven significativamente disminuida su expresión. Este efecto positivo de la GH sobre el balance de nitrógeno fue previamente estudiado en ratas hipofisectomizadas [8,40,213].

La GH actúa como una hormona anabolizante ya que promueve la lipolisis y evita la lipogénesis en el tejido adiposo, aumentado la disponibilidad de ácidos grados (FFA) para su uso como energía [8]. El E2 puede interferir en este proceso impidiendo la inducción de algunos genes relacionados con la utilización de lípidos, como es ApoC2. Por ello, las acciones del E2 en el hígado pueden afectar los efectos metabólicos periféricos de la GH.

En situaciones de poco gasto energético, como el hipotiroidismo, la deficiencia en GH, E2 o el envejecimiento, se observa una estimulación de la lipogenesis [214]. Nuestros análisis del contenido lipídico del hígado revelan que las ratas TXOX presentan un aumento significativo de los niveles de ácidos grasos saturados totales (SFA) en comparación con el grupo intacto de ratas. El tratamiento con E2 no modifica los niveles de expresión de mRNA de los principales reguladores de la lipogenesis hepática [i.e., Sterol regulatory element binding protein (SREBP)1c, acetyl-Co A carboxylase alpha (ACC), fatty-acid synthase (FAS)] [215], mientras que sí activa el programa transcripcional de PPARα, que promueve el catabolismo de los ácidos grasos en el hígado [216,217]. Esto es evidente por el aumento de la expresión del gen de PPARα y sus genes diana involucrados en la β/ω-oxidación de ácidos grasos (i.e., CTE-I, CPT-2, Fasd6, Fasd1, Fasd2, Scd1, ACOX1, ECH1, BAAT, FGF21, CYP4A1, CYP4A3) mediante el E2. La terapia con E2 produce

una disminución significativa de los SFAs. En conjunto nuestros hallazgos sugieren la existencia de una relación positiva entre E2 y PPARα, la cual esta respaldada por múltiples estudios independientes [136,159,218,219]. Sorprendentemente, a pesar del aumento de la expresión de genes involucrados en β-oxidación, detectamos un aumento significativo del contenido de TG hepáticos en las ratas TXOX tratadas con E2, lo cual probablemente pueda ser explicado por efectos en el transporte de lípidos. El primer paso en el en la absorción de ácidos grasos de cadena larga es su translocación a través de la membrana plasmática. Particularmente, el E2 aumenta la transcripción de varios genes activados por PPARα que codifican proteínas que están implicadas en la absorción y activación de los ácidos grasos como CD36, ACSL4 and SLC27A5 (FATP5) [220,221]. En estudios publicados previamente por nuestro laboratorio demostramos que el transportador de ácidos grasos CD36 se expresa predominantemente en los hígados de las ratas hembras y propusimos que este dimorfismo sexual depende de le patrón de secreción de la GH, el cual se puede ver influenciado por el tratamiento con E2. Además, el E2 aumenta la transcripción de genes que codifican para FATP5, como SLC27A5, y transportadores de los ácidos grasos como acil-CoA-sintetasa, que cataliza la conjugación de los ácidos biliares con aminoácidos antes de su excreción en los canalículos biliares [222]. Tras la absorción de los ácidos grasos, el primer paso para la utilización intracelular de los ácidos grasos de cadena larga es su esterificación con CoA. Esta reacción esta catalizada por las acil-CoA-sintetasas, como por ejemplo la ACSL4 la cual es inducida por E2 en los hígados de las ratas TXOX. Los acil-CoA producidos son sustratos para la ß-oxidación, pero también participan en la síntesis de triglicéridos (TG), fosfolípidos, CE y ceramidas y por lo tanto también son una fuente primaria de moléculas de señalización [223]. La idea de que E2 pueda regular la formación de intermediarios de la señalización lipídica se apoya en la estimulación de la expresión de la elongasa 5 de ácidos grasos (Elovl5). Elovl5 utiliza los ácidos grasos insaturados para general muchos de los PUFAs de cadena

larga que son asimilados por los lípidos celulares (i.e., 20:4n-6 and 22:6n-3). Sin embargo, merece la pena mencionar que la administración de E2 no altera el metabolismo de VLCPUFA, porque los niveles de 20:4n-6, 20:5n-3 and 22:6n-3 son similares a los observados para el grupo de TXOX. Se ha descrito que E2 puede desempeñar un papel crítico en la lipogénesis y la transcripción de SCD1 [136], gen que codifica para la enzima que limita la generación de MUFAs como 18:1 n−9 and 16:1 n−7. Estudios previos revelan que la ausencia de E2 o ERα en ratas da lugar un profundo incremento en la lipogénesis y en la transcripción de SCD1 [137], lo cual sugiere que el E2 inhibe la transcripción de SCD1. Se ha descrito que el efecto antilipogénico de la terapia con E2, manteniendo la eficiencia de la exportación de TG, depende del ERα hepático [219,224]. Nuestro estudio muestra que el E2 aumenta la expresión de SCD1 y que este efecto es paralelo a una reducción del contenido hepático 18:0 y a un aumento de 18:1 n−9 (producto principal de las reacciones de SCD), y lípidos neutrales en comparación con los TXOX, lo cual indica que E2 modula la actividad de SCD1 en los hígados de las ratas TXOX. Sorprendentemente, el E2 disminuye además la expresión de SCD2 en los hígados de las ratas TXOX. La importancia de esta regulación transcripcional opuesta de los genes SCD es desconocida, pero los niveles transcripcionales de SCD1 son aproximadamente 1800 veces más altos que los de SCD2 en el hígado de las ratas [225]. Esto último indica que los cambios en 18:1n-9 y 18:0 deben atribuirse a las variaciones de la expresión de SCD1. En general, los cambios en la composición lipídica y en el perfil de expresión genético que se observan en las ratas TXOX tratadas con E2 sugieren que las interacciones funcionales E2 - PPARα juegan un papel fisiológico clave en la regulación del metabolismo hepático de los lípidos.

El E2 es capaz de reducir colesterol (CHO) en mujeres y modelos animales alimentados con dieta alta en grasa [226]. Sin embargo, el E2 es incapaz de revertir eficazmente la hipercolesterolemia o la hipertriglicemia en las ratas TXOX.

Esto se debe al hecho de que el E2 reduce la expresión de diversos transportadores de CHO, incluyendo Apob y ABCA1 en las ratas TXOX, los cuales contribuyen al mantenimiento y aumento de los niveles hepáticos de CE. Además, el E2 induce la movilización intracelular de CHO modulando las enzimas involucradas en las síntesis/movilización de CE y CHO [214,227]. Diferentes enzimas catalizan la conversión de CHO en CE en el hígado. Un claro ejemplo es la lecitina:colesterol aciltransferasa (LCAT), la cual usa fosfatidilcolina (PC) y ACAT, que es usada por acyl-CoA. Debido a que los niveles de lisofosfatidilcolina (LPC) son indetectables en todos los grupos, nuestra conclusión inicial es que el E2 estimula la reacción de ACAT2 para aumentar CE. Aunque no se detectaron en los niveles de expresión de ACAT, esto no descarta la posible modificación traduccional de enzimas del ciclo de CE en el hígado de las ratas TXOX tratadas con E2. El aumento de CE hepático junto con el aumento de TG y la disminución del contenido hepático de FFA en las ratas TXOX tratadas con GH, son similares a los efectos del E2 en la composición hepática lipídica lo cual sugiere que algunos efectos del E2 podrían estar mediados por la GH. Una consecuencia llamativa de la terapia combinada de E2 y GH es la completa recuperación de los niveles de MUFA de los lípidos totales y neutrales. Este efecto se le atribuye al aumento de 18:1n9, probablemente mediante la alteración de la expresión de la Δ9 desaturasa. Además GH y E2 aumentan los CE hepáticos y el efecto combinado de las dos hormonas es aditivo con respecto a CE, ya que sus niveles son el doble de los encontrados en los animales intactos, y son aproximadamente un 30% superiores a los encontrados en los grupos tratados con E2 o con GH por separado, lo cual es un indicativo de un metabolismo hepático de CHO más eficiente. Por consiguiente, en presencia de E2 y GH el nivel hepático de contenido en CHO se ve reducido no sólo respecto al grupo TXOX sino también respecto a los grupos tratados con E2 o GH. La GH aumenta significativamente el contenido de TG hepáticos en las ratas TXOX tratadas previamente con E2, lo cual sugiere que el tratamiento combinado de E2 y GH aumenta drásticamente la lipogénesis. Actualmente, es conocido que

en contra de este efecto lipolítico de la GH en los tejidos adiposos, la GH ejerce efectos lipogénicos en el hígado a través de la estimulación de SREBP1, la cual normalmente se ve acompañada por un aumento de la secreción de los TG hepáticos (VLDL) [8]. Nuestro análisis del perfil lipídico sugiere que la administración intermitente de GH a ratas TXOX aumenta la lipognesis en el hígado, al contrario que el tratamiento continuo con GH en ratas hipofisectomizadas [40]. La administración intermitente de GH a ratas TXOX no aumenta SERBP1, mientras que sí induce diferentes genes involucrados en el transporte de ácidos grasos (e.g., FABP) y en la biosíntesis de ácidos grasos insaturados de 18:2n-6 y18:3n-3 (por ejemplo las desaturasas de ácidos grasos 4, 5 y 6). La administración intermitente de GH a ratas TXOX disminuye la expresión del gen lipina, un gen diana de SREBP1c, el cual es un regulador clave de los niveles celulares de DG y TG, además de la oxidación de ácidos grasos en el tejido adiposo, en el musculo esquelético y en el hígado [228]. Estos hallazgos apoyan la hipótesis de que un patrón femenino de administración de GH es un estimulo más eficiente para inducir efectos lipogenicos en el hígado que un patrón masculino [184,229]. Otro mecanismo mediante el que GH fomenta la lipogénesis en el hígado es a través de la disminución de la oxidación lipídica. Estudios previos de nuestro laboratorio han demostrado que la administración continua de GH a ratas hipofisectomizadas [40] y a ratas adultas intactas [46] inhibe PPARα. De acuerdo con nuestro análisis lipídico y genómico la administración intermitente de GH a ratas TXOX da lugar a la regulación negativa de la vía de señalización de PPARα. En particular, la GH reprime la expresión de PPARα, ACOX-1, CPT-1, FGF21, además de diversos miembros de la familia CYP4A, involucrados en la oxidación de ácidos grasos.

En resumen, nuestro estudio añade datos novedosos que resaltan el efecto de la administración subcutánea de E2 sobre la fisiología del hígado y su relación con la GH. Estos resultados ponen de manifiesto el papel de E2 como regulador clave del

metabolismo en el hígado de los mamíferos y añaden más peso a la hipótesis de que E2 actúa como un importante regulador de las acciones de GH en el hígado. La interacción E2-GH en el hígado es relevante debido a las diferentes funciones fisiológicas que estas hormonas tienen en mamíferos y al amplio uso de los estrógenos y los compuestos derivados de los estrógenos en humanos. En particular, este es el primer estudio que demuestra que los perfiles de lípidos hepáticos tienen marcas singulares que se pueden utilizar para separar diferentes grupos con estados hormonales alterados. Esto incluye los reemplazos hormonales (E2 o GH) que inducen cambios que se superponen en la expresión génica. Por lo tanto, el perfil lipídico del hígado puede servir para identificar deficiencias hormonales crípticas o exposición a hormonas o a sustancias similares a las hormonas.

4.2. El Supresor de la Señalización de Citoquinas-2 influencia los efectos somáticos del E2 y el transcriptoma hepatico (Artículo II; manuscrito en preparación). En el artículo I, añadimos nuevos datos que ponen de manifiesto el impacto de la administración subcutánea E2 sobre la fisiología del hígado y su interacción con GH. La interacción E2-GH en el hígado es relevante debido a las funciones fisiológicas que estas hormonas tienen en los mamíferos y el amplio uso de los estrógenos y los compuestos derivados del estrógeno en humanos. En este trabajo (artículo II), hemos llevado a cabo un análisis del transcriptoma para obtener información completa sobre los mecanismos moleculares de los efectos de E2 *in vivo* en presencia (WT) o en ausencia ($SOCS2^{-/-}$) de SOCS2. La influencia de SOCS2 en el crecimiento somático regulado por E2 y en transcriptoma hepático que observamos en el presente estudio podría contribuir a una mejor comprensión de los mecanismos moleculares implicados en las consecuencias endocrinas y metabólicas de la exposición a los estrógenos o compuestos derivados del estrógeno y su influencia sobre las acciones de la GH. Como primera aproximación para evaluar la influencia de SOCS2 sobre los efectos de E2 en el

hígado se estudiaron los cambios en las funciones somatotrópicas del hígado en la ausencia o en la presencia de SOCS2. Después de 30 días, E2 redujo la ganancia de peso corporal en los dos genotipos. Sin embargo, la reducción de peso corporal por E2 fue más prolongada y mayor en el grupo WT (datos no mostrados). Como era de esperar [118], cuando se inició el tratamiento E2 (tiempo 0), los ratones $SOCS2^{-/-}$ tenían un mayor peso corporal que los WT. De esta forma, las mediciones de ganancia de peso corporal se normalizaron por el peso corporal en el tiempo 0. Esta normalización demostró claramente que el tratamiento con E2, en comparación con los ratones tratados con el vehículo, causa una mayor reducción el peso corporal en los ratones WT que en los ratones $SOCS2^{-/-}$ (Fig. 1B). A punto final, observamos que el tratamiento con E2 produjo cambios significativos en la longitud del cuerpo (Fig. 1C) y el fémur (Fig. 1D) pero sólo en los ratones WT. Además, el E2 produjo un aumento de los niveles del mRNA hepático de IGF-I (Fig. 1E) en ambos genotipos sin cambios en los niveles circulantes de IGF-I (Fig. 1F).

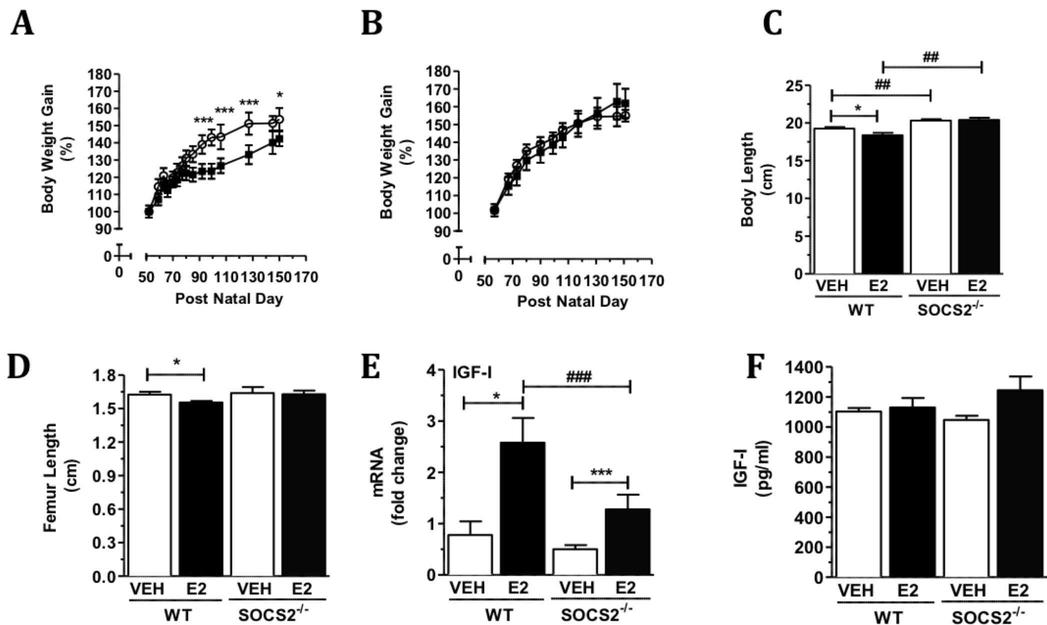

Figure 1. Influencia de SOCS2 sobre el crecimeinto regulado por E2 en el hígado. Ratones WT y SOCS2$^{-/-}$ se trataron con E2 benzoateo(E2B) (50µg/kg/48h) o vehiculo (VEH) durante 90 dias tal y como se describe ne el Material y Metodos. Peso corporal, normalizado a dia 50, de los ratones WT (A) and SOCS2$^{-/-}$ (B) se monitorizo cada 7 dias en ausencia de E2B (símbolo blanco) o en presencia de E2B (simbolo negro). Longitud corporal (C) y del femur (D), niveles hepaticos de expression de IGF-I mRNA levels (E), y nivel circulante de IGF-I (F) se midieron a teimpo fianl. Los resultados se expresan como media ± SEM de 6 individuos por grupo. La media de la expression de IGF-I en el VEH WT group se define como 1, y los demás valores de expression se relativizan respect a este valor. *P<0.05; **P<0.01, *** P<0.001; # P<0.05, ## P<0.01, ### P<0.001 para comparacion entre los dos genotipos

Estudios previos han demostrado que el E2 induce la expresión de SOCS2 en el hígado, que a su vez regula negativamente la actividad transcripcional mediada por STAT5 [61,62,193]. Por lo tanto, la consecuencia molecular de la deleccion del gen de SOCS2 es una activación prolongada de STAT5 [230]. En este estudio realizamos además un análisis cuantitativo de la expresión de los mRNA de SOCS2, CIS, SOCS1 y SOCS3, todos reguladores negativos de GHR-STAT5 [48,210]. El tratamiento con E2 aumentó los niveles hepáticos del mRNA de SOCS2 (Fig. 2A) en los ratones WT, mientras que éste no pudo ser detectado en

ratones SOCS2$^{-/-}$. En ratones WT, el E2 incrementó la expresión hepática de los mRNAs de CIS (Fig. 2B) y SOCS1 (Fig. 2C), mientras que no alteró la expresión del mRNA de SOCS3 (Fig.2D). Cabe destacar que en los ratones SOCS2$^{-/-}$ tratados con vehículo, los niveles de expresión del mARN de CIS (Fig. 2B) y SOCS3 (Fig.3D) se redujeron significativamente mientras que los de SOCS1 (Fig. 2C) se mantuvieron sin cambios. Sin embargo, el tratamiento con E2 fue capaz de incrementar los niveles de expresión del mRNA de CIS, SOCS1 o SOCS3 en los ratones SOCS2$^{-/-}$.Estos resultados demuestran que la eliminación del gen de SOCS2 influye drásticamente en la actividad transcripcional activada por el E2 en el hígado.

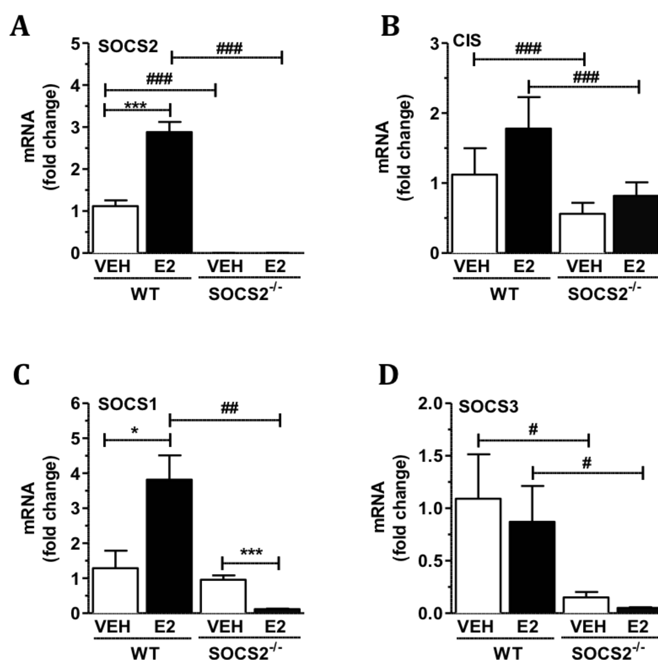

Figure 2. Influencia de SOCS2 sobre la regulacion ejercida por E2 en la expression hepatica de los Suprresores de la Señalización de Citoquinas. SOCS2 (A), CIS (B), SOCS1 (C), y SOCS3 (D) se midieron por qPCR tras el sacrificio. Los resultados se expresan como media ± SEM de 6 individuos por grupo. La media de la expression del VEH WT group se define como 1, y los demás valores de expression se relativizan respect a este valor. . *P<0.05; **P<0.01, *** P<0.001; # P<0.05, ## P<0.01, ### P<0.001 para comparacion entre los dos genotipos

La mayoría de los estudios anteriores se han centrado en la influencia de los estrógenos sobre la secreción hipofisaria de GH [34] y sobre la influencia del género en esta secreción, demostrado que ésta tiene un gran impacto en la regulación transcripcional hepática [33]. Teniendo en cuenta lo anteriomente mencionado, en el presente estudio investigamos la influencia del E2 en los genes regulados por la GH y relacionados con el dimorfismo sexual hépatico [33] en ratones WT y SOCS2$^{-/-}$. Al tratar con E2 se observó una reducción en la expresión del mRNA del gen MUP (un gen específicamente de machos) (Fig. 3A), mientras que los niveles del mRNA de Cyp2B9 y A1BG (dos genes específicos de las hembras) (Fig. 3B) se vieron incrementados. Estos hallazgos sugieren que la ausencia de SOCS2 es suficiente por sí misma para feminizar el hígado macho e influir en los efectos de E2 el dimorfismo sexual. En ambos genotipos, el tratamiento con E2 produjo un descenso de expresión del mRNA de MUP (Fig. 3A), mientras que los niveles de mRNA de Cyp2b9 (Fig. 3B) y A1BG (Fig. 3C) se vieron aumentados. Esto último sugiere que el tratamiento con E2 feminiza el hígado adulto. En conjunto, estos resultados apoyan nuestra hipótesis de que SOCS2 puede influir en los efectos del E2 sobre el eje somatotrópico-hígado.

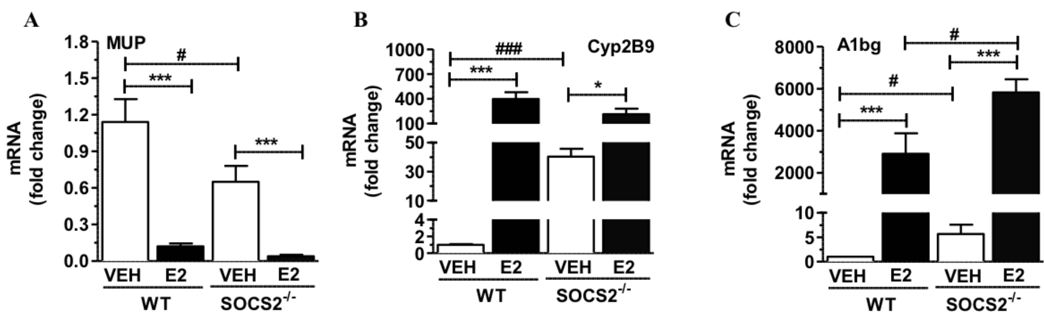

Figure 3. Influencia de SOCS2 sobre la regulacion de E2 en el dimorfismo del hígado. MUP (A), Cyp2b9 (B), and A1bg (C) se midieron por qPCR tras el sacrificio. Los resultados se expresan como media ± SEM de 6 individuos por grupo. La media de la expression del VEH WT group se define como 1, y los demás valores de expresion se relativizan respect a este valor. . *P<0.05; **P<0.01, *** P<0.001; # P<0.05, ## P<0.01, ### P<0.001 para comparacion entre los dos genotipos

Varios estudios sugieren que la señalización mediada por E2 puede tener un papel importante en el control del metabolismo lipídico y de la glucosa [136,137]. Estudios realizados tanto en humanos como en roedores sugieren que niveles alterados de E2 o de sus receptores pueden dar lugar a un síndrome similar al síndrome metabólico (es decir, resistencia a la insulina, adiposidad y la dislipidemia). Como se ha mencionado en esta Tesis (Introducción), la influencia beneficiosa del E2 en relación con la normalización de los niveles de lípidos y de la homeostasis de la glucosa es evidente a partir de los resultados obtenidos a partir de experimentos realizados con ratones ob/ob (carentes del gen que codifica para al leptina: ratones obesos) y con ratones alimentados con dieta alta en grasa (modelo de diabetes tipo II) [158,159]. Además, el tratamiento de ratones ob/ob con el agonista selectivo del ERα, propil-pirazol-triol, mejora la tolerancia a la glucosa y la sensibilidad a la insulina, lo que apoya el papel crítico del ERα en el control de la homeostasis de la glucosa. En la ausencia de tratamiento con E2, los ratones $SOCS2^{-/-}$ presentaron niveles más bajos de TG (Fig. 4B), leptina (Fig. 4C), y la grasa perirrenal (Fig.4D) que los ratones WT. De acuerdo con los datos descritos [124], estos resultados demuestran que los ratones $SOCS2^{-/-}$ presentan una reducida adiposidad. Curiosamente, el E2 reduce además los niveles circulantes de TG (figura 4b) y leptina (Fig. 4C) en ratones WT, pero no en ratones $SOCS2^{-/-}$. Por otro lado, también se observó una reducción de los niveles de CHO (Fig. 4A) en los ratones $SOCS2^{-/-}$ tratados con E2, pero no en los ratones WT.

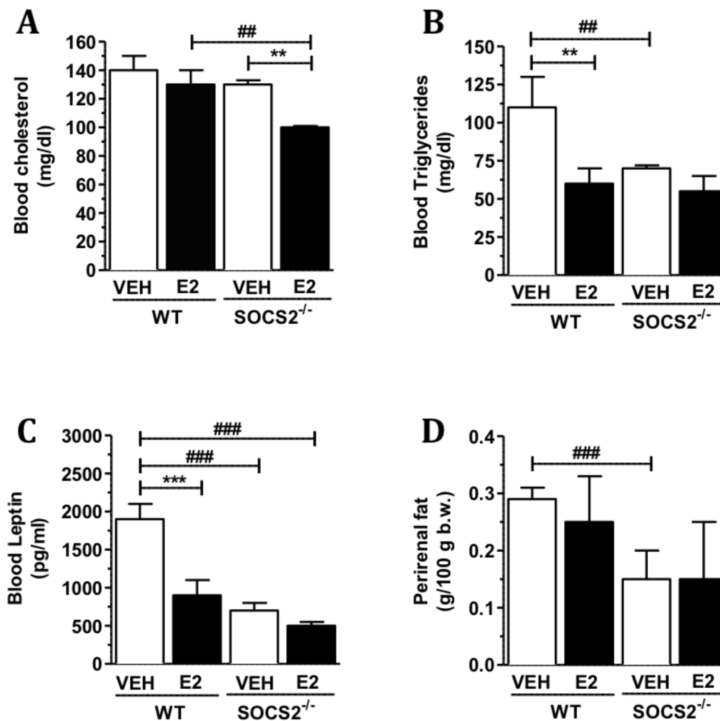

Figure 4. Influenncia de SOCS2 sobre los efectos de E2 en los lipidos y al adiposidad. and adiposity index. Niveles de colesterol (A), triglieridos (B), leptina (C), y grasa perirenal (D). Los resulatos se expresan como media ± SEM (n=6). . *P<0.05; **P<0.01, *** P<0.001; # P<0.05, ## P<0.01, ### P<0.001 para comparacion entre los dos genotipos

Independientemente del genotipo, el tratamiento con E2 incrementó los niveles hepáticos de FAT/CD36 (Fig. 5F), receptor implicado en la captacion de ácidos grasos de cadena larga y que se expresa, predominantemente, en hígado de rata hembra [231]. Además, se observó que el tratamiento con E2 redujo, en ambos genotipos, los niveles de dos receptores de lípidos que desempeñan un papel fundamental en el metabolismo de los lípidos [224], como son LXR (Fig. 5D) y PPAR (Fig. 5E). Sin embargo, el tratamiento con E2 reguló de manera diferencial la expresión de L-FABP (Fig.5B) y FAS (Fig.5C) en los ratones WT con respecto a los $SOCS2^{-/-}$. Por otro lado, el tratamiento con estradiol no afectó los niveles de expresión de un gen implicado en el control de la lipogénesis [233] como es

SREBP1c (Fig.5A), Por todo ello, en esta Tesis Doctoral aportamos un conjunto de hallazgos que sugieren que SOCS2 puede influir en los efectos del E2 sobre el metabolismo de los lípidos en el hígado.

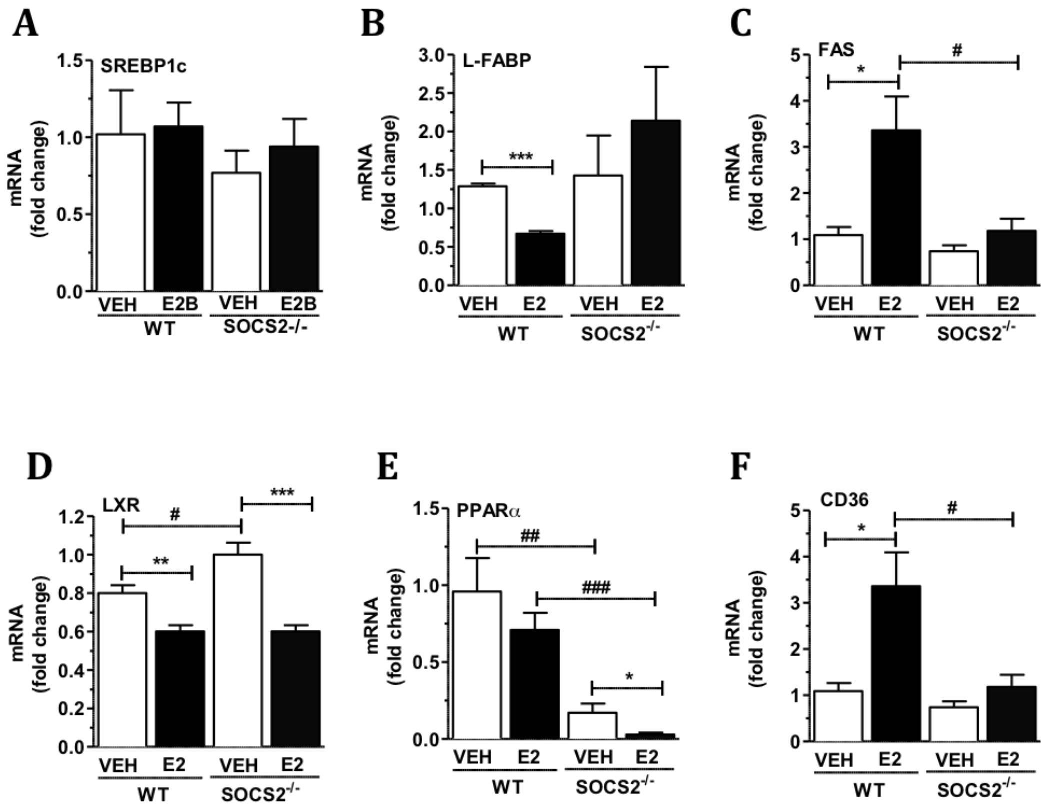

Figure 5. Influenncia de SOCS2 sobre los efectos de E2 en la expression de genes lipidicos en el higado. Wild-type (SOCS2$^{+/+}$) and SOCS2$^{-/-}$ male mice were treated with E2 benzoate (EB) (50µg/kg/48h) or vehicle (VEH) during 90 days as described in Material and Methods. SREBP1c (A), L-FABP (B), FAS (C), LXR (D), PPARα (E), y CD36 (F) se midieron por qPCR tras el sacrificio. Los resultados se expresan como media ± SEM de 6 individuos por grupo. La media de la expression del VEH WT group se define como 1, y los demás valores de expresion se relativizan respect a este valor. . *P<0.05; **P<0.01, *** P<0.001; # P<0.05, ## P<0.01, ### P<0.001 para comparacion entre los dos genotipos

En el presente trabajo se investigó, además, los efectos del E2 sobre la homeostasis de la glucosa. Como se describió con anterioridad [124], los ratones SOCS2$^{-/-}$ poseen niveles basales aumentados de glucosa con respecto a los ratones WT (Fig.

6A). Curiosamente, tras la administración de glucosa el máximo pico de glucosa en sangre se detectó 20 min antes en los ratones SOCS2$^{-/-}$ (Fig.6C) que en los WT, y se mantuvo elevado hasta los 30 minutos (Fig.6D) tras la administración de glucosa. Estas diferencias entre los genotipos en lo que se refiere a los niveles de glucosa también fueron detectadas cuando se analizó el área bajo la curva (AUC) (Fig.6E). Cabe destacar que, independientemente del genotipo, el tratamiento con E2 no mejoró la tolerancia oral a la glucosa.

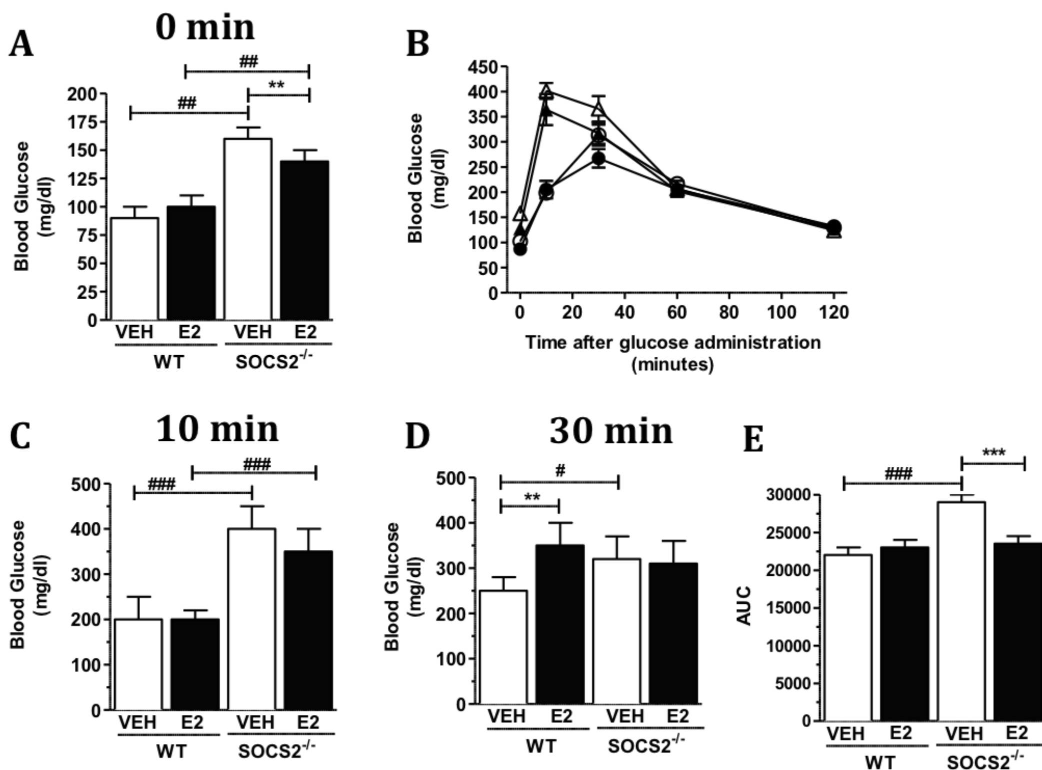

Figure 6. Influencia de SOCS2 sobre la regulacion de E2 en la homeostasis de la glucose. Glucosa Basal (A), ipGTT (WT (circulo) y SOCS2$^{-/-}$ (triangulo), VEH (simbolo blanco) y tratemineto con E2B (simbolo negro)) (B) , niveles de glucose en sangre a los 10 min en el ipGTT (C), niveles de glucose en sangre a los 30 min en el ipGTT (D) y AUC (E). *P<0.05; **P<0.01, *** P<0.001; # P<0.05, ## P<0.01, ### P<0.001 para comparacion entre los dos genotipos

A continuación, se estudió el papel de SOCS2 sobre los efectos del E2 sobre la sensibilidad a la insulina. El estudio de ipITT mostró que ambos genotipos tenían una sensibilidad similar a la insulina (Fig. 7A). De hecho, la ausencia de diferencias significativas en el AUC (figura 7B) sugieren que ambos genotipos responden de manera similar a la insulina y al E2. Sin embargo, los ratones SOCS2$^{-/-}$ tuvieron al final del test (120 min) unos niveles de glucosa en sangre dos veces más altos que los WT (Fig. 7C), lo que sugieren que son más resistentes a la hipoglucemia exógena inducida por la insulina que los WT.

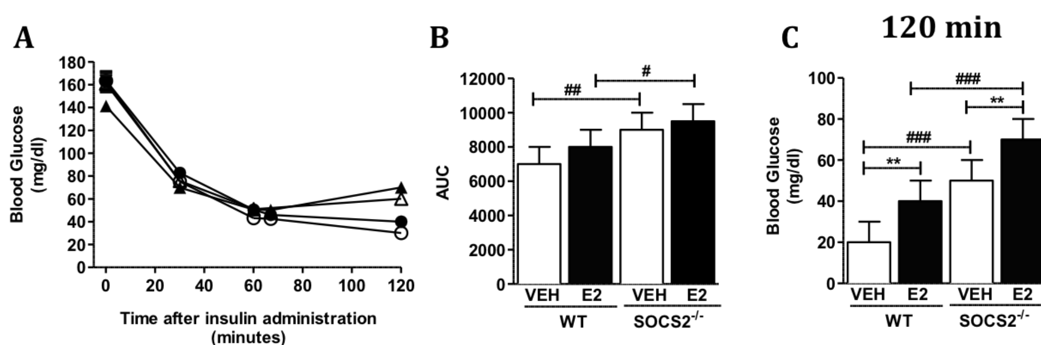

Figure 7. Influencia de SOCS2 sobre la regulacion de E2 en la sensibilidad a la insulina. ipGITT (WT (circulo) y SOCS2$^{-/-}$ (triangulo), VEH (simbolo blanco) y tratemineto con E2B (simbolo negro)) (A) , AUC (B) y niveles de glucose en sangre a los 120 min en el ipITT (C). *P<0.05; **P<0.01, *** P<0.001; # P<0.05, ## P<0.01, ### P<0.001 para comparacion entre los dos genotipos

Por último, se realizó un análisis profundo de expresión génica para comprender mejor la influencia de SOCS2 en la fisiología hepática regulada por el E2 . En este trabajo se identificaron 4.631 genes (3.356 inducidos / 1.275 reducidos) regulados diferencialmente en ratones WT después de dos días de tratamiento con E2. Sin embargo, el E2 reguló un 50% menos de genes (2330) en los ratones SOCS2$^{-/-}$ (1.425 inducidos / 905 reducidos) en comparación con los ratones WT. A continuación, se identificaron los procesos biológicos a partir de los perfiles de expresión mediante GO análisis de enriquecimiento (GSEA) [202,203] (Fig. 8-9). El análisis GSEA mostró que los grupos de genes regulados después de dos días de tratamiento con E2 fueron similares en SOCS2$^{-/-}$ y WT. Sin embargo, algunas

funciones GO sí que fueron significativamente diferentes. Particularmente, el E2 produjo una marcada disminución de la respuesta inmune hepática en ausencia de SOCS2 mientras que ésta se vio incrementada en los WT. Sin embargo, el ciclo celular y la proliferación se vieron estimuladas por el E2 en ambos genotipos. Además, el tratamiento con E2 produjo en los ratones SOCS$^{-/-}$ un aumento de la expresión de varios GPCR que pertenecen a la familia olfativa.

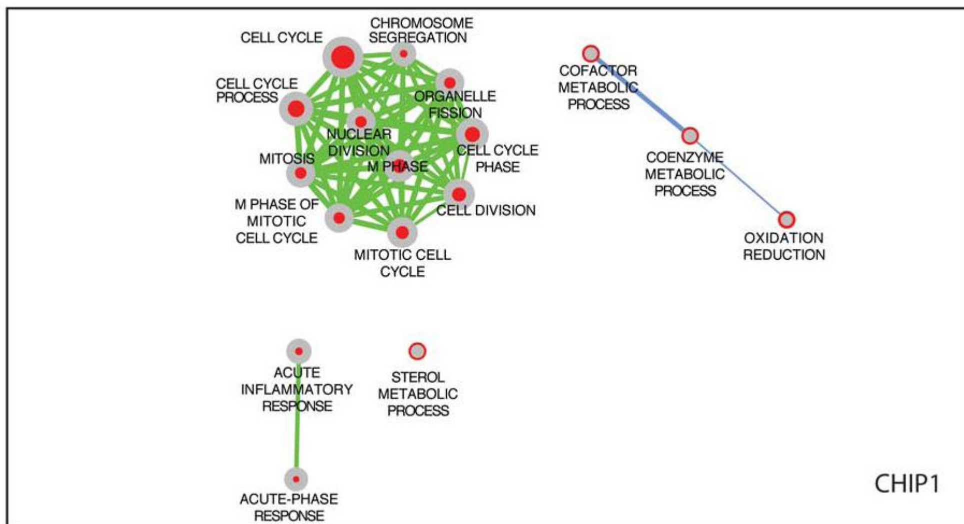

Figure 8. Analisis de los efectos biologicos de 2 días de tratamiento E2 en el transcriptoma del hígado en ratones WT. Mediante microarray de DNA analizamos las diferencias de expression de los genes hepaticos como se describe en Material y Métodos. Represnetamos los resulatdos del analisis functional y biologico (DAVID) usando Cytoscape. Nodo (círculo interno) el tamaño corresponde al número de genes regulados positivamente por E2; parte exterior de el Nodo (círculo exterior) el tamaño corresponde al número de genes regulados negativamente por E2; color del nodo y de la parte exterior corresponde a la importancia del conjunto de genes, sobreexpresados o inhibidos respectivamente, (rojo oscuro = enriquecido significativamente, rojo claro = enriquecido no significativamente, gris = ausente); El grosor de las lineas conectoras corresponde al número de genes que se superponen entre las dos situaciones analizadas. Lineas verdes corresponden a los genes sobreexpresados y lineas azules corresponden a los genes inhibidos.

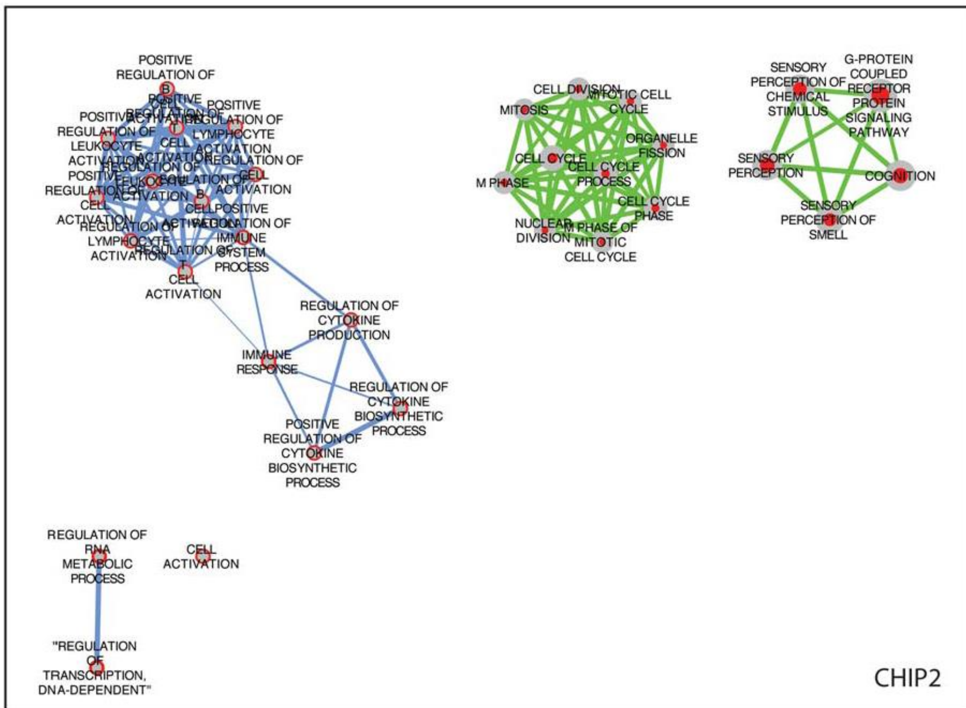

Figure 9. Analisis de los efectos biologicos de 2 días de tratamiento E2 en el transcriptoma del hígado en ratones SOCS2$^{-/-}$. Mediante microarray de DNA analizamos las diferencias de expression de los genes hepaticos como se describe en Material y Métodos. Represnetamos los resulatdos del analisis functional y biologico (DAVID) usando Cytoscape. Nodo (círculo interno) el tamaño corresponde al número de genes regulados positivamente por E2; parte exterior de el Nodo (círculo exterior) el tamaño corresponde al número de genes regulados negativamente por E2; color del nodo y de la parte exterior corresponde a la importancia del conjunto de genes, sobreexpresados o inhibidos respectivamente, (rojo oscuro = enriquecido significativamente, rojo claro = enriquecido no significativamente, gris = ausente); El grosor de las lineas conectoras corresponde al número de genes que se superponen entre las dos situaciones analizadas. Lineas verdes corresponden a los genes sobreexpresados y lineas azules corresponden a los genes inhibidos.

Cabe destacar que 625 y 678 genes fueron inducidos y reprimidos, respectivamente, por el E2, en los ratones SOCS2$^{-/-}$ pero no en animales WT (véase la Tabla 1-2 una pequeña representación). En general, estos resultados revelan que el E2 produce una profunda re-programación de la fisiología del hígado a corto plazo y que SOCS2 influencia notablemente los efectos del E2 *in vivo*.

ID Probe	Unigene/ Refseq	Symbol	Gene description	R		q(%)
				mean ± SD		
A_55_P1995537	NM_010824	Mpo	Myeloperoxidase	3,41	0,85	0,00
A_52_P15388	NM_008522	Ltf	Lactotransferrin	2,13	0,61	0,67
A_55_P1983921	NM_021352	Crybb3	Crystallin, beta B3	1,95	0,93	1,49
A_51_P167292	NM_009892	Chi3l3	Chitinase 3-like 3	1,93	0,55	0,67
A_55_P2163098	NM_134066	Akr1c18	Aldo-keto reductase family 1, member C18	1,90	0,36	0,00
A_51_P461067	ENSMUST00000 103420	G1m	Immunoglobulin heavy constant gamma 1	1,85	0,64	0,93
A_55_P1953387	NM_001272097	Fabp5	Fatty acid binding protein 5, epidermal	1,74	1,31	4,03
A_51_P199168	NM_007702	Cidea	Cell death-inducing DNA fragmentation factor, alpha subunit-like effector A	1,73	0,72	0,99
A_52_P213889	NM_172476	Tmc7	Transmembrane channel-like gene family 7	1,66	1,08	3,07
A_51_P488196	NM_028472	Bmper	BMP-binding endothelial regulator	1,65	0,28	0,00
A_55_P2024155	NM_001033324	Zbtb16	Zinc finger and BTB domain containing 16	1,65	1,16	3,38
A_51_P520650	NM_177639	Dlgap1	Discs, large (Drosophila) homolog-associated protein 1	1,62	1,26	4,03
A_55_P2002975	NM_205795	Mrgprb4	MAS-related GPR, member B4	1,55	0,96	2,80
A_55_P2054913	NM_001011863	Olfr406	Olfactory receptor 406	1,54	0,56	0,99
A_55_P2149921	TC1780716	Q80XJ7	Aldo-keto reductase family 1, member A4	1,52	0,91	2,80
A_55_P2185900	NM_032002	Nrg4	Neuregulin 4	1,49	0,87	2,80
A_51_P295896	NM_028934	4930452B06 Rik	RIKEN cDNA 4930452B06 gene	1,47	0,56	0,99
A_55_P2290388	NM_001043354	Rorb	RAR-related orphan receptor beta	1,46	0,53	0,99
A_55_P2001553	NM_020043	Igdcc4	Immunoglobulin superfamily, DCC subclass, member 4	1,45	0,16	0,00

Table 1. Lista representative de los genes hepaticos sobreexpresados en los ratones SOCS2-/- pero no en los WT tras 2 dos dias de tratamiento con E2. Mediante microarray de DNA analizamos las diferencias de expression de los genes hepaticos como se describe en Material y Métodos. Usamos el analisis estadistico SAM y discriminamos los genes que se expresan usando un FDR menor a 5% y log2 > |0.58|. La tabla se compone de probe ID, Unigene/Refseq, gene symbol, gene description, R (log2 E2-treated OXSOCS2-/- / vehicle-treated SOCS2-/-), and q (%).

ID Probe	Unigene/Refseq	Symbol	Gene description	R		q(%)
				mean ± SD		
A_55_P2408588	NM_007489	Arntl	Aryl hydrocarbon receptor nuclear translocator-like	-2,29	1,22	1,93
A_52_P480044	XR_105914	BC023105	cDNA sequence BC023105	-2,21	0,72	0,94
A_55_P2213968	NR_045840	4933416M07Rik	RIKEN cDNA 4933416M07 gene	-2,14	0,88	1,17
A_66_P120728	NM_001011791	Olfr193	Olfactory receptor 193	-2,10	0,90	1,49
A_51_P107315	NM_145423	Slc5a8	Solute carrier family 5 (iodide transporter), member 8	-2,02	0,52	0,94
A_65_P11137	NM_019541	Cts8	Cathepsin 8	-1,96	0,22	0,00
A_55_P2056714	NM_001042612	Nlrp9c	NLR family, pyrin domain containing 9C	-1,95	0,90	1,57
A_55_P2151591	ENSMUST00000032909	Pde3b	Phosphodiesterase 3B, cGMP-inhibited	-1,94	0,73	1,09
A_51_P210031	NM_001252679	Smr2	Submaxillary gland androgen regulated protein 2	-1,90	0,94	1,93
A_55_P2121042	NM_001082531	Pla2g2a	Phospholipase A2, group IIA (platelets, synovial fluid)	-1,85	0,53	0,94
A_52_P122649	NM_175647	Dmrta1	Doublesex and mab-3 related transcription factor like family A1	-1,81	0,67	1,09
A_52_P669922	NM_032541	Hamp	Hepcidin antimicrobial peptide	-1,80	0,28	0,00
A_55_P2095859	NR_037604	Rdh18-ps	Retinol dehydrogenase 18, pseudogene	-1,78	0,49	0,94
A_52_P186751	NM_198677	BC061237	cDNA sequence BC061237	-1,77	0,58	1,09
A_51_P267969	NM_011259	Reg3a	Regenerating islet-derived 3 alpha	-1,77	0,60	1,09
A_55_P2073024	NM_001034859	Gm4841	Predicted gene 4841	-1,76	0,46	0,94
A_51_P322473	NM_172417	2310042D19Rik	RIKEN cDNA 2310042D19 gene	-1,74	0,07	0,00
A_55_P2051082	XR_168616	Gm11634	Predicted gene 11634	-1,72	0,25	0,00

Table 2. Lista representative de los genes hepaticos inhibidos en los ratones SOCS2-/- pero no en los WT tras 2 dos dias de tratamiento con E2. Mediante microarray de DNA analizamos las diferencias de expression de los genes hepaticos como se describe en Material y Métodos. Usamos el analisis estadistico SAM y discriminamos los genes que se expresan usando un FDR menor a 5% y log2 > |0.58|. La tabla se compone de probe ID, Unigene/Refseq, gene symbol, gene description, R (log2 E2-treated OXSOCS2-/- / vehicle-treated SOCS2-/-), and q (%).

4.3. Relación entre SOCS2, esteatosis hepatica y resistencia a la insulina en una Dieta alta en grasa (HFD) en ratones (Paper II; FASEB J 2012, 26:3282-3291). En este estudio analizamos al respuesta metabólica de los ratones SOCS2$^{-/-}$ a una dieta hipercalórica y rica en grasa (HFD). Los ratones SOCS2$^{-/-}$ muestraron un aumento de la expresión hepática de secreción de TG y están más protegidos de la esteatosis hepática inducida por HFD (Fig.1). Sin embargo, éstos presentan una severa resistencia sistémica a la insulina asociada a una hiperinsulinemia y a una peor sensibilidad a la insulina en el hígado en comparación con los WT alimentados con la misma dieta. Los ratones SOCS2$^{-/-}$ alimentados con HFD presentan además un aumento de la expresión hepática de citoquinas inflamatorias, sugiriendo así un novedoso papel de SOCS2 en la regulación negativa de la activación de macrófagos bajo condiciones de estrés, como es la HFD. Es importante señalar que el fenotipo de los ratones SOCS2$^{-/-}$ es claramente diferente a los fenotipos de los ratones deficientes en SOCS3 y SOCS1 en el hígado [234,235], los cuales muestran un aumento de la estatosis hepática. Esto último pone de manifiesto la existencia de diferencias funcionales entre los diferentes miembros de la familia SOCS en la regulación del metabolismo hepático.

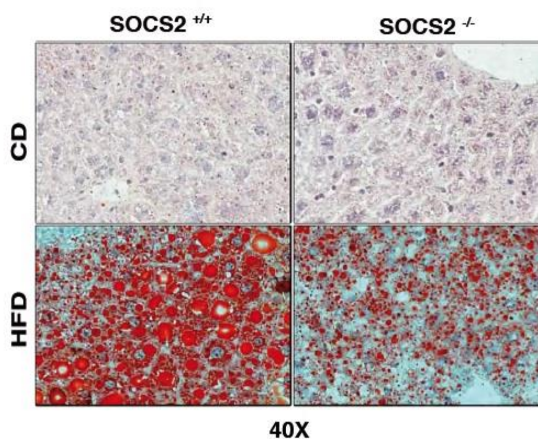

Figure 1. Analisis de el contenido lipidico en higado de ratones, WT y SOCS$^{-/-}$, alimentados 4 meses con CD o HFD (A), tinción Oil Red de secciones de higado (imagenes magnificadas 40x)

En estudios previos realizados en nuestro laboratorio hemos demostrado que SOCS2 es regulador negativo de la señalización activada por la GH a través de su receptor en el hígado [116]. La reducida esteatosis observada en los ratones SOCS2$^{-/-}$ (HFD) en comparación con los WT (HFD), está en concordancia con la conocida acción de la GH fomentando la movilización de lípidos hepáticos [236] y sugiere, claramente, que el aumento de la señalización de GH en el hígado es en parte responsable de estos efectos. De hecho, en el presente estudio encontramos en los ratones SOCS2$^{-/-}$ un incremento de los genes involucrados en la formación y secreción de TG, regulados por la GH, como son ApoB, FABP1 y DGAT2. Un análisis detallado realizado en ratones con una delección hepática específica de JAK2 ha dado a conocer otro mecanismo mediante el cual la GH controla el contenido de grasa del hígado [170]. Estos ratones, llamados JAK2L, presentan elevados niveles de GH circulante, que a su vez da lugar a un aumento de la lipólisis en el tejido adiposo. Esto aumenta el suministro de FFA al hígado y se produce la esteatosis [170]. Al contrario que los JAK2L, los ratones SOCS2$^{-/-}$ tienen unos niveles de GH circulante reducidos y muestran un incremento en el tejidos graso, lo que sugiere que la reducida lipólisis en el tejido adiposo de los ratones SOCS2$^{-/-}$ podría contribuir a la reducida acumulación de TG en el hígado. En este estudio, no detectamos cambios significativos en los niveles circulantes de ácidos grasos libres (FFA) ni en la expresión hepática de CD36 en los ratones SOCS2$^{-/-}$ en comparación con lo previamente descrito en los ratones JAK2L. Teniendo en cuenta todo lo anteriormente comentado, consideramos que se necesitan experimentos adicionales para analizar la influencia de la GH y la lipólisis del tejido adiposo en los hígados de los ratones SOCS2$^{-/-}$.

Sorprendentemente, los ratones SOCS2$^{-/-}$ muestran una respuesta exagerada de empeoramiento de la sensibilidad a la insulina provocada por HFD, mientras que ante una dieta normal encontramos pocas diferencias entre los SOCS2$^{-/-}$ y los WT en la señalización activada por la insulina. Esto sugiere que la hiperactividad de la

señalización hepática del receptor de GH por sí sola, tal como se observa en los ratones SOCS2$^{-/-}$, no es suficiente para explicar el deterioro observado en este estudio en lo que se refiere a la homeostasis de la glucosa inducida por la HFD. Una explicación más plausible podría ser que los efectos anti-insulinicos de la GH se ven aumentados por mecanismos relacionados con la dieta bajo el control de SOCS2. En este estudio proporcionamos evidencias de tres de estos mecanismos: hiperinsulinemia, adiposidad periférica y excesiva producción de citoquinas inflamatorias. Los receptores tipo Toll fomentan la activación de macrófagos en el hígado y en el tejido adiposo mediante la producción de citoquinas inflamatorias inducidas por la HFD. Este proceso tiene que darse para que se produzca la resistencia hepática a la insulina inducida por dieta alta en grasa [237]. La perdida de SOCS2 da lugar a respuestas alteradas ante la HFD en ratones, produciendo un incremento en la expresión de citoquinas inflamatorias y un aumento de la activación de NFκB. Esto parece estar mediado por acciones directas de SOCS2 sobre la activación de los macrófagos, como demostramos en experimentos *ex vivo*, en los que BMDM de los ratones SOCS2$^{-/-}$ mostraban un incremento en la actividad fagocitica *in vitro* y además hiper-responden a la estimulación con lipopolisacárido (LPS) produciendo la expresión de *IL-6, iNOS, IL-1β* and *INF-γ.* Algunos estudios previos describen el papel antiinflamatorio de SOCS2 mediando la actividad de lipoxinas activadas por la aspirina [133]. Nuestros datos sugieren que el efecto antiinflamatorio de SOCS2 podría estar mediado a través de la inhibición de la respuesta al LPS en los macrófagos mediante la activación de NFκB. Otros estudios han demostrado que ratones transgénicos que presentan un aumento de la actividad hepática de NFκB muestran una resistencia a la insulina fomentada por la inflamación en ausencia de acumulación de TG [238], lo cual nos recuerda al modelo de ratones SOCS2$^{-/-}$ alimentados con HFD.

Las medidas de producción hepática de citoquinas proporciona evidencias de que las células no parenquimales del hígado participan en la respuesta a la HFD en los

ratones SOCS2$^{-/-}$. Sin embargo, se desconoce si esta reactividad alterada es debida a la GH o a otros intermediarios. En relación con la GH, en estudios previos de nuestro laboratorio hemos demostrado que el tratamiento con GH en ratones SOCS2$^{-/-}$ da lugar a un aumento de la expresión hepática de genes regulados por citoquinas [116,118]. Además la GH aumenta la respuesta inflamatoria producida en respuesta al tratamiento con LPS en roedores y empeora las condiciones de pacientes en estado crítico [88]. Por tanto, se precisa de más investigación para clarificar el rol de la señalización activada por GH en diferentes tejidos y tipos celulares en ratones SOCS2$^{-/-}$ alimentados con dieta HFD.

Diferentes estudios realizados en humanos y roedores sugieren que el E2 protege del desarrollo de la diabetes, la esteatosis y del incremento de grasa producido por la HFD [226,239,240,241]. Los datos de nuestro laboratorio sugieren un dimorfismo sexual en respuesta a la HFD en los ratones SOCS2$^{-/-}$ (manuscrito en preparación). Curiosamente, cuando se mantienen los ratones SOCS2$^{-/-}$ con dieta estándar (CD) no encontramos diferencias entre machos y hembras en la tolerancia a la glucosa (ipGTT). Sin embargo la HFD produce una intolerancia a la glucosa más marcada en las hembras que en los machos SOCS2$^{-/-}$ (Fig. 1).

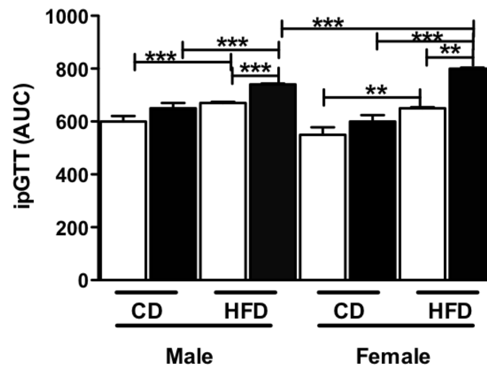

Figure 1. Dimorfismo en la tolerancia a la glucosa en ratones SOCS2$^{-/-}$ con HFD. WT (columnas blancas) y SOCS2$^{-/-}$ (comlumnas negras) se alimentaron con Dieta control (CD) o alta en grasa (HFD) como se describe en material y metodos. Dos semanas antes del sacrificio se realize un test de tolerancia a la glucose (ipGTT). Los resultados se expresan como media ± SEM (n=6). *P<0.05, **P<0.001, ***P<0.0001

El test de sensibilidad a la insulina (ipITT), muestra que ante la dieta CD ambos genotipos (SOCS2$^{-/-}$ y WT), independientemente del genero, se comportan de manera similar. Sin embargo, tal como se describió anteriormente, ambos grupos de ratones SOCS2$^{-/-}$ (machos y hembras) tratados con HFD muestran una menor repuesta hipoglucémica a la insulina exógena que los WT. Si bien es cierto que las hembras SOCS2$^{-/-}$ (HFD) presentan una mejor sensibilidad a la insulina si se las compara con los machos SOCS2$^{-/-}$ (HFD) (Fig. 1B).

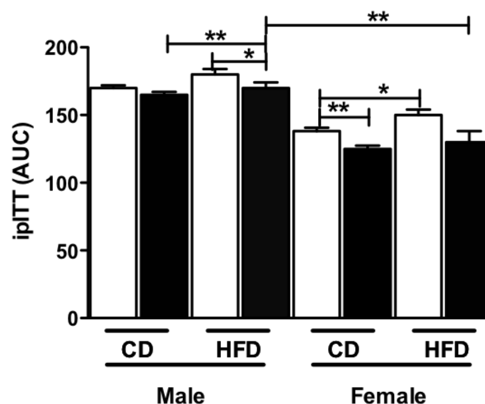

Figure 2. Dimorfismo en la sensibilidad a la insulina en ratones SOCS2$^{-/-}$ con HFD. WT (columnas blancas) y SOCS2$^{-/-}$ (comlumnas negras) se alimentaron con Dieta control (CD) o alta en grasa (HFD) como se describe en material y metodos. Dos semanas antes del sacrificio se realize un test de tolerancia a la insulina (ipITT). Los resultados se expresan como media ± SEM (n=6). *P<0.05, **P<0.001, ***P<0.0001

Además, la HFD produce un menor grado de esteatosis en las hembras que en los machos SOCS2$^{-/-}$ con un menor contenido hepático de TG. Finalmente, las hembras SOCS2$^{-/-}$ (HFD) en comparación con los machos SOCS2$^{-/-}$ (HFD) presentan 2.5 veces más leptina circulante y unos niveles 9 veces inferiores de marcadores inflamatorios plasmáticos (i.e., IL6, INFγ, RANTES, TNFα, and IL1-β) (Fig. 2B-C). Nuestros datos sugieren que el E2 protege de la esteatosis y la resistencia a la insulina inducidos por la HFD en ausencia de SOCS2.

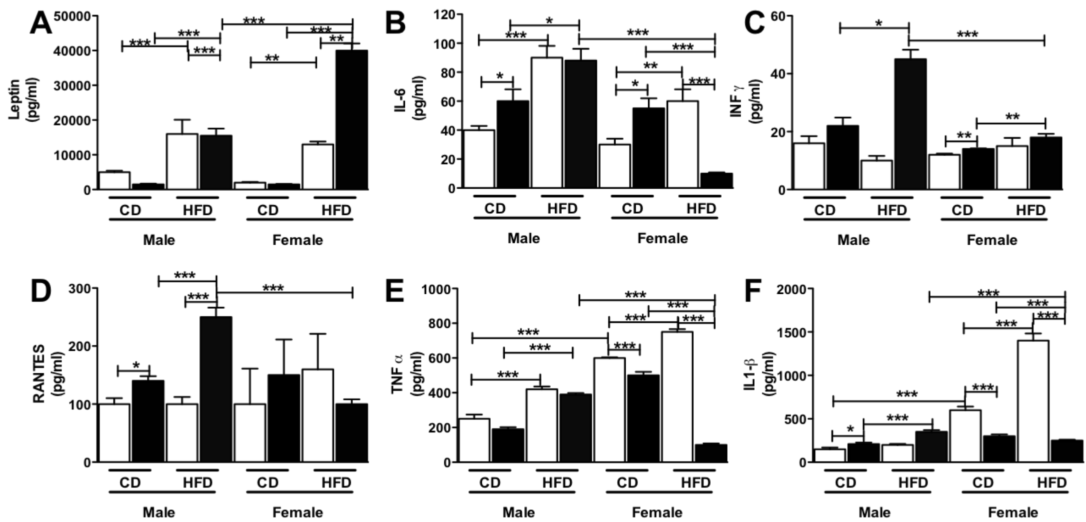

Figure 3. Dimorfismo en la respuesta inflamatori en ratones SOCS2$^{-/-}$ con HFD. WT (columnas blancas) y SOCS2$^{-/-}$ (comlumnas negras) se alimentaron con Dieta control (CD) o alta en grasa (HFD) como se describe en material y metodos. Se analizaron los niveles circulantes de leptina (A), IL-6 (B), INFγ (C), RANTES (D), TNFα (E), y IL1β (F). Los resultados se expresan como media ± SEM (n=6). *P<0.05, **P<0.001, ***P<0.0001

Desde el punto de vista fisiológico nuestros datos sugieren que los ratones deficientes en SOCS2 pueden representar un novedoso modelo para el estudio del síndrome metabólico con características únicas que son relevantes para las enfermedades humanas.

Los ratones SOCS2$^{-/-}$ no muestran un fenotipo metabólico claro. De esta forma, los mencionados ratones no muestran alteraciones en la sensibilidad a la insulina [118], al contrario que los ratones transgénicos que carecen de GH en los que se desarrolla una marcada resistencia a la insulina [59,77,242]. Sin embargo, cuando los ratones SOCS2$^{-/-}$ son alimentados con HFD desarrollan una gran resistencia a la insulina comparándolos con los WT (HFD). Los niveles de insulina plasmática en ayunas se ven aumentados en los ratones SOCS2$^{-/-}$ HFD, pero no se observan diferencias en los niveles pancreáticos de insulina ni en la morfología de los islotes, lo cual sugiere que el deterioro de la sensibilidad a la insulina no es causada por

alteraciones en las funciones de las células-β. Además la HFD produce una regulación positiva de la expresión génica de las citoquinas pro-inflamatorias hepáticas en los ratones SOCS2$^{-/-}$, lo que sugiere que SOCS2 ejerce un efecto protector ante el desarrollo de la resistencia a la insulina, probablemente suprimiendo la expresión de citoquinas pro-inflamatorias.

Para concluir, nuestros resultados demuestran que SOCS2 juega un importante papel en la regulación de la respuesta al estrés inducida por la HFD. Además, los ratones SOCS2$^{-/-}$ nos proporcionan un novedoso modelo para poder entender la compleja relación que existe entre inflamación, acción de la GH y nutrición en el control de la homeostasis hepática de la glucosa y los lípidos. La utilización futura de este modelo permitirá ayudar a distinguir la contribución de los diferentes mecanismos en el desarrollo de la resistencia a la insulina inducida por la grasa y los que son debidos a la sobrecarga de lípidos y poder compararlos con los que se relacionan con el stress inflamatorio.

4.4. La supresión de SOCS2 afecta al desarrollo de la diabetes inducida por MLDSTZ (Paper IV; manuscrito en preparación). La eliminación experimental de las diferentes SOCSs permite demostrar que cada proteína SOCS tiene funciones fisiológicas especificas, sugiriendo que las proteínas SOCSs son potentes reguladoras de la señalización activada por insulina y de las funciones de las células beta pancreáticas. La eliminación de SOCS1 incrementa los niveles de interferón y potencia la muerte de los islotes celulares [177] mientras que SOCS3 es un potente protector del desarrollo de Diabetes Mellitus tipo I (DM) a través de la supresión de IL-b y TNF en las células beta pancreáticas [178,179]. Las células beta SOCS3$^{-/-}$ muestran una gran resistencia a la muerte celular por apoptosis [180]. Por otro lado SOCS2, está implicado en la señalización activada por la GH y la PRL. En estudios previos hemos identificado a SOCS2 como una regulador negativo de la GH [119] y además los ratones SOCS2$^{-/-}$ crecen más sin elevar su concentración de GH [118]. Esta gran sensibilidad de los ratones deficientes en SOCS2 a la GH es debida a la mayor cantidad de GHR que presentan. Debido a la elevada sensibilidad de los ratones SOCS2$^{-/-}$ a la GH y a la PRL es lógico que analicemos las celulas β en el presente estudio en el cual investigamos cómo afecta la eliminación de SOC2 al desarrollo de la diabetes en un modelo de diabetes autoimmune y de destrucción de células β (múltiples dosis de estreptozotocina (MLDSTZ)) [194], en ratones alimentados con dieta normal.

Para analizar el papel de SOCS2 en la homeostasis de la glucosa y en el metabolismo, desarrollamos un modelo de diabetes autoinmune y de destrucción de células- β *in vivo* usando MLDSTZ [194]. Los parámetros del desarrollo de la diabetes fueron monitorizados, y ambos genotipos, tanto SOCS2$^{-/-}$ como WT, desarrollaron diabetes tras 9 días desde la primera dosis de STZ, mientras que los no tratados permanecieron con una glucemia normal (Fig. 1). Sin embargo, los ratones SOCS2$^{-/-}$ mostraron más resistencia al desarrollo de la diabetes.

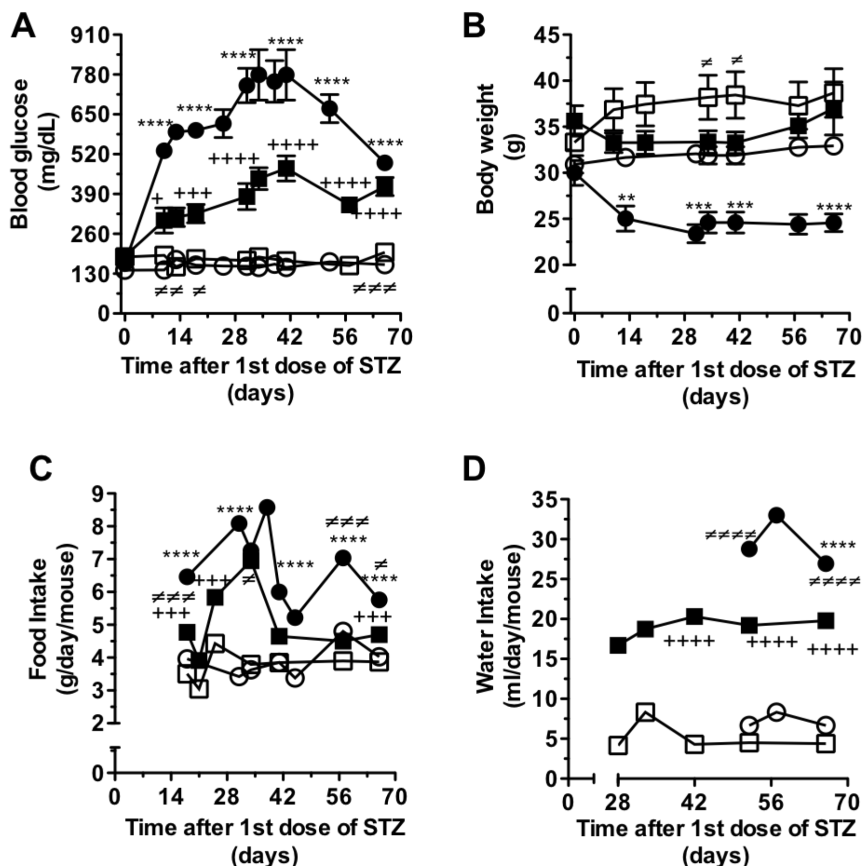

Figure 1. MLDSTZ-induce diabetes en ratones WT y SOCS2 $^{-/-}$. WT (circulo) y SOCS2$^{-/-}$ (cuadrado) recivieron 5 injections de vehiculo (simbolo blanco) o STZ (50 mg/kg b.w.) (simbolo negro) como se describe en material y metodos. Se monitorizó la glucose en sangre (A), el peso corporal (B), la ingesta (C), y la ingesta de agua (D). Se expresan los resultados como media ± SEM. WT-Veh vs WT-STZ: *P<0.05, **P<0.001, ***P<0.0001; SOCS2-/- Veh vs SOCS2-/- STZ: +P<0.05, ++P<0.001, +++P<0.0001; WT-Veh vs SOCS2-/- Veh: ≠P<0.05, ≠≠P<0.001, ≠≠≠P<0.0001.

Nuestros resultados muestran un aumento en la secreción de insulina inducida por la glucosa en los animales SOCS2$^{-/-}$ tratados con MLDSTZ. Además, encontramos niveles más bajos de glucosa en los SOCS2$^{-/-}$ tratados con MLDSTZ que los encontrados en los ratones WT diabéticos. En el test de tolerancia oral a la glucosa (ipGTT) (Fig. 2 A) observamos que el área bajo la curva de los ratones SOCS2$^{-/-}$ tratados con MLDSTZ fue menor que la de los WT (Fig. 2 B). Puesto que observamos cambios en la tolerancia a la glucosa en al suprimir SOCS2,

decidimos realizar un test de tolerancia a la insulina (ipITT) para estudiar la sensibilidad a esta hormona. Los ratones SOCS2$^{-/-}$ diabéticos por el tratamiento con MLDSTZ (Fig. 2 C y D), presentaron una menor respuesta hipoglucémica a la insulina exógena, lo cual es un indicativo de un mayor grado de sensibilidad a la insulina debido a la ausencia de SOCS2.

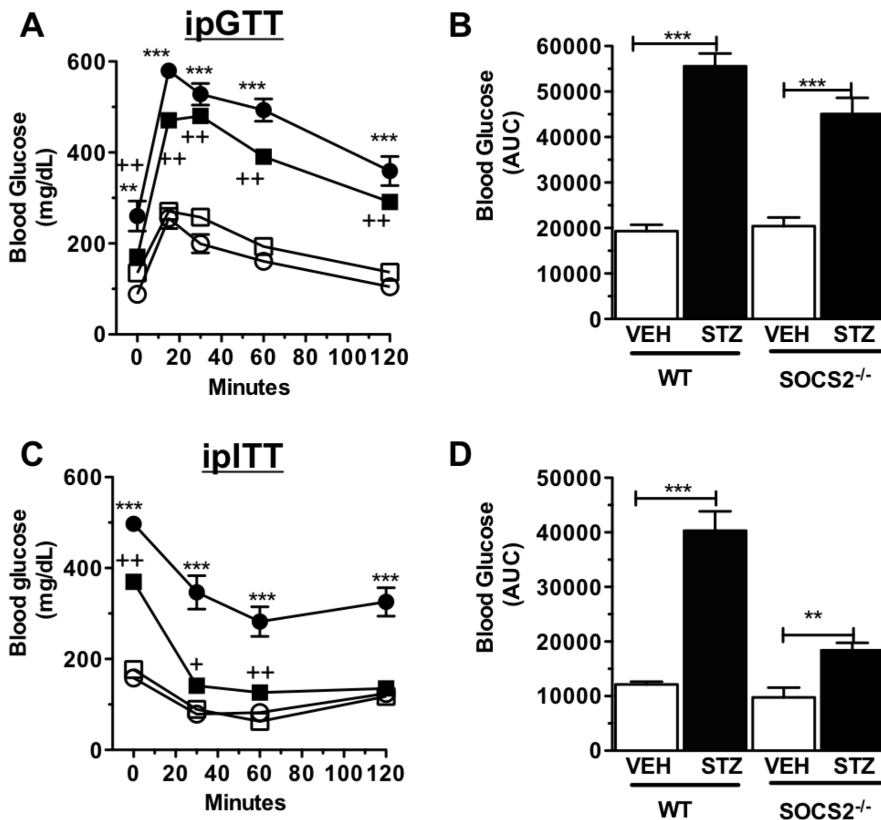

Figure 2. MLDSTZ-induce diabetes en ratones WT y SOCS2 $^{-/-}$. Circulos negros WT-STZ, circulos blancos WT-Veh, Cuadrados negros SOCS2$^{-/-}$STZ, cuadrados blancos SOCS2$^{-/-}$Veh. ipGTT y ipTT se realizaron el dia 58 y 53 tras la primera dosis de STZ. (A) ipGTT, (B) Area bajo la curva (AUC) de ipGTT, ipITT (C) y (D) Area bajo la curva (AUC) de ipITT, wt-Veh vs wt STZ: *P<0.05, **P<0.001, ***P<0.0001. T-student analysis for SOCS2-/- Veh vs SOCS2-/- STZ: +P<0.05, ++P<0.001, +++P<0.0001. T-student analysis for wt-Veh vs SOCS2-/- Veh≠P<0.05, ≠≠P<0.001, ≠≠≠P<0.0001.

Los resultados de sensibilidad a la insulina anteriormente comentados, fueron confirmados al observar un mayor nivel basal de insulina plasmática así como un

mayor índice HOMA-IR en los ratones SOCS2$^{-/-}$. De esta forma, observamos como el índice HOMA-IR de los ratones SOCS2$^{-/-}$ tratados con MLDSTZ fue mayor que el de los WT-STZ, lo cual es probablemente debido a que los primeros son más resistentes a la insulina exógena porque están segregando más insulina, reflejándose esto en un HOMA-IR mayor (Fig. 3 A-B). Estos resultados sugieren que la eliminación de SOCS2 parece compensar la destrucción de células beta inducida por MLDSTZ.

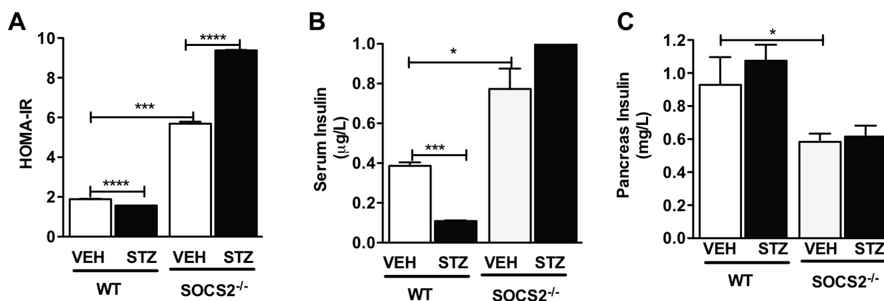

Figure 3. MLDSTZ-induce diabetes en ratones WT y SOCS2 $^{-/-}$. (A) Indice HOMA-IR index y (B) Insulin, medidos tras una noche de ayuno (Dia 58 tras la primera dosis de STZ). C) contenido de insulin por gramo de pancreas. *P<0.05, **P<0.001, ***P<0.0001.

Un aumento de células beta ha sido descrito durante el embarazo [244]. Este fenómeno esta causado por la estimulación de los receptores de GH/PRL [245]. Algunos estudios muestran que los ratones SOCS2$^{-/-}$ tienen más tejido pancreático, una arquitectura normal de los islotes pancreáticos [246], un contenido similar de insulina y un HOMA-B [124] similar con respecto a los ratones WT. Los ratones SOCS2$^{-/-}$ presentan además una mayor masa de células beta [62], como comprobamos en las tinciones con insulina de las células beta extraídas de nuestros animales (Fig. 5). Estos resultados parecen explicar el aumento de insulina sérica que observamos en los SOCS2$^{-/-}$ (Fig. 4).

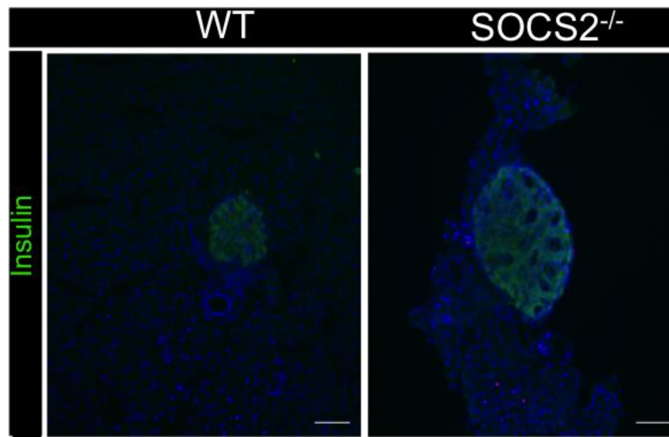

Figure 4. Analisis immunohistocquimico de insulina en pancreas de ratones de cepa salvaje (WT) y deficientes en SOCS2 (SOCS2$^{-/-}$).

La presencia de SOCS2 en las células beta da lugar a hiperglucemia e intolerancia al glucosa [175]. Los islotes pancreáticos de los ratones SOCS2$^{-/-}$ responden más a la PRL y GH que los de los WT, porque la ausencia de SOCS2 da lugar a un mayor contenido en receptores de GH y PRL. Para confirmar la mayor sensibilidad de los animales SOCS2$^{-/-}$ a la GH/PRL [59], realizamos una immunohistoquimica frente a GHR y PRLR en secciones pancreáticas obtenidas de los dos grupos experimentales. Nuestros resultados demuestran un mayor contenido de GHR y PRLR en el páncreas de los ratones SOCS2$^{-/-}$ (Fig. 5) con respecto al de los ratones WT. El mayor contenido pancreático de GHR, en ausencia de SOCS2, da lugar a una activación constitutiva de la vía de señalización JAK2/STAT5. Además, actualmente sabemos que el crecimiento, supervivencia y producción de insulina de las células beta pancreáticas esta estimulado por la GH a través de la activación de GHR/JAK2/STAT5b [174,247,248,249,250]. Por todo ello, los mayores niveles de GHR/PRLR encontrados en los ratones SOCS2$^{-/-}$ podrían explicar la mayor masa de células beta y la mayor producción de insulina observada en los páncreas de los ratones SOCS2$^{-/-}$ con respecto al de los WT.

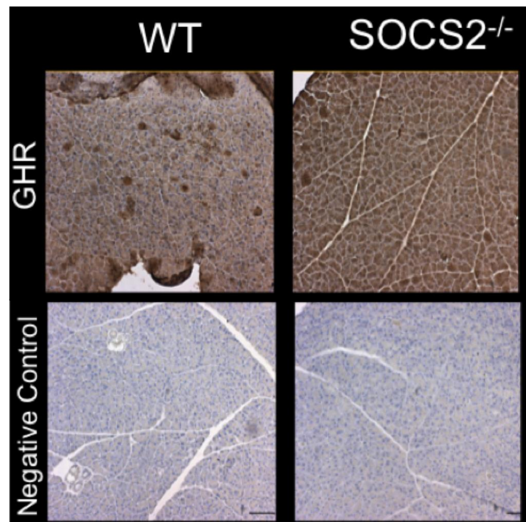

Figure 5. Analisis immunohistocquimico en pancreas de GHR enratones de cepa salvaje (WT) y deficientes en SOCS2 (SOCS2$^{-/-}$).

En conclusión, este estudio permite identificar a SOCS2 como un importante regulador de la homeostasis de la insulina *in vivo* y sugiere que la inhibición de SOCS2 podría usarse como una diana terapéutica para retrasar el desarrollo de la diabetes [251].

5. CONCLUSIONES

Los estudios que componen esta tesis doctoral añaden nuevos datos que enfatizan el impacto de la administración subcutánea de E2 sobre la fisiología del hígado y su interacción con GH. Nuetros resultados ponen de manifiesto el papel de E2 como un regulador crítico del metabolismo en hígado de los mamíferos y añaden más peso a la hipótesis de que E2 actúa como un importante regulador de las acciones de GH en el hígado. La relación E2-GH en el hígado es relevante debido a las diferentes funciones fisiológicas que estas hormonas ejercen en los mamíferos y al amplio uso de los estrógenos y los compuestos relacionados con los estrógenos en humanos.

Nuestros hallazgos muestran que el E2 induce una feminización del hígado de las ratas hipotiroideas castradas adultas (TXOX). En primer lugar, se observó que la administración de E2 a ratas TXOX incrementó la síntesis y secreción de GH desde la pituitaria, emulando un "patrón femenino", y dando lugar al aumento de la regulación de los genes femeninos. En segundo lugar, descubrimos que el efecto del E2 sobre las acciones de la GH en hígado de las ratas TXOX se puede atribuir a la interacción del E2 con la vía de señalización GH-JAK2-STAT5 a través de la inducción de los reguladores negativos de esta vía de señalización, como son SOCS2, la CIS y FGF21 (Articulo I). Nuestros resultados muestran claramente que el E2, directa o indirectamente, puede modificar los efectos de la GH sobre la fisiología del hígado. Curiosamente, los efectos del E2 producen una alteración de la expresión de los genes hepáticos relacionados con el crecimiento corporal y el metabolismo de los lípidos. Estos efectos dan lugar a una reducción en la longitud corporal en el grupo de los WT tratado con E2, mientras que estos efectos no resultaron significativos en los ratones SOCS2$^{-/-}$. Por otra parte, el E2 tiene la capacidad de controlar el equilibrio energético, la ingesta de alimentos y la distribución de grasa corporal. La reducción de los niveles de E2 se asocia con

cambios en el peso corporal y en la distribución de la grasa en los seres humanos, encontrándose hallazgos paralelos en los animales. En nuestros resultados los ratones WT tratados con E2, sufrieron una disminución de la ganancia de peso corporal, mientras que en los ratones SOCS2$^{-/-}$ no se observó ninguna diferencia.

Diversos estudios realizados tanto en humanos como en roedores sugieren que una reducción en los niveles E2 o sus receptores pueden dar lugar a un a un síndrome parecido al metabólico. El E2 protege de la diabetes, la esteatosis y la adiposidad inducidas por la dieta alta en grasa (HFD). Los efectos de la HFD presentan un dimorfismo de sexual, en el que las hembras están más protegidas que los machos de los daños inducidos por este tipo de dieta. Los resultados presentados en esta Tesis sugieren que SOCS2, un inhibidor fisiológico de la señalización de citoquinas, podría desempeñar un papel fisiológico en la regulación de la esteatosis hepática en ratones alimentados con HFD. Además, SOCS2 media los efectos inhibidores del E2 sobre la vía de señalización de GH-JAK2-STAT5, que controla el metabolismo hepático de los lípidos, el tamaño corporal de los mamíferos y además es una importante reguladora del metabolismo de la glucosa. De hecho, la GH tiene acción diabetogénica, ya que induce resistencia a la insulina. Los ratones SOCS2$^{-/-}$ muestran un gigantismo dependiente de GH, pero no presentan alteraciones en la sensibilidad a la insulina. A diferencia de los ratones transgénicos en GH que son altamente resistentes a la insulina. Por lo tanto, los ratones SOCS2$^{-/-}$ proporcionan un novedoso modelo que permitirá entender la compleja relación entre la inflamación, las acciones de GH y la nutrición en el control de la glucosa hepática y la homeostasis de los lípidos.

Los ratones SOCS2$^{-/-}$ presentan una mayor secreción hepática de TG y están protegidos de la esteatosis hepática inducida por HFD, estando las hembras más protegidas que los machos. Sin embargo, los ratones SOCS2$^{-/-}$ alimentados con HFD muestran una severa resistencia sistémica a la insulina, asociada a una

hiperinsulinemia y a una menor sensibilidad a la insulina en el hígado. Los ratones SOCS2$^{-/-}$ alimentados con HFD presentan también un aumento de la expresión de citoquinas inflamatorias en el hígado y la grasa, reflejando un nuevo papel de SOCS2 como regulador negativo de la activación de los macrófagos en situaciones de estrés debido a la dieta.

Por último, la eliminación experimental de SOCS2 permite identificar esta proteína como un importante regulador de la homeostasis de la insulina *in vivo*. Nuestros resultados demuestran que los ratones SOCS2$^{-/-}$ son más resistentes al desarrollo de diabetes mediante MLDSTZ. Por otra parte, observamos que la supresión de SOCS2 se relaciona con una mejor conservación de la sensibilidad a la insulina. Además, los ratones SOCS2$^{-/-}$ tienen una mayor masa de células beta y mayor tamaño de los islotes beta en el páncreas que los ratones WT. Los ratones SOCS2$^{-/-}$ son más sensibles a la GH (y probablemente a la PRL) ya que muestran mayores niveles pancreáticos de receptores GH and PRL. La GH estimula el crecimiento, la supervivencia y la producción de insulina de las células β del páncreas, a través de la vía de señalización GHR-JAK2-STAT5b. Por lo tanto, la eliminación de SOCS2 podría estar relacionada con la mejora de la sensibilidad a la insulina y con una menor resistencia a la insulina a través de una acción no regulada negativamente de la GH sobre el páncreas. Estos resultados sugieren que la inhibición SOCS2 podría ser utilizada como diana terapéutica para retrasar el desarrollo de la diabetes.

6. PERSPECTIVAS DE FUTURO

Las conclusiones que se extraen de esta tesis permiten aumentar nuestro conocimiento sobre la relación que existe entre SOCS2, E2 y GH. Entender esta relación en los diferentes estados fisiológicos y patológicos podría ayudar a prevenir enfermedades y mejorar el manejo clínico de los pacientes con trastornos del crecimiento, metabólicos y del desarrollo.

- Las consecuencias endocrinas y metabólicas de la exposición prolongada a los estrógenos o a compuestos relacionados con ellos, así como su influencia sobre las acciones de la GH son ampliamente desconocidas por parte de la población general. Por tanto, es muy importante incrementar nuestro conocimiento sobre SOCS2 y su regulación para poder comprender las relaciones entre E2 y GH. De esta forma, se requieren futuras investigaciones para poder definir con exactitud las alteraciones hormonales y ambientales que producen variaciones en la expresión de SOCS2.
- futuros experimentos en ratones SOCS2$^{-/-}$ ratones tratados con E2 permitirán elucidar con exactitud el mecanismo molecular por el que los efectos del E2,sobre el metabolismo, el crecimiento y el dimorfismo sexual, se ven afectados por SOCS2 *in vivo.*
- Es necesario seguir trabajando para aclarar el papel de la señalización de GH en los diferentes tejidos y tipos celulares en los ratones SOCS2$^{-/-}$.
- La futura utilización del modelo de ratones SOCS2$^{-/-}$ alimentados con HFD ayudará a perfilar la contribución de los diferentes mecanismos implicados en el desarrollo de la resistencia a la insulina, la sobrecarga lipídica hepática y el estrés inflamatorio, inducido por dietas ricas en grasa.
- Por último, consideramos que deben realizarse más experimentos para poder comprender la compleja interacción entre SOCS2 y Diabetes Mellitus.

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Lipid Profiling and Transcriptomic Analysis Reveals a Functional Interplay between Estradiol and Growth Hormone in Liver

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Abstract

17β-estradiol (E2) may interfere with endocrine, metabolic, and gender-differentiated functions in liver in both females and males. Indirect mechanisms play a crucial role because of the E2 influence on the pituitary GH secretion and the GHR-JAK2-STAT5 signaling pathway in the target tissues. E2, through its interaction with the estrogen receptor, exerts direct effects on liver. Hypothyroidism also affects endocrine and metabolic functions of the liver, rendering a metabolic phenotype with features that mimic deficiencies in E2 or GH. In this work, we combined the lipid and transcriptomic analysis to obtain comprehensive information on the molecular mechanisms of E2 effects, alone and in combination with GH, to regulate liver functions in males. We used the adult hypothyroid-orchidectomized rat model to minimize the influence of internal hormones on E2 treatment and to explore its role in male-differentiated functions. E2 influenced genes involved in metabolism of lipids and endo-xenobiotics, and the GH-regulated endocrine, metabolic, immune, and male-specific responses. E2 induced a female-pattern of gene expression and inhibited GH-regulated STAT5b targeted genes. E2 did not prevent the inhibitory effects of GH on urea and amino acid metabolism-related genes. The combination of E2 and GH decreased transcriptional immune responses. E2 decreased the hepatic content of saturated fatty acids and induced a transcriptional program that seems to be mediated by the activation of PPARα. In contrast, GH inhibited fatty acid oxidation. Both E2 and GH replacements reduced hepatic CHO levels and increased the formation of cholesterol esters and triacylglycerols. Notably, the hepatic lipid profiles were endowed with singular fingerprints that may be used to segregate the effects of different hormonal replacements. In summary, we provide *in vivo* evidence that E2 has a significant impact on lipid content and transcriptome in male liver and that E2 exerts a marked influence on GH physiology, with implications in human therapy.

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Introduction

17β-estradiol (E2), a major natural estrogen in mammals, has physiological actions not limited to reproductive organs in males [1,2,3,4,5,6]. Studies in patients with natural mutations in the human estrogen receptor alpha (ERα) [7,8] and aromatase [9,10] genes, and in the ERα (ERKO) and aromatase (ArKO) null mice models have shown that E2 can play a critical physiological role in males [1]. In particular, an insufficient E2 signaling in the ERKO and ArKO null mice models results in a metabolic syndrome-like phenotype with fatty liver due to a disruption in β-oxidation and increased lipogenesis, a phenotype that is reversed by physiological doses of E2. Moreover, both of these models exhibit a sexually dimorphic fatty liver that, notably, is male specific [1].

The effects of E2 in the liver can be explained through the direct actions of ER [2,11,12,13] or, indirectly, by modulating growth hormone (GH) physiology [14,15]. E2 can influence pituitary GH secretion but also GH direct actions in the liver. In particular, E2 induces the expression of Suppressor of Cytokine Signaling (SOCS)-2, which is a negative regulator of the GHR-JAK2-STAT5 signaling pathway [16]. Recently, we have identified SOCS2 as an important regulator of hepatic homeostasis (i.e., lipid and glucose metabolism and inflammation) under conditions of high-fat dietary stress [17]. The ability of GHR-JAK2-STAT5

signaling pathway to regulate hepatic lipid metabolism has also been highlighted in recent mouse genetic studies showing that hepatic inactivation of the GHR [18], its associated kinase, JAK2 [19] or its downstream signaling intermediary, STAT5b [20], leads to fatty liver. The metabolic influence of GH deficiency has also been well documented in humans by the development of a metabolic syndrome (i.e, increased visceral obesity, reduced lean body mass and fatty liver), a phenotype that is ameliorated by GH replacement therapy [21]. Notably, oral administration of pharmacological doses of E2 in humans inhibits GH-regulated endocrine (e.g., IGF-I) and metabolic (e.g., lipid oxidation, protein synthesis) effects [22,23] but these effects are attenuated when E2 is administered transdermally, suggesting that liver is the major target of regulatory cross-talk between estrogens and GH. However, the molecular characterization of the hepatic changes induced by long-term E2 treatment, when it is administered subcutaneously, and how they influence the liver response to male pattern of GH administration are not well understood.

Animal studies of hepatic effects of E2 or its interplay with GH actions have been focused on females [24,25]. Nonetheless, it is unclear if males exhibit equivalent responses, and there are reasons why such equivalence should not be presumed. In particular, gender dimorphism in GH secretion patterns develops soon after birth and the pituitary GH release maintains a sexually dimorphic liver function in adulthood [15], which may influence the nature of E2 effects in the livers of males and females. Several GH deficient models can be used to study the interplay between E2 and GH in males. Notably, the hypothyroid-orchidectomized (TXOX) rat model reaches very low or undetectable blood levels of GH and E2, which can be readily restored by hormone replacement treatment (HRT) [25,26,27], and shows systemic and hepatic metabolic disturbances with features that mimic deficiencies in E2 [2] and GH [28] (e.g., hypercholesterolemia, adiposity, fatty liver). In this study, we hypothesized that functional interplay between E2 and GH influences liver physiology in male. To test this hypothesis, we investigated the mechanisms of E2 and GH to regulate liver function at the molecular level. We studied gene expression profiles in liver tissue and correlated them with the changes in hepatic lipid content in TXOX rats before and after E2 and/or GH replacement. The results show that the interactions with GH contribute to multiple effects of E2 in male rat liver. Indeed, we found that E2 significantly influenced the GH-regulated endocrine, metabolic, immune, and gender specific responses in the liver. E2- and GH-induced changes in hepatic gene expression profiles were associated with changes in hepatic lipid composition. Finally, hepatic lipid profiles from E2- and/or GH-treated TXOX rats substantially differ from those observed in intact animals, indicating that the normal functions of thyroid glands and testes are an absolute requirement for physiological hepatic lipid homeostasis.

Material and Methods

Materials

Recombinant human GH was kindly donated by Pfizer laboratories (Spain). E2 benzoate, Tri-Reagent and, unless otherwise indicated, the rest of the products cited in this work were purchased from the Sigma Chemical Co. (St. Louis, MO).

Animal treatment

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the University of Las Palmas de Gran Canaria and conducted in accordance with European and Spanish laws and regulations. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Las Palmas de G.C. (permit number: 2006-07824). All efforts were made to minimize suffering. Adult (2–3 months old) male Sprague-Dawley rats (n = 6 per group) were used throughout these experiments. Animals were kept under a constant dark/light cycle, and in a controlled temperature (21–23°C) environment, and had free access to autoclaved standard chow (A04 SAFE Panlab, Barcelona, Spain) and tap water throughout the experiment. The generation of hypothyroid animals was performed as previously described [27,29]. The goitrogenic drug methimazole (MMI; 0.05%) was added to the drinking water for 5 weeks starting on postnatal day (PND) 59 until sacrifice on PND94. Calcium chloride (1%) was included with MMI in the water to ensure adequate dietary calcium intake because hypothyroidism decreases food intake by up to 40% [25]. The MMI-containing water was changed twice per week. Two weeks after starting MMI administration, rats were orchidectomized (OX) or sham–operated to make TXOX or testis-intact hypothyroid (TX) groups, respectively. Six rats were not treated with MMI and were subjected to sham-surgery to provide euthyroid testis-intact controls (INTACT). Four days after OX, we began HRT with E2 benzoate (50 µg/kg; sc; 5 days per week, from Monday to Friday) (TXOXE2) or vehicle (0.2 ml corn oil; sc; 5 days per week, from Monday to Friday) (TXOX) to TXOX rats for 20 days [30,31] before hormonal replacement for 7 days with either E2 plus GH (TXOXE2GH) or vehicle plus GH (TXOXGH). GH (0.3 mg/kg/day) was administered as two daily sc injections at 12-h intervals (08:00h and 20:00h) to mimic the male-specific GH secretion [32,33]. TX and TXOX control animals received equivalent amounts of the vehicle alone. Hypothyroidism status was corroborated by monitoring the body weight gain at 7 day intervals and the serum levels of T4 and T3. Twenty-four hours (in the case of E2) or twelve hours (in the case of GH) after the last injection, the animals were killed by exsanguinations. On PND94, blood samples were collected and serum stored at −80°C until analysis. Portions of the liver were snap frozen in liquid nitrogen and stored at −80°C until processed for mRNA analysis.

Serum analysis

The blood was analyzed for T3, T4, glucose, cholesterol (CHO), triacylglycerols (TG), leptin, IGF-I, E2, and testosterone (T). Serum free T3 and T4 concentrations were measured in duplicate by enzyme immunoassay (Access Systems, Beckman Coulter, Inc), with a detection limit of 0.60 ng/dl and 88 ng/dl, respectively. Serum levels of glucose, CHO, and TG were quantified by using an Olympus AU2700 chemistry analyzer (Beckman Coulter Inc.). The immunoassay method was also used to determine serum levels of E2 and T by using the UniCel DxI 800 immunoassay system (Beckman Coulter Inc). Serum levels of leptin and IGF-I were determined by using rat immunoassays (Quantikine, R&D systems) according to manufacturer recommendations. The IGF-I and leptin assays included quality controls provided by the manufacturer, and the standard curves of the assays were performed in accordance with the manufacturer's provided samples. All the samples were assayed together and each sample was assayed in duplicate.

Hepatic lipid analysis

Liver lipids were analyzed following the procedures detailed in Fabelo et al. [34]. Briefly, total lipids were extracted with chloroform/methanol (2:1 v/v) containing 0.01% butylated hydroxytoluene (BHT) as an antioxidant. Lipid classes were separated by one-dimensional double-development high-perfor-

mance thin-layer chromatography (HPTLC) using methyl acetate/isopropanol/chloroform/methanol/0.25% (w/v) KCl (5:5:5:2:1.8 by vol.) as the developing solvent system for the polar lipid classes and hexane/diethyl ether/acetic acid (22.5:2.5:0.25 by vol.) for the neutral lipid classes. Lipid classes were quantified by densitometry using a Shimadzu CS-9001PC spot scanner. Total and neutral lipid fractions were subjected to acid-catalyzed transmethylation for 16 h at 50°C using 1 ml of toluene and 2 ml of 1% sulfuric acid (v/v) in methanol. The resultant fatty acid methyl esters (FAME) were purified by TLC, and visualized by spraying with 1% iodine in chloroform [35]. FAME were separated, and quantified by using a Thermo gas chromatograph equipped with a flame ionization detector (250°C) and a fused silica capillary column Supelcowax TM 10 (30 m×0.32 mm I.D.). Individual FAMEs were identified by referring to authentic standards. Equal amounts of total lipids were used in all analyses. Throughout the manuscript, lipid nomenclature adhered LIPID MAPS classification system (http://www.lipidmaps.org/data/structure/index.html).

RNA isolation, cDNA microarray, probe preparation, and hybridization

Total RNA was isolated by homogenization of frozen rat tissues using a polytrone PT-2000 (Kinematica AG) and TriReagent (Sigma, St. Louis, MO) according to the protocol supplied by the manufacturer. All samples were treated with RNAse-free DNase (Promega, Madison, WI). RNA yields were measured by UV absorbance and the quality of total RNA was analyzed by using a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). A microarray containing 27000 rat 70-mer oligo probe sets produced at the KTH Microarray Center (www.biotech.kth.se) was used to evaluate the effects of hypothyroidism and hormonal replacement in TXOX animals on liver gene expression. Five µg of high-quality total RNA from the liver were reversed-transcribed, labeled, and hybridized following the manufacturer's protocol (Pronto Plus System, Promega). After 16 h of hybridization, the slides were washed and scanned using the GenePix Microarray Scanner (Axon Instruments, CA). Four independent hybridizations were performed comparing individual animals from the different experimental groups for a total of 4 analyses.

Microarray data processing and analysis

Image analysis was performed using the GenePix Pro 6.0 software (Axon Instruments, Union City, CA) as previously described [36]. The LOWESS (Locally Weighted Scatter Plot Smoother) method was used to normalize the raw intensity data [37]. If the measured probe sets were not present in at least 3 of the 4 chips, they were assumed to contain no information and therefore were eliminated to reduce data complexity. Differentially expressed genes were identified by using the SAM (Significance Analysis for Microarrays) statistical technique [38]. A q value was assigned for each of the detectable genes in the array. This value is similar to a P-value, measuring the lowest false discovery rate (FDR) at which differential expression of a gene is considered significant. A minimal FDR of 0.05 was assigned for each gene. In this work, a completed list of regulated genes is available as supplementary (S) files. An additional selection requirement was added to FDR based on absolute changes in the gene expression ratios. A value of 1.5 (50%) ($\log_2$ ratio $\geq |0.58|$) was chosen to describe ratios as up- or down-regulated. The microarray data discussed in this publication have been deposited in NCBIs Gene Expression Omnibus [39] and are accessible through GEO Series accession number GSE50014 (www.ncbi.nlm.nih.gov/geo). Functional and system biological network analyses were performed on the basis of the Gene Ontology (GO) enrichment of differentially expressed genes in liver using DAVID [40], and the results were depicted using Cytoscape [41]. For the graphical representation, the significance cut-off was set to a p value <0.05 and a corrected q value (Benjamini) <0.1. GO graphs interpretation: node (inner circle) size corresponds to the number of genes up-regulated by GH or E2; node border (outer circle) size corresponds to the number of genes down-regulated by GH or E2; color of the node and border corresponds to the significance of the gene set for up or down regulated genes, respectively (dark red = significantly enriched, light red = enriched no significantly; grey = absent); edge size corresponds to the number of genes that overlap between the two connected gene sets. Green edges correspond to shared up-regulated genes and blue edges correspond to shared down-regulated genes.

Analysis of gene expression by real-time quantitative-PCR (qPCR)

The mRNA expression levels of genes were measured using qPCR. Briefly, 2 µg of total RNA was treated with RNase-free DNase I (Promega) to remove genomic DNA and reverse transcribed using iScript (Bio-Rad) according to the manufacturer's instructions. Two µl of cDNA served as a template in a 20 µl qPCR reaction mix containing the primers and SYBR Green PCR Master Mix (Diagenode, Belgium). Quantification of gene expression was performed according to the manufacturer's protocol using ABI PRISM 7000 SD RT-PCR. A relative standard curve was constructed with serial dilutions (1:1, 1:10, 1:100) using a pool of the cDNA generated from all animals used in the study. The amplification program consisted of 1 cycle of 95°C for 10 min, followed by 45 cycles of 95°C for 15 s, annealing for 10 s, and 72°C for 30 s. The fluorescent intensity was measured at a specific acquisition temperature for each gene. A dissociation protocol was performed to assess the specificity of the primers and the uniformity of the PCR generated products. The amplified PCR products were subjected to agarose electrophoresis to confirm their predicted size. Data were extracted and amplification plots generated with ABI SDS software. All amplifications were performed in duplicate, and C_t scores were averaged for subsequent calculations of relative expression values. The level of individual mRNA measured by qPCR was normalized to the level of the housekeeping gene cyclophilin by using the Pfaffl method [42]. Exon-specific primers (Table S1) were designed by the Primer 3 program [43].

Statistical analysis

The significance of differences between groups was tested by one-way ANOVA, followed by post hoc comparisons of group means according to the GraphPad Prism 5 program (GraphPad Software, San Diego, CA). Statistical significance was reported if $P<0.05$ was achieved. For graphing purposes in the qPCR analysis, the relative expression levels were scaled so that the expression level of the INTACT group equaled one. Lipids classes and main fatty acids were additionally submitted to factor analysis by means of Principal Component Analysis (PCA) [44]. Variable extraction was carried out based on the proportion of total variance explained by two principal components. Factor scores for principal component 1 in multivariate analyses of lipid classes, and fatty acids from total and neutral lipid are depicted. Factor scores were further analyzed by one-way and to assess statistical differences between treatments and by two-way ANOVA to evaluate the combined effects of hormonal treatments and their interactions, as we have previously reported [45].

Results

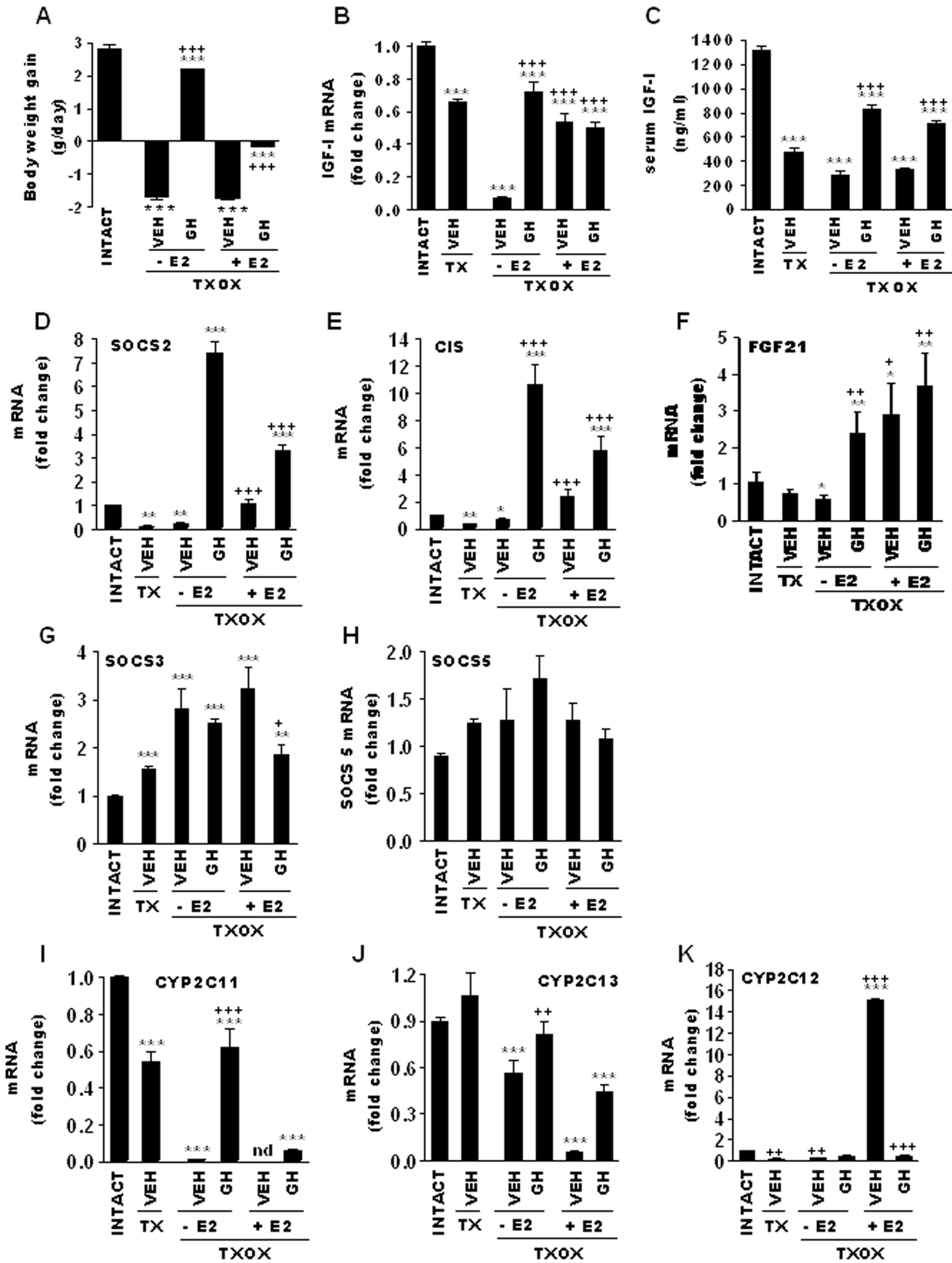
Estradiol inhibits the effects of GH on somatotropic-liver axis and induces negative regulators of GH-STAT5 signaling and a female-pattern of gene expression in adult hypothyroid-orchidectomized rat liver

Upon sacrifice on PND94, biochemical hypothyroidism was shown, and significantly ($P = 0.001$) lower or undetectable serum levels of T3 (ng/dl) [36.17±5.43 (INTACT); 7.92±4.84 (TXOX); 1.48±2.14 (TXOXE2); 0 (TXOXGH); 0.14±0.33 (TXOX-E2GH)] and T4 (ng/dl) [(1.90±0.17 (INTACT) *vs.* 0)] were found in all TXOX groups in comparison with the age-matched euthyroid control group (INTACT). Serum E2 levels (pg/ml) [6.33±5.57 (INTACT); 438.80±122.12 (TXOXE2); 363.10±107.95 (TXOXE2GH)] were increased ($P = 0.001$ *vs* INTACT) in E2-treated TXOX rats up to 5-10 times those observed in rats during pregnancy [46] or the proestrus phase of the reproductive cycle [12]. A first approach to assess the effects of E2 on TXOX rat liver was made through analysis of its influence on the somatotropic-liver axis. TXOX caused impaired growth, which was evident as a reduction in daily body weight gain (Fig. 1A). At the last measurement, on PND94, a significant difference ($P<0.001$) in body weight remained between INTACT (447±33 g) and TXOX (338±28 g) groups. Accordingly, TXOX showed reduced levels of hepatic IGF-I mRNA (Fig. 1B) and circulating IGF-I (Fig. 1C). Treatment of TXOX rats with GH, partially or totally, restored body weight gain (Fig.1A), liver IGF-I mRNA (Fig.1B), and circulating IGF-I (Fig.1C), whereas these effects were prevented in the presence of E2. Notably, E2 administration to TXOX rats increased hepatic IGF-I mRNA to the level of non-orchidectomized-hypothyroid (TX) rats (Fig.1B). Next, we carried out mRNA quantitative analysis of SOCS2, CIS, and FGF21, which are negative regulators of GHR-STAT5 signaling [47,48]. E2 treatment of TXOX rats induced the mRNA expression of SOCS2 (Fig. 1D) and CIS (Fig.1E), but only to a fraction of the levels obtained after GH treatment. Indeed, when E2 was used in combination with GH, a 2-3-fold reduced mRNA expression levels of SOCS2 (Fig. 1D) and CIS (Fig.1E) were observed compared with GH treatment alone, again demonstrating the inhibitory actions of E2 on GH hepatic actions. In contrast, the positive effect of GH on FGF21 mRNA was not affected in the presence of E2 (Fig.1F). The level of SOCS3 mRNA was induced by hypothyroidism itself (Fig.1G), whereas neither hypothyroidism nor hormonal replacement altered SOCS5 (Fig.1H). Finally, we measured GH-regulated gene markers of liver sexual dimorphism [15]. Figure 1I shows that the mRNA expression level of CYP2C11, a male-specific gene, was completely abolished in TXOX liver, whereas it was recovered by intermittent GH replacement. In contrast, E2 prevented the GH-induced mRNA expression levels of CYP2C11 (Fig.1I) and CYP2C13 (Fig.1J). Unlike intermittent GH, E2 induced the female-specific CYP2C12 gene in TXOX liver (Fig.1K). Our microarray analysis (see below) also showed that male predominant genes (e.g., CYP2C11, CYP2C13, CYP2E1, alpha-2u-globulin) were down-regulated by E2, whereas female predominant genes (e.g., CYP2C12, CYP2A1, CYP2C7) were induced [15]. Overall, these findings demonstrate that E2 influences the transcription of GHR-STAT5 targeted genes and induces a female-pattern of gene expression in TXOX rat liver.

Influence of estradiol on serum and hepatic lipids in hypothyroid-orchidectomized rats

The effects of E2 and GH on hepatic lipid composition were explored in TXOX rats by carrying out a quantitative analysis of lipid classes (Table 1) and fatty acids from total (Table 2) and neutral (Table 3) lipids. The development of a hypothyroid state in male rats was accompanied by altered circulating lipids: a 2-fold increase of total CHO and a 3-fold decrease of TG (Table 1). However, these changes were not prevented by E2 or GH replacement. Hypothyroidism increased the hepatic levels of CHO and decreased those of TG and diacylglycerols (DG) in comparison with the INTACT group (Table 1). However, the levels of free fatty acids (FFA) were not significantly affected by hypothyroidism, though the average values were lower than in the INTACT animals. Among fatty acids from neutral lipids (Table 3), TXOX rats contained increased levels of total saturated fatty acids (SFA) (due to the significant increase in18:0 content). Among monounsaturated fatty acids (MUFAs), 18:1n-9 (oleic acid), 16:1n-7 (palmitoleic acid), and 18:1n-7 (vaccenic acid) were all significantly reduced in neutral lipids (where these MUFAs are abundant) compared with INTACT animals. Interestingly, these changes paralleled the increase in 18:0. These findings suggest an alteration of Δ9 desaturase in TXOX animals. The most representative 18 carbon polyunsaturated fatty acids (PUFAs), 18:2n-6 (linoleic acid) and 18:3n-3 (linolenic acid) from total (Table 2) or neutral (Table 3) lipids, were unaffected in TXOX. Noticeably, TXOX rats exhibited increased levels of 20:4n-6 [arachidonic acid (AA)] and reduced levels of its elongated precursor 22:4n-6. This later effect on AA was a direct consequence of hypothyroidism and orquidectomy and could be restored by the hormonal therapies used here, especially when used in combination. Another physiologically relevant very long chain polyunsaturated fatty acid (VLCPUFA), namely 22:6n-3 (DHA), exhibited a significant reduction in total lipids from TXOX rats (Table 2). As expected, levels of essential fatty acids 20:4n-6 and 22:6n-3 in INTACT and TXOX animals were three-to-four times higher in total lipids than in neutral lipids, confirming their preferential location within membrane phospholipids. Moreover, the results from total lipids indicate that hypothyroidism and orquidectomy strongly affect the acylation of DHA-containing phospholipids in liver cells. These findings are physiologically relevant since depletion of membrane 22:6n-3 is known to severely impact physicochemical properties of cell membrane [45,49].

E2 treatment in TXOX rats brought about an important reduction in total SFA compared with the TXOX group, this effect was due to the significant reduction in 14:0 (myristic acid), 16:0 (palmitic acid), and 18:0 (stearic acid) from both total (Table 2) and neutral (Table 3) lipids. In parallel to these changes, FFA as a lipid class and DG were dramatically reduced by more than 75% and 38%, respectively, whereas TG increased significantly by 72% in comparison to TXOX rats. It is worth mentioning that E2 did not alter VLCPUFA metabolism in terms of the levels of 20:4n-6, 20:5n-3, and 22:6n-3, which remained similar to the values for neutral and total lipids in the TXOX group. However, E2 increased the levels of the essential n-6 precursor 18:2n-6 (but not the essential n-3 precursor 18:3n-3) in neutral lipids. Noticeably, E2 treatment partly reversed the effects of hypothyroidism on MUFAs. Thus, E2 significantly increased levels of 18:1n-9 (and also those of 16:1n-7 acid though to a lower extent) in total and neutral lipids (Table 2 and 3, respectively), which, together with the reductions in 16:0 and 18:0, point to an upregulation of stearoyl-CoA desaturase (Scd) genes in response to E2. Overall, these changes indicate that E2 stimulates β-oxidation of SFA in the



A
Bodyweight gain (g/day)
INTACT
VEH
GH
- E2
+ E2
TXOX
B
IGF-I mRNA (fold change)
TX
C
serum IGF-I (ng/ml)
D
SOCS2
mRNA (fold change)
E
CIS
F
FGF21
G
SOCS3
H
SOCS5
SOCS 5 mRNA (fold change)
I
CYP2C11
nd
J
CYP2C13
K
CYP2C12

Figure 1. E2 inhibits the effects of GH on somatotropic-liver axis and induces negative regulators of GH-STAT5 signaling and a female-pattern of gene expression in hypothyroid-orchidectomized rat liver. Euthyroid testis-intact controls (INTACT) and hypothyroid-orchidectomized (TXOX) rats were described in Material and Methods. E2 or vehicle (VEH) administration to TXOX rats was performed for 20 days. Then, GH replacement during 7 days rats was carried out in TXOX in the absence (-E2) or in the presence (+E2) of E2. On PND90, body weight gain (A), hepatic IGF-I mRNA (B), circulating IGF-I (C), SOCS2 (D), CIS (E), FGF21 (F), SOCS3 (G), SOCS5 (H), CYP2C11 (I), CYP2C13 (J), and CYP2C12 (K) were measured by qPCR. Results are expressed as mean ± S.D. (n = 6). ***, $P<0.001$ for comparison with vehicle treated INTACT group; +, $P<0.05$, ++, $P<0.01$, +++, $P<0.001$ for comparison with vehicle treated TXOX group.
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liver while it increases the depots of n-6 polyunsaturated precursors in TG. Regarding other lipid classes, namely, CHO, CHO esters (CE), and phospholipids, we observed that E2 slightly reduces hepatic CHO levels (yet not statistically significant) compared with TXOX but induces a significant increase in the formation of CE (32% and 47% over TXOX and INTACT groups, respectively). This effect on CE formation is independent of hypothyroidism but totally determined by E2 and likely reflects an up-regulation of acyl:cholesterol acyltransferase (ACAT) activity (see discussion).

To some extent, GH administration to TXOX rats resembled the effects of E2 on hepatic lipid composition described above. Thus, GH increased TG and decreased FFA hepatic contents compared with TXOX rats. However, the levels of DG were notably increased by GH treatment in the TXOX group compared with the vehicle- or E2-treated TXOX group (Table 1). TG levels were also increased in serum from GH-treated rats (Table 1). Compared with the TXOX group, the hepatic levels of 18:0, 16:0 and total SFA in neutral lipids (Table 3) were reduced in GH-treated animals. Interestingly, unlike in E2-treated animals, the levels of 18:0 and 16:0 in neutral and total lipids in GH-treated rats approached those observed in INTACT animals. Taken together, these data suggest that GH induces a lipogenic effect in hypothyroid animals by mobilizing SFAs as FFA→DG→TG. Furthermore, when compared with vehicle- or E2-treated TXOX group, GH treatment induced a significant increase in 20:4n-6 and 22:6n-3 in total lipids (Table 2), which is reflected in the high levels of VLCPUFA observed in this group of animals. Because these later effects on LCPUFA were not observed in neutral lipids, the results point to a significant effect of GH on phospholipid remodeling, though the effect of E2 in modulating phospholipid acylation-reacylation has been established [34], similar effects induced by GH or by the combined treatment E2+GH represent a novel finding. Finally, CHO levels in the GH-treated TXOX group were returned to values observed in the INTACT group, but as in the E2-treated TXOX group, an increased CE level was present in response to GH (Table 1).

In the presence of E2, GH gave rise to a complex hepatic lipid phenotype. Thus, total SFA, 14:0, 16:0, and 18:0 in total lipids (Table 2), were reduced compared with TXOX and reached the levels observed in INTACT animals. In neutral lipids (Table 3), however, the contents of these fatty acids were significantly lower than in TXOX. Among VLCPUFA, AA was also decreased well below TXOX animals to values similar to those found in the INTACT group in neutral lipids (Table 3), while as in the TXOXGH group, DHA was significantly increased in the phospholipids of the TXOXE2GH group to achieve the levels in INTACT animals. A striking effect of the combined effects of E2 and GH is the complete restoration of MUFA levels from total and neutral lipids, an effect attributable to the increase in 18:1n9, likely through alteration of Δ9 desaturase expression. Interestingly, the combined E2+GH treatment gave raise to significantly higher MUFA levels compared to E2 and GH treatments individually. Given that MUFA levels of in GH-treated rats were identical to those in TXOX rats, and that E2 treatment increased MUFA (especially 18:1n-9) as compared for GH, the results suggest a permissive action of GH on E2 effect. We have described above that E2 and GH increased hepatic CHO and CE, but the combined effect of the two hormones seemed to be additive with regard to CE, because its levels doubled those found in INTACT animals and were approximately 30% higher than in the E2 and GH groups (Table 1). Conversely, in the presence of E2, GH reduced the hepatic CHO content compared not only to the TXOX group but also in relation to the E2- or GH-treated TXOX groups, indicating an antagonistic hormonal interaction. Finally, the significant increase in hepatic TG by GH in the presence of E2, with the largest content among all groups (>96% compared with the INTACT and >133% compared with the TXOX group), points to a substantial stimulation of hepatic lipogenesis by the combination of E2 and GH.

Finally, we performed PCA to identify data that discriminate groups. Figure 2 shows the outcomes of PCA on lipid profiles for lipid classes and total and neutral lipids. For lipid classes (Fig. 2A), PC1 (principal component 1) was positively related to FFA, CHO, sphingomyelin (SM) and phosphatidylethanolamine (PE), and negatively correlated with CE and TG. On the other hand, for fatty acids from neutral (Fig. 2C) and total (Fig. 2B) lipids, PC1 was negatively related to C18 PUFA (18:2n-6, 18:3n-6 and 18:3n-3) in total lipids, and positively to saturated (14:0, 15:0 and 16:0) and n-7 MUFA. Therefore, PCA allowed a substantial simplification of lipid data for group discrimination. When we computed factor scores 1 and 2 from PC1 to obtain a group simplification for lipid classes (Fig. 2A) and fatty acids from total (Fig. 2B), and neutral lipids (Fig. 2C), these results allowed a neat discrimination between HRT conditions for all three analyses. INTACT rats are represented by a discrete cluster whereas the effects of E2 or GH on TXOX rats can be distinguished from the untreated TXOX group and display some degree of overlap with the combined E2 and GH treatments. Thus, in contrast with somatic growth in which E2 clearly prevented GH actions, the quantitative lipid analysis displays a more complex picture of the molecular changes induced by the different hormonal treatments. The E2 and GH effects on lipid contents display significant similarities but also treatment-dependent specific effects, such those leading to the regulation of DG content by GH.

Estradiol influences liver transcriptome in hypothyroid male rats

Next, we performed a genome wide gene expression analysis to better understand the influence of E2 on liver physiology. This experiment identified 634 genes that were differentially regulated in TXOX rats after E2 treatment (Table S2). Next, we identified the active biological processes from expression profiles by GO enrichment analysis [40] and system biological network [41] analysis. GO enrichment analysis of the 352 genes up-regulated by E2 revealed a significant over-representation of genes related to fatty acid metabolism whereas among the 282 down-regulated genes, over-representation of genes involved in steroid and xenobiotic metabolism was observed (Fig. 3). Accordingly, the genes up-regulated by E2 were clustered in cellular pathways (KEEG) related to the PPARα signaling (P = 1,4E-05; Bonferroni

Table 1. Effects of hormonal replacement on serum and hepatic lipid classes.

Lipid classes	INTACT		TXOX		TXOXE2		TXOXGH		TXOXE2GH	
	mean ± SEM		mean ± SEM		mean ± SEM		mean ± SEM		mean ± SEM	
Serum										
Glucose (g/dl)	141,2±4.7	a	126,8±6,2	ab	112,0±3,9	bc	107,7±7,1	bc	95,0±3,6	d
CHO (mg/dl)	67,2±3	a	126,5±10,1	b	141,2±12,9	b	129,3±12,9	b	143,5±6,4	b
TG (mg/dl)	133±16,3	a	44±5,1	b	52,6±7,5	b	43,8±6,5	b	77,3±7,3	b
Leptin (ng/ml)	2,8±0,5		2,6±1,1		1,1±0,4		2,1±0,3		1,2±0.6	
Hepatic (%)										
LPC	nd		nd		nd		nd		nd	
SM	0,9±0,0	a	1,0±0,0	a	0,5±0,0	b	0,7±0,2	ab	0,4±0,1	ab
PC	25,3±0,6	a	26,9±1,2	ab	30,6±0,6	b	28,5±0,7	ab	26,9±1,1	ab
PS	2,3±0,0	a	1,8±0,2	a	3,3±0,1	b	2,7±0,3	ab	2,9±0,2	ab
PG	5,5±0,1		5,3±0,3		5,3±0,3		4,5±0,5		5,6±0,4	
PE	17,4±0,3	a	18,1±0,6	a	16,1±0,5	ab	17,6±1,6	ab	13,5±0,5	b
PLE	0,8±0,2		0,4±0,0		0,3±0,1		0,3±0,0		0,3±0,1	
DG	1,7±0,3	a	0,9±0,2	b	0,6±0,1	b	1,8±0,1	a	1,7±0,2	a
CHO	14,7±0,7	bc	17,1±0,3	d	15,6±0,4	cd	13,2±0,2	b	11,3±0,7	a
FFA	7,9±0,5	b	7,1±0,9	b	1,8±0,3	a	2,5±0,5	a	2,0±0,3	a
TG	8,3±0,4	a	7,0±0,4	a	12,1±0,6	bc	11,5±1,0	bc	16,4±3,0	c
CE	4,1±0,3	a	4,6±0,1	a	6,0±0,1	b	6,5±0,1	b	9,5±1,1	c
TPL	63,2±0,6		62,9±1,7		63,7±0,9		64,6±0,7		59±2,8	
TNL	36,8±0,6		37,1±1,7		36,3±0,9		35,4±0,7		41±2,8	

Euthyroid testis-intact controls (INTACT) and hypothyroid-orquidectomized (TXOX) rats were performed as described in Material and Methods. Long-term administration of E2 (TXOXE2) or vehicle (VEH) to TXOX rats was performed for 20 days. Then, VEH (TXOX), E2 (TXOXE2), GH (TXOXGH) or E2 plus GH (TXOXE2GH) replacements were performed for additional 7 days. On PND90, the animals were sacrificed and serum and hepatic lipid classes were measured. Data are expressed as mean ± SEM for serum (n = 5) or hepatic (n = 5) independent samples (different animals) and each independent sample was tested twice. Values represent weight percent of total lipid. Values were submitted to ANOVA followed by post hoc Tukey's test. Values in the same row with different lowercase letters are significantly different with $P < 0.05$.

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Table 2. Effects of hormonal replacement on fatty acid composition from liver total lipids.

TOTAL LIPIDS	INTACT		TXOX		TXOXE2		TXOXGH		TXOXE2GH	
	mean ± SEM		mean ± SEM		mean ± SEM		mean ± SEM		mean ± SEM	
Fatty acids										
C 14: 0	0,5±0,1	ab	0,5±0,1	b	0,3±0,0	a	0,5±0,0	ab	0,3±0,0	ab
C 16: 0	19,8±0,5	ab	20,2±0,6	b	18,5±0,1	a	19,1±0,3	ab	18,5±0,6	a
C 16:1 n-9	0,3±0,0		0,4±0,0		0,3±0,0		0,4±0,0		0,3±0,0	
C 16: 1 n-7	2,2±0,4		1,4±0,1		1,6±0,1		1,5±0,3		1,7±0,2	
C 18: 0	13,2±0,2	a	15,8±0,4	c	14,5±0,2	b	14,7±0,2	b	12,7±0,3	a
C 18: 1 n-9	10,6±0,5	a	10,3±0,4	a	12±0,5	ab	10,4±0,5	a	13,9±0,6	b
C 18: 1 n-7	5,6±0,4	b	2,6±0,3	a	2,8±0,1	a	3,0±0,2	a	2,9±0,1	a
C 18: 2 n-6	15,8±0,9		18,2±0,7		19,1±0,3		17,1±0,9		18,7±0,9	
C 18: 3 n-6	0,2±0,0	a	0,8±0,1	b	1,4±0,1	c	0,6±0,0	b	1,3±0,3	abc
C 18: 3 n-3	0,3±0,0		0,3±0,0		0,4±0,0		0,3±0,0		0,3±0,1	
C 18: 4 n-3	0,0±0,0	a	0,0±0,0	ab	0,1±0,0	b	0,0±0,0	ab	0,1±0,0	b
C 20: 3 n-6	0,7±0,0	b	1,2±0,2	c	0,4±0,0	a	0,8±0,0	b	0,5±0,0	a
C 20: 4 n-6	19±0,9	ab	18,1±0,2	a	17,5±0,2	a	20,2±0,4	b	18,8±1,4	ab
C 20: 5 n-3	0,2±0,0	a	0,4±0,1	b	0,4±0,0	b	0,3±0,1	ab	0,2±0,1	ab
C 22: 4 n-6	0,8±0,1		0,5±0,0		0,7±0,1		0,8±0,1		0,6±0,1	
C 22: 5 n-6	1,0±0,2		0,5±0,0		1,0±0,1		0,8±0,1		0,8±0,1	
C 24: 0	0,4±0,0	a	0,6±0,0	b	0,6±0,0	b	0,6±0,1	b	0,5±0,1	ab
C 22: 6 n-3	4,4±0,2	b	3,4±0,2	a	4,0±0,0	ab	4,4±0,3	b	4,2±0,1	b
Totals										
SFA	35,3±0,3		38,7±0,8		35,2±0,2		36,1±0,4		33,2±0,6	
MUFA	19,6±1,1	b	17,3±1,9	a	17,3±0,4	ab	16,1±0,9	a	19,5±0,7	b
PUFA	43,7±1,5		43,2±2,0		46,9±0,3		47,0±10,9		46,9±0,8	
VLCPUFA	27±0,7		24,5±1,2		25,6±0,4		28,5±0,7		26,1±1,6	

Euthyroid testis-intact controls (INTACT) and hypothyroid-orquidectomized (TXOX) rats were performed as described in Material and Methods. Administration of E2 (TXOXE2) or vehicle (TXOX) to TXOX rats was performed for 20 days. Then, VEH (TXOX), E2 (TXOXE2), GH (TXOXGH) or E2 plus GH (TXOXE2GH) replacements were performed for additional 7 days. On PND90, animals were sacrificed and the liver extracted for fatty acid analyses as described in Material and Methods. Data are expressed as mean ± SEM for 5 independent samples (different animals) and each independent sample was tested twice. Values represent weight percent of total lipid. Values were submitted to ANOVA followed by post hoc Tukey's test. Values in the same row with different lowercase letters are significantly different with $P<0.05$.
doi:10.1371/journal.pone.0096305.t002

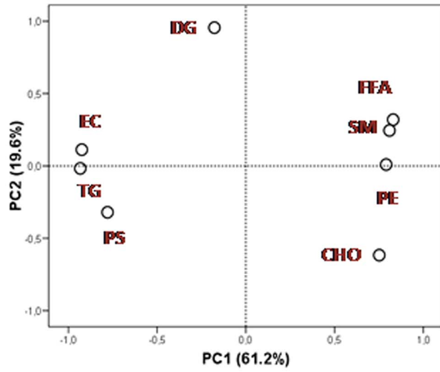
Table 3. Effects of hormonal replacement on fatty acid composition from hepatic neutral lipids.

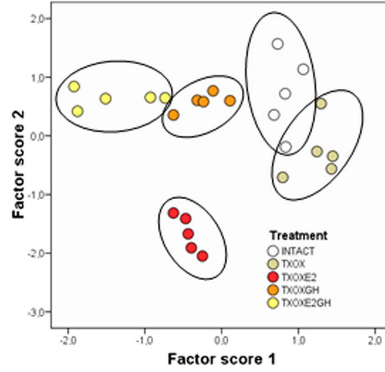
NEUTRAL LIPIDS	INTACT		TXOX		TXOXE2		TXOXGH		TXOXE2GH	
	mean ± SEM		mean ± SEM		mean ± SEM		mean ± SEM		mean ± SEM	
Fatty acids (%)										
C 14: 0	1,0±0,1	b	1,0±0,1	b	0,5±0,0	a	0,9±0,1	ab	0,6±0,1	ab
C 16: 0	25,2±0,8	b	25,4±1,0	b	22,2±0,4	ab	22,9±0,6	ab	21,6±1,1	b
C 16:1 n-9	0,6±0,1	ab	0,7±0,0	b	0,5±0,0	a	0,7±0,0	ab	0,5±0,0	ab
C 16: 1 n-7	4,1±0,8		2,5±0,1		2,8±0,2		2,8±0,5		3,3±0,6	
C 18: 0	4,3±0,3	ab	7,7±0,7	c	4,2±0,2	ab	5,2±0,5	b	3,3±0,3	a
C 18: 1 n-9	22,1±1,0	b	18,3±0,8	a	22,6±0,8	b	20,6±1,0	ab	26,1±0,3	c
C 18: 1 n-7	6,3± 0,7	b	3,3±0,3	a	3,1±0,1	a	3,5±0,3	a	3,7±0,2	a
C 18: 2 n-6	20,9±1,7	b	22,3±1,3	ab	25,2±0,8	a	23,3±1,7	ab	25,1±0,8	a
C 18: 3 n-6	0,2±0,0	a	1,1±0,1	b	2,3±0,1	c	1,1±0,1	b	1,8±0,5	abc
C 18: 3 n-3	0,7±0,1		0,7±0,1		0,8±0,0		0,7±0,1		0,8±0,1	
C 18: 4 n-3	0,0±0,0	a	0,1±0,0	abc	0,3±0,0	b	0,1±0,0	abc	0,2±0,0	c
C 20: 3 n-6	0,4±0,0	b	0,6±0,1	c	0,3±0,0	a	0,6±0,0	bc	0,5±0,1	bc
C 20: 4 n-6	5,6±0,8	a	8,6±0,3	b	7,3±0,3	ab	7,8±0,5	ab	6,0±1,2	ab
C 20: 5 n-3	0,3±0,0		0,4±0,1		0,5±0,1		0,5±0,2		0,3±0,1	
C 22: 4 n-6	1,0±0,2		0,5±0,1		0,7±0,1		1,2±0,2		0,7±0,1	
C 22: 5 n-6	0,6±0,1	a	0,3±0,1	a	0,4±0,1	ab	0,6±0,0	b	0,3±0,0	a
C 22: 6 n-3	1,5±0,2	b	1,2±0,1	ab	1,1±0,0	ab	1,4±0,2	b	0,8±0,1	a
C 24: 0	0,5±0,1	a	1,0±0,1	b	0,9±0,1	b	0,9±0,1	b	0,5±0,2	a
Totals										
SFA	32,1±0,8	b	36,5±1,2	c	28,9±0,3	ab	31,2±1,2	b	27,1±0,7	a
MUFA	34,4±2,3	b	25,8±1,1	a	29,8±0,8	ab	28,5±1,7	ab	34,6±0,8	b
PUFA	32,5±3,0		37,1±1,6		40,5±0,9		39,2±2,1		37,8±1,2	
VLCPUFA	10,1±1,4		12,6±0,4		11,2±0,5		13,0±0,7		9,5±1,4	

Euthyroid testis-intact controls (INTACT) and hypothyroid-orquidectomized (TXOX) rats were performed as described in Material and Methods. Administration of E2 (TXOXE2) or vehicle (TXOX) to TXOX rats was performed for 20 days. Then, VEH (TXOX), E2 (TXOXE2), GH (TXOXGH) or E2 plus GH (TXOXE2GH) replacements were performed for additional 7 days. On PND90, animals were sacrificed and the liver extracted for fatty acid analyses as described in Material and Methods. Data are expressed as mean ± SEM for 5 independent samples (different animals) and each independent sample was tested twice. Values represent weight percent of total lipid. Values were submitted to ANOVA followed by post hoc Tukey's test. Values in the same row with different lowercase letters are significantly different with $P<0.05$.

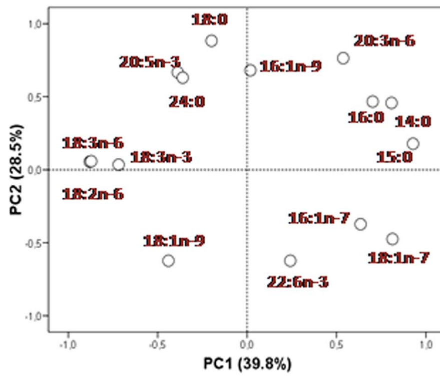
doi:10.1371/journal.pone.0096305.t003

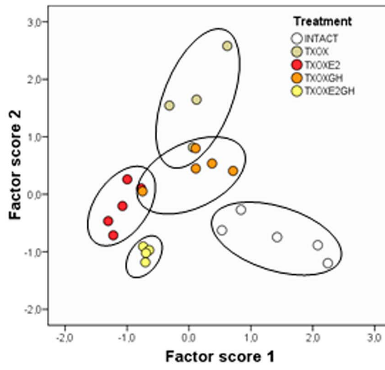
A) Lipid classes



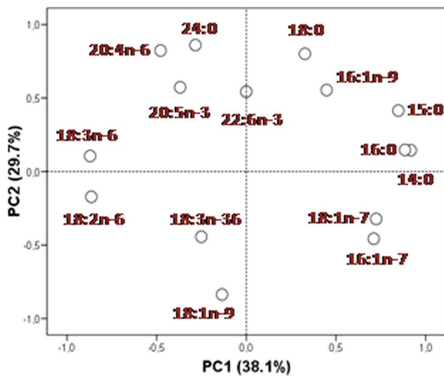


B) Fatty acids (total lipids)





C) Fatty acids (neutral lipids)



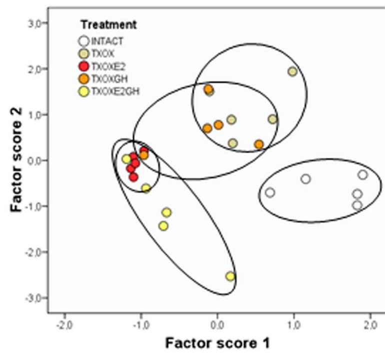


Figure 2. Principal component analysis for liver lipid composition. (A) Lipid classes, (B) Total lipids and (C) Neutral lipids. Left panels represent the factor loadings of principal components 1 (PC1) and 2 (PC2), and right panels the factor scores plots for PC1. Percent values in parentheses indicate the proportion of overall variance explained by each principal component. Each ellipse denotes a hormonal cluster. SM: sphingomyelin, PS: phosphatidylserine, PE: phosphatidylethanolamine, CHO: cholesterol, SE: sterol esters, FFA: free fatty acids, DG: diacylglycerols, TG: triacylglycerols.
doi:10.1371/journal.pone.0096305.g002

= 0.002) and biosynthesis of unsaturated fatty acids (P = 8,2E-04; Bonferroni = 0.02). Among the genes up-regulated by E2 we observed PPARα and PPARα target genes [48,50] such as CYP4A1, CYP4A3, FGF21, carnitine palmitoyltransferase 2 (CPT-2), Scd1, LCFA-CoA ligase 4, acyl-CoA oxidase (ACOX1), acyl-CoA synthetase (ACS), fatty acid translocase (FAT/CD36), angiopoietin-like 4 (ANGPTL4), ELOVL5, and BAAT. The fatty acid desaturases FAD6 and FAD1 were also upregulated by E2. In contrast, genes involved in the metabolism of C21-steroid hormones (P = 1,6E-05; Bonferroni = 0.001), metabolism of xenobiotic by cytochrome 450 (P = 1,2E-04; Bonferroni = 0.006), glutathione (P = 0.002; Bonferroni = 0.04), and androgen and estrogen

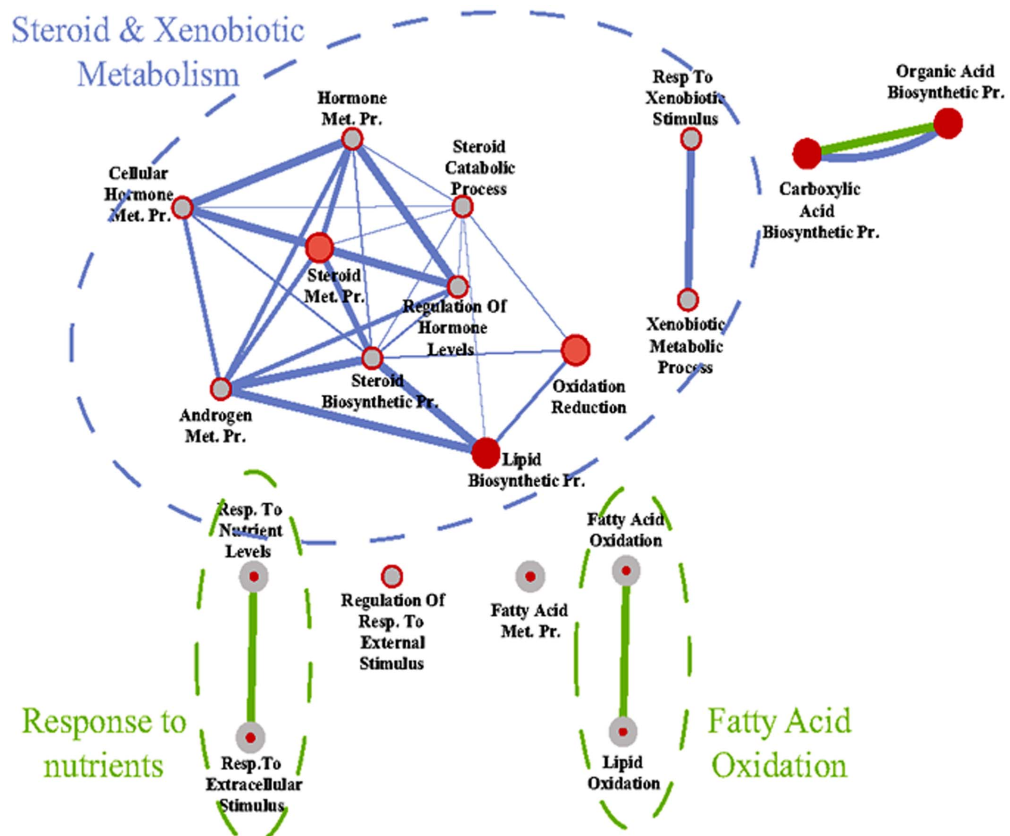


Figure 3. System biological network analyses of the effects of E2 on liver transcriptome in hypothyroid-orchidectomized rats. The genes differentially-expressed in the livers were identified by DNA microarrays as described under Material and Methods. Then, functional and system biological network analysis were performed on the basis of the GO enrichment of differentially-expressed genes in liver using DAVID, and the results depicted using Cytoscape. Node (inner circle) size corresponds to the number of genes up-regulated by E2; node border (outer circle) size corresponds to the number of genes down-regulated by E2; color of the node and border corresponds to the significance of the gene set for up or down regulated genes, respectively (dark red = significantly enriched, light red = enriched no significantly; grey = absent); edge size corresponds to the number of genes that overlap between the two connected gene sets. Green edges correspond to shared up-regulated genes and blue edges correspond to shared down-regulated genes.
doi:10.1371/journal.pone.0096305.g003

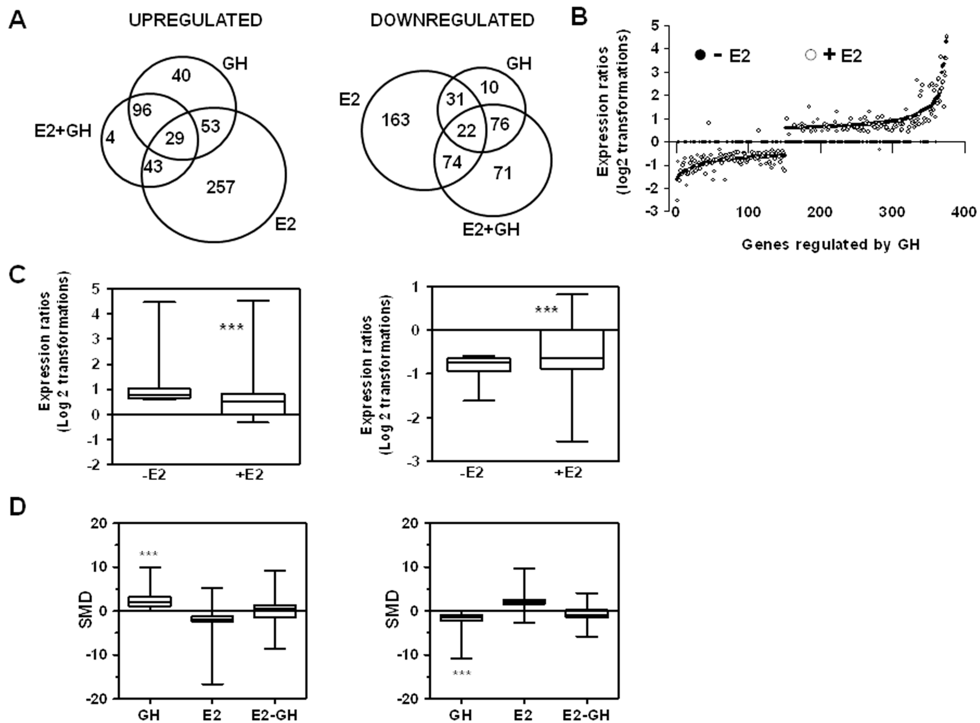


Figure 4. E2 influences the gene expression profiling regulated by GH in hypothyroid-orchidectomized rat liver. Hypothyroid-orchidectomized (TXOX) rats were injected with GH for 7 days in the absence (−E2) or in the presence (+E2) of E2. Differently expressed genes in the livers were identified by DNA microarrays as described under Material and Methods. (A) The number of genes regulated by GH in the absence of E2, by E2, or GH in the presence of E2. The overlapping areas show genes for which expression was altered by GH in the absence or presence of E2. (B) Individual genes are arranged along the X axis according to the value order of decreases and increases in gene expression measured in GH-treated TXOX rats in the absence of E2. The Y axis shows the log 2 ratio of the transcript signals in GH-treated TXOX rats in the absence (−E2) and in the presence (+E2) of E2. (C) Box plot shows a statistical evaluation of the differences in the mean expression changes induced by GH in the absence (−E2) or in the presence (+E2) of E2 for the set of genes induced and repressed by GH treatment in the absence of E2. (D) SAM multiclass analysis was performed to identify GH regulated genes whose mean expression (SMD) values were significantly different from E2 or E2 plus GH-treated TXOX rats. In Box plots, the lines connect the medians, the boxes cover the 25th to 75th percentiles, and the minimum and maximum values are shown by the ends of the bars. ***, $P<0.001$.
doi:10.1371/journal.pone.0096305.g004

(P = 0.002; Bonferroni = 0.05) were significantly down-regulated by E2 [i.e., aldo-keto reductase 1D1 (Akr1d1), epoxide hydrolase 1; glutathione S-transferases (GST) (mu 2, mu 3, mu 4, mu 7, pi 1), CYP3A18, CYP2C23, CYP2E1, CYP17A1, 3β-HSD, 11β-HSD1, and estrogen sulfotransferase (SULT1E)]. Overall, these results reveal an extensive re-programing of liver's transcriptome by long-term E2 treatment of TXOX rats, particularly genes involved in the metabolism of fatty acids and endo-xenobiotics.

Estradiol influences GH-regulated liver transcriptome in hypothyroid male rats

Finally, we performed a genome wide gene expression analysis to better understand the interplay between E2 and GH in liver. We first defined the gene expression changes induced by GH replacement in TXOX rats (Table S3). Second, we analyzed the similarities in gene expression changes induced by treatment with E2 or GH in TXOX rats and identified 869 significantly (FDR < 5%) regulated transcripts in both cases. A Spearman rank correlation test comparing the E2 and GH effects on these genes yields a positive correlation (r = 0.6287; $P<0.0001$) indicative of strong similarities between E2 and GH effects in the TXOX liver. Accordingly, 94% of these genes were regulated by E2 and GH in the same direction. Third, we obtained transcription profiles from TXOX rats simultaneously treated with both E2 and GH (Table S4). A comparative analysis of genes with altered expression levels in TXOXGH or TXOXE2GH groups revealed considerable reduction in the presence of E2 of the effects induced by GH in TXOX rats (Fig. 4A–B). This was a general phenomenon that affected a large fraction of GH-regulated genes. Accordingly, in the absence of E2, the average expression changes (log_2) across four independent hybridizations were 0.98 ± 0.04 and -0.82 ± 0.02 for GH-induced (Fig. 4C) and GH-repressed (Fig. 4D)

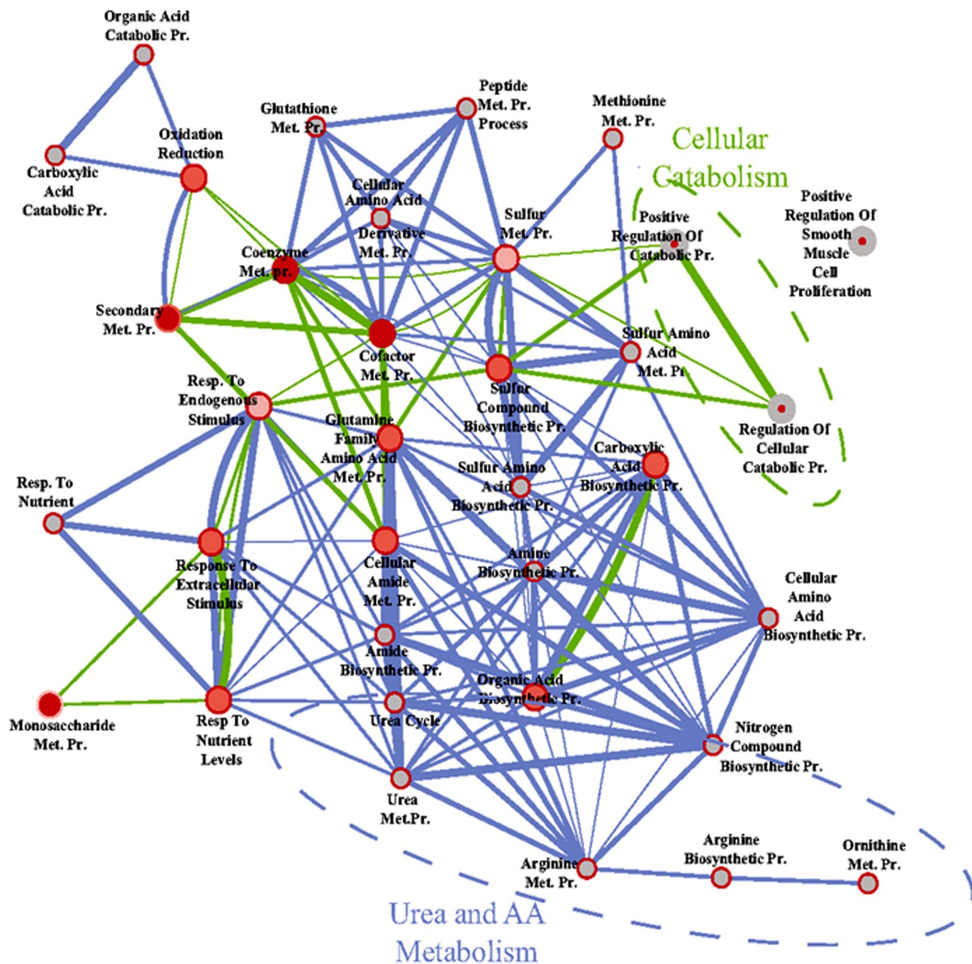


Figure 5. System biological network analyses on GH effects on liver transcriptome in hypothyroid-orchidectomized rats. The differentially-expressed genes in the livers were identified by DNA microarrays as described under Material and Methods. Then, functional and system biological network analysis were performed on the basis of the GO enrichment of differentially-expressed genes in liver using DAVID, and the results depicted using Cytoscape. Node (inner circle) size corresponds to the number of genes up-regulated by GH; node border (outer circle) size corresponds to the number of genes down-regulated by GH; color of the node and border corresponds to the significance of the gene set for up or down regulated genes, respectively (dark red = significantly enriched, light red = enriched no significantly; grey = absent); edge size corresponds to the number of genes that overlap between the two connected gene sets. Green edges correspond to shared up-regulated genes and blue edges correspond to shared down-regulated genes.
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genes, respectively, whereas the average fold regulation for the same set of genes in the presence of E2 was 0.60 ± 0.05 (Fig. 4C) and -0.61 ± 0.04 (Fig. 4D). These differences were significant ($P < 0.001$) and demonstrate an inhibition by E2 of the hepatic response to GH treatment. Finally, SAM multiclass analysis [38] identified genes regulated by GH whose mean expression values were significantly different from those in E2- or E2 plus GH-treated TXOX rats (Fig. 4E and 4F and Table S5) (e.g., Snapc2, SLC13A2, Apo-H, TNFR, SULT1E1, CYP2C11, CYP2C12, EGFR, Hsd3b6, SPI, alpha 2u-globulin, FTO, Acacb, PPARα, ACOX1, SOCS5, Akr1c14).

Functional analysis [40] revealed that the biological processes over-represented in the list of genes up-regulated by GH in TXOX liver, were connected with the positive regulation of

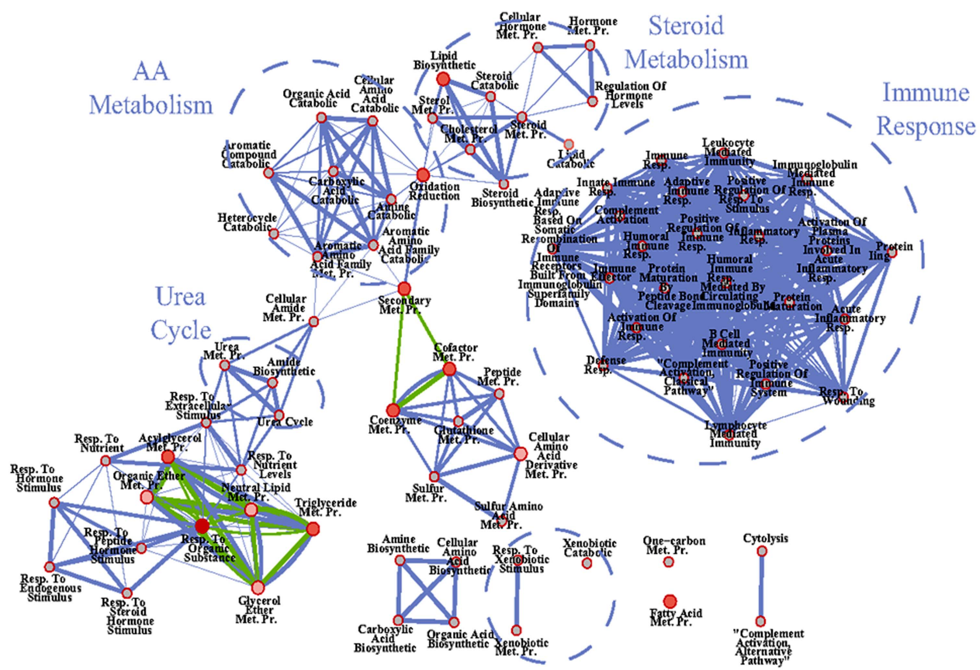

Figure 6. System biological network analyses on GH effects on liver transcriptome in E2-treated hypothyroid-orchidectomized rats. The differentially-expressed genes in the livers were identified by DNA microarrays as described under Material and Methods. Then, functional and system biological network analysis were performed on the basis of the GO enrichment of differentially-expressed genes in liver using DAVID, and the results depicted using Cytoscape. Node (inner circle) size corresponds to the number of genes up-regulated by E2-GH; node border (outer circle) size corresponds to the number of genes down-regulated by E2-GH; color of the node and border corresponds to the significance of the gene set for up or down regulated genes, respectively (dark red = significantly enriched, light red = enriched no significantly; grey = absent); edge size corresponds to the number of genes that overlap between the two connected gene sets. Green edges correspond to shared up-regulated genes and blue edges correspond to shared down-regulated genes.
doi:10.1371/journal.pone.0096305.g006

cellular catabolism (e.g., GCLC, GCK, ABHD5, IGF-I, APOC2, HSP90AB1) whereas the metabolism of aminoacids and urea (e.g., CTH, ARG1, ASS1, OTC, ASL, CPS1, MAT1A, MTR, BHMT, PAH) was significantly down-regulated (Fig. 5). When these changes were mapped to cellular pathways (KEEG), we confirmed a significant connection with the metabolism of aminoacids and urea, metabolism of xenobiotics by P450, or PPARα signaling (Table 4). In contrast to E2, GH repressed the PPARα signaling pathway. Notably, in the presence of E2, the biological functions and network regulated by GH varied notably (Fig. 6). Thus, whereas GH still reduced the expression of genes involved in urea and amino acid metabolism in the presence of E2 [e.g., carbamoyl-phosphate synthetase-1 (CPS1), ornithine carbamoyltransferase (OTC); argininosuccinate synthase (ASS1)], it was no longer able to induce the expression of genes related to cell catabolism [e.g., glutamate-cysteine ligase (GCLC) and APOC2] (Table 5 and Table S4). In contrast, genes involved in the metabolism of steroids and xenobiotics (e.g., Gst2, Gst-m3, Gst-m4, EPHX1, CYP2E1, Gst-p1, Gst-m7) downregulated by E2 but not by GH alone are even further inhibited by the combined treatment. Finally, when E2 was present, GH exerted a significant influence on immune system response (Fig. 6). In particular, the mRNA expression levels of genes involved in complement activation, lymphocyte immune response, wounding or acute inflammatory response were significantly reduced (e.g., C9, C4a, AHCY, ASS1, MASP1, C5, CLU, CST3, A1I3, C4BPB, SERPING1, C1S, C4BPA, C8B, SDC1, IL10RB, IldR1, SLC7A2, PIGR, sialomucin, IGFBP1, or CFI) (Table 5 and Table S4). Taken together, these data indicate that E2 and GH may be effective regulators of immune system contributing to the maintenance of immune homeostasis under conditions of immunological stress to reduce the susceptibility to stress-induced disease by negative immunoregulators.

Discussion

In this study, we show that E2 and GH replacements in hypothyroid male rats have a significant impact on lipid content and transcriptome in the liver and that E2 exerts a marked influence on GH-regulated endocrine, metabolic, immune, and gender specific responses in the liver.

Hypothyroidism impaired body weight gain and decreased circulating levels of IGF-I and biological markers of GH-STAT5b signaling activity in the liver (i.e., mRNA levels of IGF-I, ASL,

Table 4. Cellular pathways regulated by GH in hypothyroid-orchidectomized rat liver.

Pathway	up-regulated genes	down-regulated genes	*P*	B
Arginine and proline metabolism	GLUD-1	CPS-1, mitochondrial; OCT; ASS-1; arginase; aminotransferase	2,6E-05	3,1E-03
Urea cycle and metabolism of amino groups		CPS-1, mitochondrial; OCT; ASS-1; arginase	1,3E-03	3,8E-02
Glycine, serine and threonine metabolism	choline kinase α; glycyl-tRNA synthetase	serine dehydratase; betaine-homocysteine methyltransferase; cystathionase; glycine N-methyltransferase	1,6E-03	3,7E-02
Drug metabolism - Metabolism of xenobiotics by P450	CYP2C11; CYP2D2	CYP2A1; CYP2C23; ADH 1; GST (k1 and A3)	1,0E-03	4,0E-02
Methionine metabolism	methionine-tRNA synthetase	methionine adenosyltransferase I, alpha; betaine-homocysteine methyltransferase; cystathionase; 5-methyltetrahydrofolate-homocysteine methyltransferase	9,4E-04	5,4E-02
Retinol metabolism	CYP2C11	CYP2A1; CYP2C23; ADH 1; Dgat2	4,8E-03	9,1E-02
Caffeine metabolism	NAT-2	CYP2A1; urate oxidase	1,1E-02	1,7E-01
PPAR signaling	CPT-2; ANGPTL4	CYP27A1; CYP8B1; PEPK 1; ACOX-1	1,3E-02	1,8E-01
Cysteine metabolism	LDH-A	serine dehydratase; cystathionase	2,8E-02	2,9E-01
p53 signaling pathway	IGF-1; GADD-inducible, α and γ	cyclin B3; sestrin 1	4,4E-02	3,9E-01

The genes differentially-expressed in the livers were identified by DNA microarrays as described under Material and Methods. DAVID was used to identify the hepatic KEEG pathways that were affected by GH. The table shows pathway name, up- and down-regulated genes, P value, and corrected P value (Benjamini). Abbreviations: carbamoyl-phosphate synthetase-1 (CPS1); ornithine carbamoyltransferase (OTC); argininosuccinate synthase (ASS1); acyl-CoA oxidase 1, palmitoyl (ACOX-1); angiopoietin-like 4 (ANGPTL4); diacylglycerol O-acyltransferase 2 (Dgat2); glutamate dehydrogenase 1 (GLUD-1); Lactate dehydrogenase A (LDH-A); stearoyl-CoA desaturase (Scd); estrogen sulfotransferase (SULT).
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SOCS2, CIS, and CYP2C11) [51]. These changes were totally or partially restored by intermittent GH administration to TXOX rats. However, the effects of GH were largely prevented by E2 which is in line with the negative effects of estrogens on continuously GH administration in hypophysectomized female rats [52]. The positive effects of E2 on hepatic SOCS2, CIS, and FGF21 transcripts (see Fig.1) suggest that E2 might prevent the activation of GH-STAT5b signaling in liver through induction of these negative regulators of GH signaling [14,48]. Similarly, estrogen administration in humans can prevent the GH-induced increase in IGF-I, IGFBP-3, lipid oxidation, and protein synthesis [22,23]. The effects of hypothyroidism on growth are associated, in part, with an increased hepatic amino acid catabolism and urea synthesis [53]. Biological network analysis shows that intermittent GH administration to TXOX rats causes a positive regulation of cellular catabolism, whereas the genes involved in the metabolism of amino acids and urea (i.e., OTC, ASS1, aminotransferases, and methyltransferases) are significantly down-regulated. This is in line with the positive effects of GH on nitrogen balance, which have been previously studied in hypophysectomized rats [54,55,56].

GH serves as an anabolic hormone that promotes lipolysis and prevents lipogenesis in adipose tissue, which increases the availability of FFA for energy expenditure [56]. E2 is also able to interfere with this process by preventing the induction of some genes related to fat utilization, such as ApoC2, which activates the enzyme LPL that hydrolyzes TG. Therefore, E2 actions in liver can impact the peripheral metabolic actions of GH.

Lipogenesis is often increased in situations of reduced energy expenditure such as hypothyroidism, GH deficiency, E2 deficiency, or aging [57]. Accordingly, our analysis of the hepatic lipid content revealed that TXOX rats contained significantly increased levels of total SFA compared to INTACT rats. E2 replacement did not modify the mRNA expression levels of key regulators of hepatic lipogenesis [i.e., Sterol regulatory element binding protein (SREBP)1c, acetyl-Co A carboxylase alpha (ACC), fatty-acid synthase (FAS)] [58], whereas it activated a PPARα transcriptional program that promotes fatty acid catabolism in liver [50,59]. This was evidenced by the E2 increased expression of the PPARα gene itself and the PPARα target genes involved in the β/ω-oxidation of fatty acids (i.e., CTE-I, CPT-2, Fasd6, Fasd1, Fasd2, Scd1, ACOX1, ECH1, BAAT, FGF21, CYP4A1, CYP4A3) (Table S2). Accordingly, E2 replacement caused a significant reduction in SFAs. Overall these findings are indicative of a positive crosstalk between E2 and PPARα that is supported by multiple independent studies [2,60,61,62]. Interestingly, despite the increased expression of genes involved in β-oxidation, we detected a significant increase in hepatic TG content in E2 treated TXOX rats, which is likely explained by effects on lipid transport. The first step of long chain fatty acids uptake is its translocation across the plasma membrane. Notably, E2 increased the transcription of several known PPARα activated genes encoding proteins that have been implicated in fatty acids uptake and activation such as CD36, ACSL4 and SLC27A5 (FATP5) [63,64]. We have previously demonstrated that the fatty acid transporter CD36 is predominantly expressed in female rat livers and proposed that this sexual dimorphism depends on the GH secretion pattern, which can be influenced by E2 treatment. E2 also increased transcripts of the SLC27A5 gene which encodes FATP5, an fatty acid transporter that is an acyl-CoA synthetase (bile acid ligase) that catalyzes the conjugation of bile acids with amino acids before excretion into bile canaliculi [65]. Following fatty acids uptake, the first step for the intracellular use of long chain fatty acids is its esterification with CoA. This reaction is catalyzed by acyl-CoA synthetases such as ACSL4 which was also induced by E2 in TXOX rat liver. The produced

Table 5. E2 influences the cellular pathways regulated by GH in hypothyroid-orchidectomized rat liver.

Pathway name	up-regulated genes	down-regulated genes	*P*	B
Drug metabolism-Metabolism of xenobiotics by P450	FMO 3; CYP2C11; CYP2C24; NAT	CYP2A1; CYP2C23; CYP2E1; UGT (2B17; 2B36 and 1A); ADH 1; GST (mu 2; mu 3; mu 4; mu 7; pi 1; A3); uridine phosphorylase 2; epoxide hydrolase 1, microsomal	9,6E-10	1,2E-07
Complement and coagulation cascades	fibrinogen α chain	complement [C1; C4; C5; C8; C9]; serine (or cysteine) peptidase inhibitor; mannan-binding lectin serine peptidase 1	6,5E-07	2,8E-05
Retinol metabolism	CYP2C11; CYP2C24	CYP2A1; CYP2C23; CYP2A1; UGT (2B17; 2B36; and 1A); ADH A1; retinal pigment epithelium 65	1,0E-06	3,3E-05
Nitrogen metabolism	GLUD 1	CPS-1, mitochondrial; carbonic anhydrase (3 and 8); cystathionase; histidine ammonia lyase	1,7E-04	4,5E-03
PPAR signaling	CPT-1a; CPT-2; ANGPTL4	CYP7A1; CYP8B1; PEPK 1; CD36; LCA-CoA synthetase 1; Scd2	2,6E-04	5,7E-03
Fatty acid metabolism	CPT-1a; CPT-2	glutaryl-CoA dehydrogenase; ADH 1; enoyl CoA hydratase, short chain, 1, mitochondrial; ADH 2 (mitochondrial); LCA-CoA synthetase 1	4,4E-04	8,0E-03
Arginine and proline metabolism	GLUD1; spermidine synthase	CPS-1, mitochondrial; OCT; ASS-1; aminotransferase; ADH-2	1,5E-03	2,2E-02
Steroid hormone biosynthesis		CYP7A1; UGT (2B17; 2B36; 1A); SULT; HSD11B1	3,4E-03	4,3E-02
Tryptophan metabolism		tryptophan 2,3-dioxygenase; glutaryl-CoA dehydrogenase; kynurenine 3-monooxygenase; catalase; enoyl CoA hydratase, short chain, 1, mitochondrial; ADH-2	3,4E-03	4,3E-02
Glutathione metabolism	spermidine synthase	isocitrate dehydrogenase 1 (NADP+), soluble; GST (A3; mu2; mu 3; mu 4; mu 7; pi 1)	6,6E-03	7,4E-02

The genes differentially-expressed in the livers were identified by DNA microarrays as described under Material and Methods. DAVID was used to identify the hepatic KEEG pathways that were affected by GH in the presence of E2. The table shows pathway name, up- and down-regulated genes, P value, and corrected P value (Benjamini). Abbreviations: carbamoyl-phosphate synthetase-1 (CPS1); argininosuccinate synthase (ASS1); acyl-CoA oxidase 1, palmitoyl (ACOX-1); angiopoietin-like 4 (ANGPTL4); diacylglycerol O-acyltransferase 2 (Dgat2); glutamate dehydrogenase 1 (GLUD1); ornithine carbamoyltransferase (OTC); stearoyl-CoA desaturase (Scd); estrogen sulfotransferase (SULT).

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acyl-CoAs are substrates for β-oxidation but also can prime the synthesis of TG, phospholipids, CE, and ceramides and therefore are also a primary source of signaling molecules [66]. The notion that E2 may regulate the formation of lipid signaling intermediaries is supported by the stimulation of fatty acids elongase-5 (Elovl5). Elovl functions with fatty acid desaturases to generate many of the long-chain PUFAs assimilated into cellular lipids (i.e., 20:4n-6 and 22:6n-3). However, it is worth mentioning that E2 administration did not alter VLCPUFA metabolism because the levels of 20:4n-6, 20:5n-3 and 22:6n-3 remained similar to values in the TXOX group. It has been reported that E2 might play a critical role in lipogenesis and Scd1 transcription [2], a gene that encodes a rate-limiting enzyme to generate MUFAs such as 18:1 n−9 and 16:1 n−7. Previous studies have reported that the absence of E2 or ERα in rats provoked a profound increase in lipogenesis and Scd1 transcription [67], which suggests that E2 inhibits Scd1 transcription. Interestingly, the antilipogenic effect of E2 therapy, while maintaining efficient TG export and reduced phospholipid transfer protein, has been reported to depend on hepatic ERα [13,61]. Our study, however, shows that E2 increased the Scd1 gene expression and that this effect was paralleled by reduced hepatic content of 18:0 and increased of 18:1 n−9 (the main product of SCD reaction) contents, in total and, especially, in neutral lipids compared with TXOX animals, which indicates that E2 modulates SCD1 activity in TXOX liver. Surprisingly, E2 also downregulated Scd2 gene expression in TXOX rat livers. The significance of this opposed transcriptional regulation of Scd genes is unknown, but given that transcript levels of Scd1 are about 1800 times higher than that of Scd2 in the rat liver [68], changes in 18:1n-9 and 18:0 must be entirely attributed to variations in Scd1 gene expression. Overall, the changes in the lipid composition and gene expression profile seen in E2-treated TXOX rats support the finding that E2-PPARα functional interactions play a physiological role in the regulation of hepatic lipid metabolism.

E2 has the ability to reduce circulating CHO in women and in animal models fed on a high-fat diet [69]. However, E2 was unable to efficiently reverse hypercholesterolemia or hypotriglyceridemia in TXOX rats. This result may be due to the fact that E2 reduced expression levels of several transporters of CHO (and CE), including ApoB and ABCA1 in TXOX rats, which most likely contributed to maintaining an increased hepatic level CE. E2 may also induce intracellular CHO mobilization by modulating enzymes involved in CE and CHO synthesis and/or turnover [57,70]. Distinct enzymes can catalyze the CHO to CE conversion in liver: lecithin:cholesterol acyltransferase (LCAT), which uses phosphatidylcholine (PC) as a source of acyl changes and ACAT, which uses acyl-CoA. Because the levels of lysophosphatidylcholine (LPC) were undetectable in all groups, our initial conclusion was that E2 stimulated the ACAT2 reaction to increase CE. However, we did not detect changes in the expression level of the ACAT gene, which did not discard posttranslational modification of enzymes in the CE cycle in the liver from E2-treated TXOX rats.

An increased level of hepatic CE, together with the increased TG and decreased FFA hepatic contents in GH-treated TXOX rats, resemble the effects of E2 on hepatic lipid composition and suggest that some effects of E2 might be GH mediated. A striking consequence of the combined replacement with E2 and GH is the complete restoration of MUFA levels from total and neutral lipids, an effect attributable to the increase in 18:1n9, likely through alteration of Δ9 desaturase expression. Moreover, GH and E2 increased hepatic CE and the combined effect of the two hormones were additive with regard to CE because its levels doubled those found in INTACT animals and were approximately 30% higher than in the E2 or GH groups which indicates a more efficient hepatic CHO metabolism. Accordingly, in the presence of E2, GH reduced hepatic CHO content compared not only to the TXOX group but also in relation to the E2- or GH-treated TXOX groups. The hepatic content of TG was, however, significantly increased by GH in E2-pretreated TXOX rats, which suggests that combined treatment by E2 and GH dramatically enhances lipogenesis. It is known that in contrast with its lipolytic effects in adipose tissue, GH exerts lipogenic actions in liver through stimulation of SREBP1, which is usually accompanied by increased hepatic TG (VLDL) secretion [56]. Indeed, our lipid profiling analysis suggested that intermittent GH administration to TXOX rats increased lipogenesis in the liver. However, in contrast to the effects of a continuous infusion of GH in hypophisectomized rats [55], intermittent GH administration to TXOX rats did not increase SERBP1, whereas several genes involved in fatty acids transport (e.g., FABP) and the biosynthesis of unsaturated fatty acids from 18:2n-6 and 18:3n-3 (e.g., fatty acid desaturases 4, 5 and 6) were induced. Interestingly, intermittent GH administration to TXOX rats down-regulated the expression of the lipin gene, an SREBP1c target gene, which is critical in the regulation of cellular levels of DG and TG and a key regulator of fatty acid oxidation in adipose tissue, skeletal muscle, and liver tissue [71]. These findings support the hypothesis that the female pattern of GH administration is a more efficient stimulus to induce lipogenic effects in the liver than the male pattern [72,73]. Another mechanism whereby GH might promote lipogenesis in the liver is through the down-regulation of lipid oxidation. We have previously shown that continuous GH administration to hypophysectomized [55] and to old-intact [74] male rats inhibited PPARα. Accordingly, our lipidomic and genomic analysis showed that intermittent GH administration to TXOX rats also leads to down-regulation of the PPARα signaling pathway. In particular, GH represses the expression of PPARα itself, ACOX-1, CPT-1, FGF21, and several members of the CYP4A family, which are involved in fatty acid oxidation.

In summary, our study adds novel data that highlight the impact of subcutaneous E2 administration on liver physiology and its interplay with GH. These results highlight the role of E2 as a critical regulator of liver metabolism in mammals and add further weight to the hypothesis that E2 acts as an important regulator of GH actions in the liver. The E2-GH interplay in the liver is relevant because of the physiological roles that these hormones have in mammals and the widespread use of estrogen and estrogen-related compounds in human. Notably, this is the first study to demonstrate that hepatic lipid profiles are endowed with singular fingerprints that may be used to segregate different groups with altered hormone status. This includes different hormonal replacements (E2 or GH) that induced overlapping changes in gene expression. Therefore, liver lipid profiling can serve to identify cryptic hormone deficiencies or exposure to hormones or hormone-like substances.

Supporting Information

Table S1 Genes and primer sequences (5′- 3′) used for real-time PCR.
(PDF)

Table S2 Hepatic genes regulated by E2 in hypothyroid-orchidectomized rats. The administrations of vehicle (TXOX) or E2 (TXOXE2) to TXOX rats were described in Material and Methods. Then, differently expressed genes in the livers were identified by DNA microarrays. The analysis is based on the SAM statistical technique and differentially-expressed genes were discovered using a FDR less than 5% and a mean ratio of log2>|0.58|. The table shows ENSEMBL gene ID, Unigene/Refseq, gene symbol, gene description, R (TXOXE2/TXOX), SD, and q (%).
(PDF)

Table S3 Hepatic genes regulated by GH in hypothyroid-orchidectomized rats. The administrations of vehicle (TXOX) or GH (TXOXGH) to TXOX rats were described in Material and Methods. Then, differently expressed genes in the livers were identified by DNA microarrays. The analysis is based on the SAM statistical technique and differentially-expressed genes were discovered using a FDR less than 5% and a mean ratio of log2>|0.58|. The table shows ENSEMBL gene ID, gene symbol, gene description, R (TXOXGH/TXOX), SD, and q (%).
(PDF)

Table S4 Hepatic genes regulated by GH in E2-treated hypothyroid-orchidectomized rats. The administrations of **v**ehicle (TXOX) or E2 plus GH (TXOXE2GH) in TXOX rats were described in Material and Methods. Then, differently expressed genes in the livers were identified by DNA microarrays. The analysis is based on the SAM statistical technique and differentially-expressed genes were discovered using a FDR less than 5% and a mean ratio of log2>|0.58|. The table shows ENSEMBL gene ID, gene symbol, gene description, R (TXOX-E2GH/TXOX), SD, and q (%).
(PDF)

Table S5 Hepatic genes regulated by GH whose mean expression values are different from those in E2- and E2 plus GH-treated hypothyroid-orchidectomized rats. Hormonal replacements with GH (TXOXGH), E2 (TXOXE2) or E2 plus GH (TXOXE2GH) in TXOX rats were described in Material and Methods. Then, differently expressed genes in the livers were identified by DNA microarrays. SAM multiclass analysis was performed to identify GH regulated genes (TXOXGH) whose mean expression values were significantly different from E2 (TXOXE2)- or E2 plus GH (TXOXE2GH)-treated TXOX rats. The table shows ENSEMBL ID, Unigene/Refseq, gene symbol, gene description, R (mean expression), and q (%).
(PDF)

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Author Contributions

Conceived and designed the experiments: LF-P MD AF-M DI JCD-C. Performed the experiments: LF-P RS-F MM-G CM-D IG BG MD. Analyzed the data: LF-P MM-G BG MD DI AF-M JCD-C. Wrote the paper: LF-P BG MD DI AF-M JCD-C.

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SOCS2 deletion protects against hepatic steatosis but worsens insulin resistance in high-fat-diet-fed mice

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ABSTRACT **Hepatic steatosis is a prominent feature in patients with growth hormone (GH) deficiency. The ubiquitin ligase SOCS2 attenuates hepatic GH signaling by inhibiting the Janus kinase 2 (JAK2)–signal transducer and activator of transcription 5b (STAT5b) axis. Here, we investigated the role of SOCS2 in the development of diet-induced hepatic steatosis and insulin resistance. SOCS2-knockout (SOCS2$^{-/-}$) mice and wild-type littermates were fed for 4 mo with control or high-fat diet, followed by assessment of insulin sensitivity, hepatic lipid content, and expression of inflammatory cytokines. SOCS2$^{-/-}$ mice exhibited increased hepatic TG secretion by 77.6% (*P*<0.001) as compared with wild-type control mice and were protected from high-fat-diet (HFD)-induced hepatic steatosis, showing 49.3% (*P*<0.01) reduction in liver TG levels compared to HFD-fed wild-type littermates. In contrast, we found that HFD-triggered attenuation of systemic insulin sensitivity was more marked in SOCS2$^{-/-}$ mice. Livers from the HFD-fed SOCS2$^{-/-}$ mice showed increased NF-κB activity as well as elevated expression of genes for the inflammatory cytokines IFN-γ and IL-6. An inhibitory role of SOCS2 on Toll-like receptor 4 signaling was demonstrated in macrophages obtained from the SOCS2$^{-/-}$ and wild-type mice. This study identified SOCS2 as an important regulator of hepatic homeostasis under conditions of high-fat dietary stress.—Zadjali, F., Santana-Farre, R., Vesterlund, M., Carow, B., Mirecki-Garrido, M., Hernandez-Hernandez, I., Flodström-Tullberg, M., Parini, P., Rottenberg, M., Norstedt, G., Fernandez-Perez, L., Flores-Morales, A. SOCS2 deletion protects against hepatic steatosis but worsens insulin resistance in high-fat-diet-fed mice. *FASEB J.* 26, 000–000 (2012). www.fasebj.org**

Key Words: growth hormone · inflammation · suppressor of cytokine signaling

Mechanisms that drive the progression of nonalcoholic fatty liver diseases (NAFLDs) from simple steatosis to steatohepatitis and cirrhosis are poorly understood. Lipotoxicity, inflammation, and insulin resistance are believed to play a role, but the relative individual importance of each of these factors has been difficult to assess, because they are often manifested simultaneously and share related mechanisms of action (1–3). A better understanding of how this complex process is regulated by endogenous factors is essential for identification of effective therapeutic targets.

Abbreviations: ALT, alanine transaminase; BMDM, bone marrow-derived macrophage; CD, control diet; DMEM, Dulbecco's modified Eagle's medium; FFA, free fatty acid; GH, growth hormone; HFD, high-fat diet; HOMA-β, homeostatic model of assessment for β-cell function; HOMA-IR, homeostatic model of assessment for insulin resistance; IFN, interferon; IL, interleukin; ipGTT, intraperitoneal glucose tolerance test; ipITT, intraperitoneal insulin tolerance test; JAK, janus kinase; LDL, low-density lipoprotein; LDLR, low-density lipoprotein receptor; LPS, lipopolysaccharide; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; RANTES, regulated on activation normally T-cell expressed and secreted; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription; TG, triglyceride; TNF, tumor necrosis factor; WT, wild type

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The suppressor of cytokine signaling (SOCS) family (SOCS 1–7 and CIS) is a family of adaptor proteins that act as the substrate recognition subunits of Cullin/Ring ubiquitin ligases (4). They are generally thought to act as negative regulators of cytokine and growth factor signaling. Experimental gene inactivation in mice has unveiled highly specific physiological functions for these proteins, connected to their ability to recognize distinct molecular targets. *In vivo*, SOCS1 has a prominent function as a negative regulator of IFN-γ receptor activity while SOCS3 targets the gp130 cytokine receptor subunit, and as such regulates interleukin 6 (IL-6) actions. SOCS3 also has activity toward the leptin receptor (5, 6).

A role for SOCS proteins in the etiology of NAFLD is starting to emerge. Obese *db/db* mice treated with SOCS1 and SOCS3 antisense oligonucleotides show improvement in insulin signaling and hepatic lipid accumulation (7, 8). In contrast, SOCS3 inactivation in liver leads to increased food intake and hepatosteatosis, while SOCS1$^{-/-}$ mice exhibit severe steatohepatitis concomitant with a multiorgan inflammatory disease (9, 10). The role of SOCS2 in the regulation of liver metabolic disease has not yet been investigated, but it is suggested by studies showing changes in *SOCS2* mRNA levels in human steatotic livers (11). Also of possible relevance for the development of NAFLD is the role of SOCS2 as an inhibitor of growth hormone (GH) actions, an important regulator of hepatic triglyceride (TG) metabolism (12–15). SOCS2 acts as an ubiquitin ligase for the GH receptor inhibiting GH signaling (16). Consequently, SOCS2-deficient (SOCS2$^{-/-}$) mice are characterized by marked gigantism due to increased GH sensitivity (13). Additional physiological actions have been described for SOCS2, including the regulation of dendritic cell and CD4$^+$ T-helper 2 cell function, which could potentially influence its function in liver (17–19).

In this study, we investigated how SOCS2 regulates the hepatic metabolic response to high-fat feeding. We found that the SOCS2$^{-/-}$ mice are protected from high-fat-diet (HFD)-induced steatosis. Paradoxically, the HFD-fed SOCS2$^{-/-}$ mice showed worsening of glucose tolerance and exacerbated inflammatory response that was manifested by enhanced production of inflammatory cytokines in liver and fat tissues.

MATERIALS AND METHODS

Animals

SOCS2$^{-/-}$ mice (C57BL/6J; ref. 14) were housed under controlled temperature (23°C) and lighting (12-h light-dark cycle) conditions with free access to water and food. Male SOCS2$^{-/-}$ and wild-type (WT) littermates (8–10 wk old) were fed for 4 mo with a standard control diet (CD; SAFE-diet A04; Panlab SLU, Barcelona, Spain) or HFD (D12492; Open-Source Diets, New Brunswick, NJ, USA) containing 34.9% fat, 26.3% carbohydrate, and 26.2% protein, which corresponds to 60, 20, and 20% of total caloric content, respectively. To study the activation of insulin signaling pathways in tissues, some mice were sacrificed after an intraperitoneal (i.p.) injection of 0.75 U/kg human recombinant insulin (Actrapid; Novo Nordisk, Copenhagen, Denmark) or saline as a control. Tissues were stored at −80C until analysis. Pieces of pancreas and liver were excised for histological examination. Blood samples were collected through cardiac bleeding, and plasma was used to measure various analytes. All animal experiments were conducted in accordance with European Union laws and regulations.

Intraperitoneal glucose tolerance test (ipGTT) and intraperitoneal insulin tolerance test (ipITT)

For ipGTT, mice were unfed for 16 h overnight, followed by an i.p. injection of D-(+)-glucose (20% in 0.9% NaCl) at a dose of 2 g/kg body weight. Blood glucose levels were measured using a glucometer (Roche Diagnostics, Basel, Switzerland). Insulin was measured in blood samples collected at food withdrawal time point 0 during ipGTT. For ipITT, mice were unfed for 4 h, then intraperitoneally injected with human insulin (0.75 U/kg body weight, and glucose levels were determined as explained above. Homeostatic model of assessment for insulin resistance (HOMA-IR) was calculated as follows: fasting insulin (ng/ml) × fasting glucose (mM). HOMA for β-cell function (HOMA-β) was calculated as follows: 20 × fasting insulin (μU/ml)/fasting glucose (mM) − 3.5.

Liver histology

Frozen livers were cryosectioned and subjected to standard hematoxylin and eosin staining. Frozen sections were also stained with oil red O to visualize fat deposits in the liver, as described previously (20). Three sections per animal were visualized under same light illumination, and images were captured at different magnifications.

Histochemical analyses of pancreas

After sacrifice, total pancreatic weights were measured. One-third of the mass from the tail was fixed overnight in 4% paraformaldehyde at 4°C, washed with 70% ethanol, and paraffin embedded. Tissues were sectioned 6 μm thick, 3 sections on each glass slide for control, insulin, and glucagon immunostaining. Primary antibodies against insulin and glucagon were obtained from DakoCytomation (Stockholm, Sweden). Primary antibodies were detected with a biotinylated secondary antibody (anti-guinea pig IgG) in conjunction with Vectastatin ABC peroxidase kit (Vector Laboratories, Carpenteria, CA, USA) and chromogen diaminobenzidine (Sigma-Adrich, St. Louis, MO, USA). Slides were counterstained in hematoxylin. Images were captured by bright-field microscope under the same magnification and light illumination. One-third of the mass from the tail was digested and homogenized in 1 ml acidic ethanol (70% ethanol and 37% HCl) for insulin quantification per unit weight pancreas. Insulin extractions were carried overnight on homogenized pancreas at 4°C under constant shaking, followed by centrifugation. Supernantants were diluted 1000 times, and insulin was determined using an ELISA kit (Ultra Sensitive Rat Insulin ELISA; Crystal Chem, Downers Grove, IL, USA).

Plasma analysis

ELISA kits were used to measure plasma insulin and leptin levels (Crystal Chem Inc.), growth hormone (Millipore, Bed-

ford, MA, USA), and IGF-1 (high-sensitivity kit; Immunodiagnostic Systems, Inc., Fountain Hills, AZ, USA). Plasma alanine transaminase (ALT) enzyme activity was measured using a commercial ALT assay kit (Cayman Chemical Co., Ann Arbor, MI, USA). The mouse Bio-Plex suspension array (Bio-Rad, Hercules, CA, USA) was used to measure 5 cytokines: IL-1β, IL-6, interferon γ (IFN-γ), tumor necrosis factor α (TNF-α), and regulated on activation normally T-cell expressed and secreted (RANTES). Lipoprotein separation, by size-exclusion chromatography on individual samples from each animal, and lipid content calculations were performed as described previously (21).

In vivo TG secretion rate

The *in vivo* TG secretion rate (μmol TG/min) was measured after intravenous administration of Triton WR-1339 (Sigma), as described previously (22). Plasma TG levels were analyzed, and total plasma TG content was calculated using 0.071 ml/g as an estimate of plasma volume in the male mice (22). The hepatic TG secretion rate was expressed as total micromoles of TG per minute.

Generation of mouse bone marrow-derived macrophages (BMDMs)

Mouse BMDMs were obtained from 6- to 10 wk-old mice. Mice were euthanized, and the femur and tibia of the hind legs were dissected. Bone marrow cavities were flushed with 5 ml cold, sterile PBS. The bone marrow cells were washed and resuspended in Dulbecco's modified Eagle's medium (DMEM) containing glucose and supplemented with 2 mM L-glutamine, 10% FCS, 10 mM HEPES, 100 μg/ml streptomycin, 100 U/ml penicillin (all from Sigma), and 20 to 30% L929 cell-conditioned medium (as a source of macrophage-colony stimulating factor). Bone marrow cells were passed through a 70-μm cell strainer, plated, and incubated for 6 d at 37°C, 5% CO_2. BMDM cultures were then washed vigorously to remove nonadherent cells, trypsinized, counted, and cultured for 1 d at 37°C in culture plates. BMDMs were cultured in DMEM with sterile crushed carbon particles for 8 h before fixation in 4% formaldyhyde. Plates were washed and stained with eosin before microscopical analysis. Totals of 7 areas/plate were used to count percentages of cells with ingested carbon to total cells. Similarly, macrophages were cultured for medium collection and protein and RNA extraction after lipopolysaccharide (LPS) stimulation at a dose of 10 ng/ml. Media were used to measure cytokine levels using the Bioplex assay as above.

Western blot analysis and immunomultiplex assay

Protein extraction and SDS-PAGE were performed as described previously (13). Antibodies against Akt and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). pAKT (Thr308), pACC1, ACC1, IκBα, pJNK, JNK, pNF-κB, and NF-κB (p65) antibodies were from Cell Signaling Technology (Beverly, MA, USA) and low-density lipoprotein receptor (LDLR) antibody purchased from Cayman Chemical (Ann Arbor, MI, USA). The Milliplex MAP Cell Signaling assay was performed according to the manufacturer's protocol to quantify phosphorylated and total JNK (Thr183/Tyr185) and IRS-1 (panTyr) in liver and muscle lysates (Millipore).

Hepatic cholesterol, glycogen, free fatty acid (FFA), and TG contents

Free and total cholesterol, FFA, TG, and glycogen contents were determined by colorimetric enzymatic assays (Biovision, Mountain View, CA, USA), according to the supplier's protocols.

Gene expression analysis

Total RNA was isolated from 50–100 mg of frozen tissue using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). cDNA synthesis and quantitative real-time PCR were performed as described previously (18). All primer sequences are listed in Supplemental Table S1.

Statistical analysis

For ipGTT, 2-way ANOVA was performed to test for significant differences in the multiple effects of different time points among the 4 mouse groups. *Post hoc* analysis was performed accordingly. One-way ANOVA was performed to test for significant differences among the mean values of the 4 mouse groups. Student's *t* test was used to test for the significant differences between two groups. Values of $P < 0.05$ were considered significant.

RESULTS

HFD induced liver steatosis with effects on hepatic gene expression

To analyze SOCS2 metabolic functions, we first studied TG accumulation in the HFD-fed SOCS2$^{-/-}$ and WT mice. Histochemical analysis showed that the HFD-fed WT mice developed macrovesicular steatosis, whereas the HFD-fed SOCS2$^{-/-}$ mice showed less extensive steatosis with a microvesicular pattern (**Fig. 1*A***). High-fat feeding of the WT mice significantly increased the hepatic TG, cholesterol, and FFA contents. In contrast, the SOCS2$^{-/-}$ mice exhibited significantly lower hepatic TG accumulation when fed HFD (Fig. 1*B*). No differences were observed in hepatic FFA, cholesterol, or glycogen contents between the HFD-fed mouse groups (Supplemental Fig. S1*A*–*C*). These results indicate that SOCS2$^{-/-}$ mice are partially protected from HFD-induced hepatic steatosis.

To better understand the molecular basis for these effects, we analyzed the expression of genes involved in lipid metabolism (**Table 1**). The mRNA levels of genes involved in β-oxidation, *PPARl*α, *PGC1*α, *ACOX1*, and *CPT1*α, did not increase in HFD-fed SOCS2$^{-/-}$ mice, suggesting that enhanced fatty acid catabolism is unlikely to account for the reduced levels of steatosis in these mice. In contrast, expression levels of lipogenic genes *ACC*α, *FAS*, and *SCD1*, as well as genes involved in TG synthesis, *DGAT2* and *GPAT1*, were diminished in all HFD-fed mice, but the SOCS2$^{-/-}$ mice showed higher expression levels of *ACC*α and *DGAT2* than the WT mice. In line with these results, we also observed reduced *ACC*α phosphorylation on high-fat feeding, indicating increased

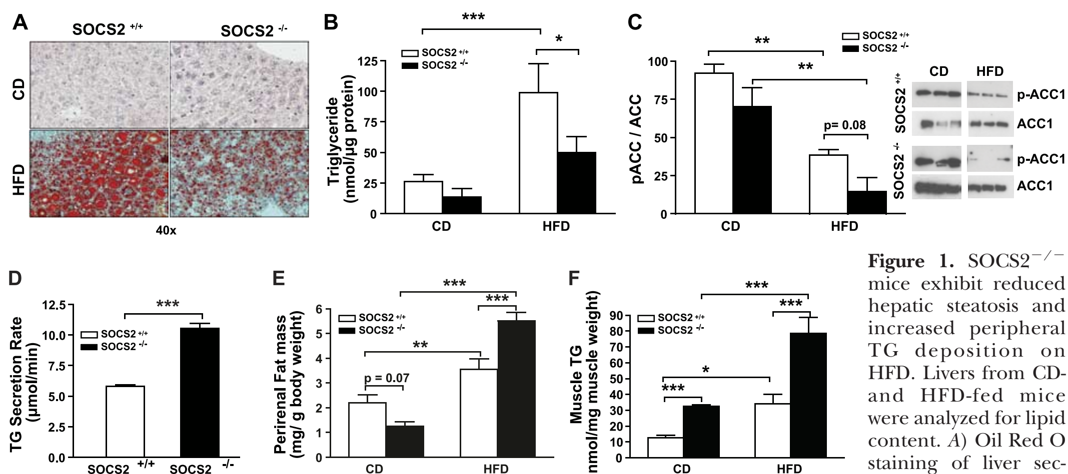


Figure 1. SOCS2$^{-/-}$ mice exhibit reduced hepatic steatosis and increased peripheral TG deposition on HFD. Livers from CD- and HFD-fed mice were analyzed for lipid content. *A*) Oil Red O staining of liver sections at high-magnification view (×40). *B*) Hepatic TG contents per microgram of liver proteins (n=9–10 mice/group). *C*) Liver extracts were immunoblotted with antibodies against ACC1 and phospho-ACC1, followed by the densitometric measurement of band intensities. All samples were run on the same gel and transferred to the same PVDF membrane. *D*) Hepatic TG secretion rates were determined and calculated as described in text (n=5–6 mice/group). *E)* Perirenal fat mass per gram of body weight. *F*) Intramuscular TG content per milligram of muscle tissue weight. $^*P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$; Student's t test.

activity, an effect more pronounced in the SOCS2$^{-/-}$ mice (Fig. 1*C*). Genes involved in TG assembly, *FABP1* and *ApoB*, had higher expression levels in the HFD-fed SOCS2$^{-/-}$ mice. This finding suggests that enhanced TG secretion in the HFD-fed SOCS2$^{-/-}$ mice may contribute to the reduced steatosis. These conclusions are supported by the observation that SOCS2$^{-/-}$ mice exhibited enhanced *in vivo* hepatic TG secretion (Fig. 1*D*). Changes in LDLR protein levels did not correlate with hepatic TG content (Supplemental Fig. S1*D*), which suggests that change in low-density lipoprotein (LDL) uptake is not the leading mechanism protecting the SOCS2$^{-/-}$ mice from HFD-induced steatosis.

Despite reduced hepatic steatosis, the HFD-fed SOCS2$^{-/-}$ mice showed no differences in blood TG levels compared with the HFD-fed WT mice (**Table 2**), which suggests that excessive fat is located in peripheral tissues. Indeed, a significant increase in the perirenal fat mass was observed in the SOCS2$^{-/-}$ mice compared with the WT mice after HFD feeding (Fig. 1*E*). We also observed increased TG accumulations in the muscles of the CD-fed SOCS2$^{-/-}$ mice, which further increased after HFD feeding (Fig. 1*F*).

HFD-induced glucose intolerance and insulin resistance in SOCS2$^{-/-}$ mice

Next, we examined the *in vivo* effect of different TG deposition in liver and peripheral tissue on glucose homeostasis in the SOCS2$^{-/-}$ and WT mice. No differences were observed in food intake between the two mouse groups when fed *ad libitum* for 4 mo with CD or HFD (Supplemental S2*A*). The CD-fed SOCS2$^{-/-}$ mice showed no difference in glucose tolerance compared with the CD-fed WT mice. In contrast, high-fat feeding induced glucose intolerance in both mouse groups, with SOCS2$^{-/-}$ mice showing more marked intolerance than the WT mice (**Fig. 2*A*, *B***). Next, we performed ipITT to assess insulin sensitivity. When maintained on CD, the SOCS2$^{-/-}$ mice behaved similarly to the WT mice, but when fed HFD, they showed a significantly lower hypoglycaemic response to exogenous insulin, indicative of a higher degree of insulin resistance (Fig. 2*C*). This condition was further confirmed by higher fasting plasma insulin and HOMA-IR index observed in the HFD-fed SOCS2$^{-/-}$ mice compared with the HFD-fed WT mice (Fig. 2*D*, *E*).

Liver and muscle tissues are key determinants of whole body insulin sensitivity. Therefore, we analyzed insulin signaling in these tissues by measuring the phosphorylation status of the key insulin signaling intermediate IRS-1 after acute insulin stimulation. IRS-1 phosphorylation after insulin treatment was impaired in the liver and muscle tissues of the HFD-fed mice. This effect was greater in the SOCS2$^{-/-}$ mice than in the WT mice, particularly in liver tissues (Fig. 2*F*). The evaluation of pancreatic morphology by immunohistochemistry showed normal pancreatic islet architecture in all mouse groups (**Fig. 3*A***). No significant alterations were observed in the insulin content or the HOMA-β index of islet function among the two mouse groups (Fig. 3*B*, *C*). These results suggest that SOCS2-associated deterioration in glucose homeostasis after high-fat feeding is not a consequence of a major impairment in β-cell function.

TABLE 1. *Hepatic gene expression data of different metabolic and inflammatory genes in WT and SOCS2$^{-/-}$ mice fed CD or HFD*

	WT		SOCS2$^{-/-}$	
Gene	CD	HFD	CD	HFD
Lypolysis				
PPARα	1.36 ± 0.22	1.39 ± 0.47	1.65 ± 0.25	1.67 ± 0.40
PGC-1	1.03 ± 0.36*	0.53 ± 0.15*	1.17 ± 0.25*	0.45 ± 0.14*
ACOX1	2.14 ± 0.28$^{\#}$	1.71 ± 0.38	0.98 ± 0.16$^{\#}$	1.77 ± 0.46
CPT1-α	1.17 ± 0.16	1.19 ± 0.15$^{\#}$	0.93 ± 0.07	0.71 ± 0.12$^{\#}$
Lipogenesis				
FAS	1.29 ± 0.40*	0.15 ± 0.05*	0.92 ± 0.41	0.23 ± 0.07
SCD1	1.78 ± 0.73$^{*,\#}$	0.10 ± 0.07*	4.57 ± 1.28$^{**,\#}$	0.21 ± 0.11**
DGAT2	0.74 ± 0.19$^{**,\#}$	0.17 ± 0.06$^{\#,**}$	0.41 ± 0.11$^{\#}$	0.30 ± 0.08$^{\#}$
GPAT1	1.12 ± 0.48*	0.34 ± 0.13*	0.69 ± 0.20	0.34 ± 0.10
ACCα	0.82 ± 0.25*	0.20 ± 0.06$^{\#,*}$	0.90 ± 0.31	0.46 ± 0.12$^{\#}$
TG assembly and lipid uptake				
MTTP	1.44 ± 0.15	1.54 ± 0.47	2.14 ± 0.49	1.97 ± 0.37
ApoB	1.18 ± 0.26	1.66 ± 0.49$^{\#}$	1.70 ± 0.43*	3.23 ± 0.20$^{*,\#}$
FABP1	3.66 ± 0.80	2.97 ± 0.91$^{\#}$	3.91 ± 1.02*	7.31 ± 1.29$^{*,\#}$
LDL receptor	3.19 ± 0.99$^{\#\#}$	2.89 ± 0.87$^{\#\#}$	0.22 ± 0.02$^{\#\#}$	0.20 ± 0.02$^{\#\#}$
Proinflammatory signaling and immune cell markers				
F4/80	1.05 ± 0.19	1.56 ± 0.52	1.22 ± 0.17	1.69 ± 0.41
CCL2	1.80 ± 0.94	1.19 ± 0.38	1.69 ± 0.40	0.95 ± 0.30
CD4	2.47 ± 0.68	2.51 ± 0.43	2.85 ± 0.61	1.73 ± 0.87
CD8A	1.70 ± 0.37	1.41 ± 0.26	1.51 ± 0.20	1.74 ± 0.25
RANTES	0.85 ± 0.23	1.00 ± 0.35$^{\#\#}$	1.26 ± 0.49**	5.20 ± 1.18$^{**,\#\#}$
iNOS	1.19 ± 0.38	0.73 ± 0.20$^{\#}$	1.81 ± 0.44*	2.56 ± 0.54$^{*,\#}$
NOX	1.05 ± 0.19	1.08 ± 0.26$^{\#}$	1.22 ± 0.17*	1.94 ± 0.38$^{*,\#}$
α-SMA	1.74 ± 0.36	1.62 ± 0.21	1.32 ± 0.24	1.95 ± 0.30
IL-1β	0.89 ± 0.25	1.18 ± 0.71	0.35 ± 0.18	0.79 ± 0.33
IL-6	2.46 ± 0.84	2.81 ± 1.28$^{\#\#}$	3.45 ± 0.71**	19.73 ± 7.07$^{**,\#\#}$
TNF-α	1.62 ± 0.36	2.85 ± 0.93	2.94 ± 0.90	3.42 ± 0.50
IFN-γ	0.81 ± 0.36	0.97 ± 0.41$^{\#}$	0.91 ± 0.39*	7.78 ± 4.5$^{*,\#}$
GH signaling				
SOCS3	1.70 ± 0.25	1.77 ± 0.56	2.25 ± 0.27	1.48 ± 0.32
SOCS2	1.58 ± 0.35*	3.86 ± 1.63*	—	—
IGFBP3	1.59 ± 0.74	1.88 ± 0.76	1.34 ± 0.48	2.01 ± 0.58
IGF1	2.01 ± 0.19	1.69 ± 0.12$^{\#}$	2.17 ± 0.16	2.7 ± 0.29$^{\#}$

Data are shown as means ± SE normalized to S18 rRNA expression (n=5). $^{*}P \leq 0.05$, $^{**}P \leq 0.001$ between diets within genotype group; $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.001$ between genotypes within diet group.

Enhanced inflammatory response to HFD in SOCS2$^{-/-}$ mice

Because of the dissociation between hepatic lipid content and insulin sensitivity described above, we studied mechanisms that might contribute to the onset of insulin resistance in the HFD-fed SOCS2$^{-/-}$ mice. Hepatic insulin resistance induced by high-fat feeding requires the actions of inflammatory cells and cytokines. We then analyzed the livers of the SOCS2$^{-/-}$ mice for expression of inflammatory marker genes (Table 1). Expressions of the macrophage cell surface marker *F4/80* and the chemokine *CCL2* (*MCP-1*) were not significantly elevated in the SOCS2$^{-/-}$ mice. Furthermore, expression of T-cell surface markers, such as *CD4* and *CD8A*, did not differ among the mouse groups. This is consistent with the observation that our hepatic histological analysis failed to detect any overt infiltration of inflammatory cells in the SOCS2$^{-/-}$ mice on either diet. No enhanced fibrosis or overt liver damage was observed, which is consistent with the measurements of hepatic *α-SMA* expression (Table 1) and plasma ALT activity (Table 2).

In contrast, significantly increased mRNA levels of *IL-6*, *IFN-γ*, *RANTES* (*CCL5*), *iNOS*, and *NOX* were observed in the livers of the HFD-fed SOCS2$^{-/-}$ mice compared with those in the mice belonging to the other groups. We also observed increased *TNF-α* and *IL-1β* mRNA levels associated with HFD, although these differences in levels between the mouse groups were not statistically significant. Analysis of liver tissues showed significantly enhanced p65 NF-κB Ser536 phosphorylation levels and decreased levels of IκBα, negative regulator of NF-κB, in SOCS2$^{-/-}$ mice, an effect that was exacerbated with high-fat feeding (**Fig. 4*A***). Similar to the liver, we found that proinflammatory cytokine mRNA levels increased in the adipose tissues of the HFD-fed mice, and expression of *IFN-γ*, *CCL2*, and *IL-1β* was higher in the HFD-fed SOCS2$^{-/-}$ mice (Supplemental Fig. S3*A*). These experiments identify the adipose tissues as a source of inflammatory cytokines and a possible contributor to the development of

TABLE 2. *Plasma levels of cytokines and lipids*

Cytokine or lipid	WT CD	WT HFD	SOCS2$^{-/-}$ CD	SOCS2$^{-/-}$ HFD
GH (ng/ml)	6.81 ± 0.84$^{##}$	3.84 ± 0.78	0.16 ± 0.05$^{**,##}$	2.56 ± 0.59**
IGF1 (ng/ml)	237.72 ± 8.29**	267.00 ± 5.16$^{**,#}$	233.00 ± 4.26	248.85 ± 3.78$^{#}$
IL6 (pg/ml)	37.15 ± 10.41*	93.04 ± 20.20*	65.82 ± 20.29	92.10 ± 23.20
INFγ (pg/ml)	16.74 ± 5.53	12.24 ± 3.91$^{##}$	23.55 ± 7.65*	44.25 ± 8.01$^{*,##}$
IL1β (pg/ml)	152.70 ± 31.41	191.81 ± 86.92$^{##}$	198.32 ± 29.27**	361.62 ± 49.68$^{**,##}$
TNFα (pg/ml)	244.31 ± 68.01	426.94 ± 51.57	173.81 ± 52.13	381.09 ± 62.36
RANTES (pg/ml)	102.66 ± 15.35	101.25 ± 18.56$^{##}$	143.42 ± 15.37**	250.98 ± 37.96$^{**,##}$
ALT (U/L)	5.43 ± 0.24	6.73 ± 0.66	6.03 ± 0.56	7.10 ± 0.72
Triglycerides (nM)	123.26 ± 33.21*	61.63 ± 15.40*	111.02 ± 20.76*	40.16 ± 6.03*
FFA (mM)	0.38 ± 0.05	0.32 ± 0.03	0.27 ± 0.03	0.26 ± 0.02
Glucose (mg/dl)	110.11 ± 4.87*	130.6 ± 6.62$^{*,##}$	108.86 ± 7.32**	151.11 ± 3.91$^{**,##}$
Endotoxin (EU/ml)	0.17 ± 0.02	0.17 ± 0.02	0.19 ± 0.02	0.19 ± 0.03

Data are means ± SE (n=9–10/group). EU, endotoxin unit. All parameters measured in plasma after 4 h food withdrawal except for glucose, which was measured after overnight food withdrawal. *$P \leq 0.05$, **$P \leq 0.001$ between diets within genotype group; $^{#}P \leq 0.05$, $^{##}P \leq 0.001$ between genotypes within diet group.

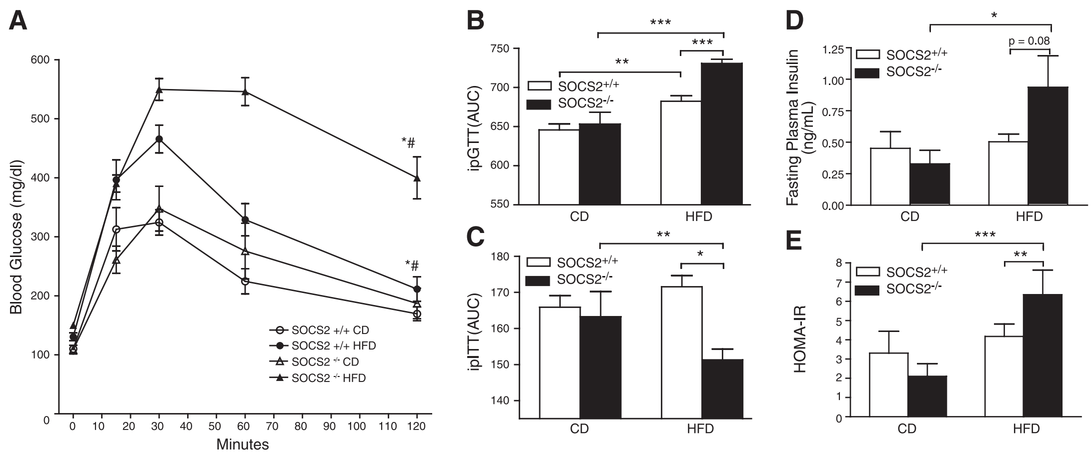
systemic and hepatic insulin resistance in these mice. We also assessed the levels of several circulating cytokines and hormones (Table 2). The SOCS2$^{-/-}$ mice exhibited reduced GH levels compared with the WT mice, whereas the amount of circulating IGF-I, a protein produced in the liver in response to GH, showed very minor differences between the groups. The SOCS2$^{-/-}$ mice fed HFD had elevated circulating levels of IFN-γ, RANTES, and IL-1β compared with the WT mice. Overall, our findings demonstrate that SOCS2$^{-/-}$ mice exhibit enhanced inflammatory signaling in liver and adipose tissue, which are exacerbated by HFD and may contribute to the development of insulin resistance.

High levels of circulating cytokines, taken together with the increased cytokine mRNA levels in the liver and adipose tissue, suggest that liver resident and peripheral macrophages in the SOCS2$^{-/-}$ mice are hyperresponsive to inflammatory stimuli. To directly test this hypothesis, we analyzed cytokine production in response to LPS stimulation as well as the phagocytic activity of BMDMs from the SOCS2$^{-/-}$ and WT mice. BMDMs from the SOCS2$^{-/-}$ mice showed increased secretion of IFN-γ, IL-1β, and TNF-α in response to LPS (Fig. 4*B*) in parallel to increased proinflammatory cytokine mRNA expression (Supplemental Fig. S3*B*). Our experiments also showed enhanced phagocytic activity of BMDMs from the SOCS2$^{-/-}$ mice (Fig. 4*C*). As shown in Fig. 4*D*, these BMDMs had higher basal levels of p65–NF-κB compared with BMDMs from the WT mice. LPS stimulation resulted in increased NF-κB phosphorylation in BMDMs from WT mice, an effect that is enhanced in BMDMs from the SOCS2$^{-/-}$ mice. In parallel, we observed decreased levels of the NF-κB inhibitor IkBα, but no changes were observed in JNK phosphorylation. These results unveil a previously unrecognized function of SOCS2 as a negative regulator of Toll-like receptor-driven macrophage activation and provide a plausible explanation for the increased insulin resistance observed in the SOCS2$^{-/-}$ mice after high-fat feeding.

DISCUSSION

We analyzed the metabolic response of the SOCS2$^{-/-}$ mice to a hypercaloric, fat-rich diet. The SOCS2$^{-/-}$ mice exhibited enhanced hepatic TG secretion and were protected from HFD-induced liver steatosis. However, they displayed severe systemic insulin resistance associated with hyperinsulinemia and worsened insulin sensitivity in the liver compared with the WT mice fed a similar diet. The HFD-fed SOCS2$^{-/-}$ mice also exhibited enhanced expression of inflammatory cytokines in liver, demonstrating a novel role of SOCS2 as a negative regulator of macrophage activation under conditions of high-fat dietary stress. Notably, the SOCS2$^{-/-}$ mouse phenotype is clearly different from the liver-specific SOCS3- and SOCS1-knockout mouse phenotypes (9, 23, 24), which show enhanced liver steatosis, highlighting the functional differences between members of the SOCS family in the regulation of hepatic metabolism.

In previous studies, we showed that SOCS2 is a negative regulator of hepatic GH receptor signaling (12, 13). The reduced steatosis observed in the HFD-fed SOCS2$^{-/-}$ mice compared with the HFD-fed WT mice is in agreement with the well-known actions of GH in promoting hepatic lipid mobilization (15, 25) and strongly suggests that enhanced GH signaling in the liver is in part responsible for these effects. Indeed, we found mRNA levels of the known GH-regulated genes *ApoB*, *FABP1*, and *DGAT2*, which are involved in TG assembly and secretion, to be increased in the SOCS2$^{-/-}$ mice. Detailed analysis of mice with a hepatic-specific deletion of JAK2 has unveiled another mechanism whereby GH controls liver fat content (26). These mice, denoted JAK2L, have elevated levels of circulation GH, which in turn leads to increased adi-



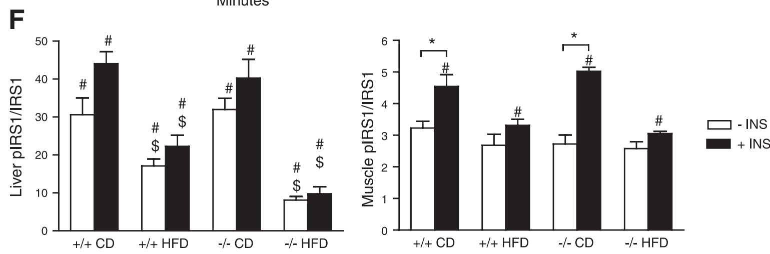


Figure 2. SOCS2$^{-/-}$ mice show severe glucose intolerance and insulin resistance after high-fat feeding. *A*) ipGTT. Blood glucose after overnight food withdrawal and after an i.p. injection of glucose (2 g/kg body weight; n=9–10 mice/group). $^{*}P < 0.05$ between diets within genotype group, $^{\#}P < 0.05$ between genotypes within diet group; 2-way ANOVA. *B*, *C*) Area under the curve (AUC) analysis for ipGTT (*B*) and ipITT (*C*). AUC for ipITT was generated from percentage reduction of plasma glucose after insulin administration. *D*) Plasma insulin after overnight food withdrawal. *E*) HOMA-IR index measured after overnight food withdrawal. *F*) Mice, unfed for 4 h, were administered an i.p. injection of saline (−INS) or insulin (+INS; 0.75 U/kg body weight) for 10 min before tissue collection. Intensities of pIRS-1 corrected to those of total IRS-1 were measured by a luminex multiplex assay (n=4–5/treatment group) in liver and muscle lysates, respectively. Data are shown as means ± SE. $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$; Student's *t* test (*B–E*) or 1-way ANOVA *post hoc* analysis performed separately in the −INS and +INS groups (*F*). separately. $^{\#}P < 0.05$ between diets within genotype group; $^{\$}P < 0.05$ between genotypes within diet group.

pose tissue lipolysis. This condition increases FFA supply to the liver, leading to steatosis (26). The SOCS2$^{-/-}$ mice have reduced levels of circulating GH and, in opposition to JAK2L, exhibit increased fat mass, which suggests that reduced adipose tissue lipolysis in SOCS2$^{-/-}$ mice may also contribute to reduced hepatic TG accumulation. Noticeably, we did not detect significant changes in circulating FFA or hepatic expression of CD36 in SOCS2$^{-/-}$ mice compared to what has been reported in the JAK2L mice. Further experi-

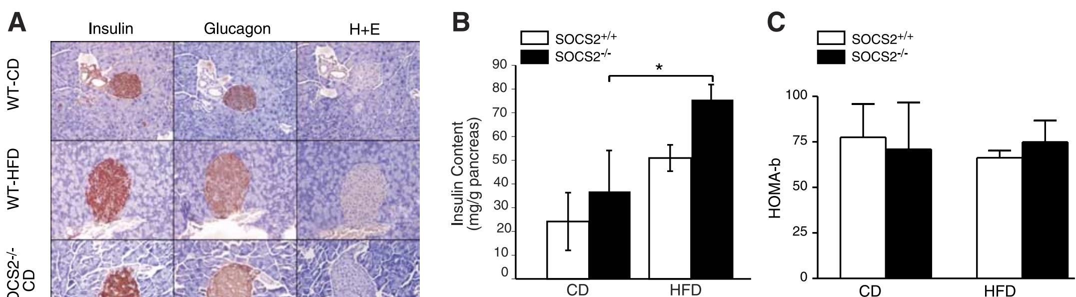


Figure 3. Morphological and functional analysis of pancreatic islets from SOCS2$^{-/-}$ mice. *A*) Pancreatic histology and immunohistochemical staining for insulin and glucagon in sections from the WT and SOCS2$^{-/-}$ mice after 4 h food withdrawal. Slides were counterstained with hematoxylin and eosin. *B*) Insulin content per gram pancreas weight (3–4 mice/group). *C*) HOMA-β as a product of plasma insulin and blood glucose after overnight food withdrawal (n=9–10 mice/group). $^{*}P < 0.05$; Student's *t* test.

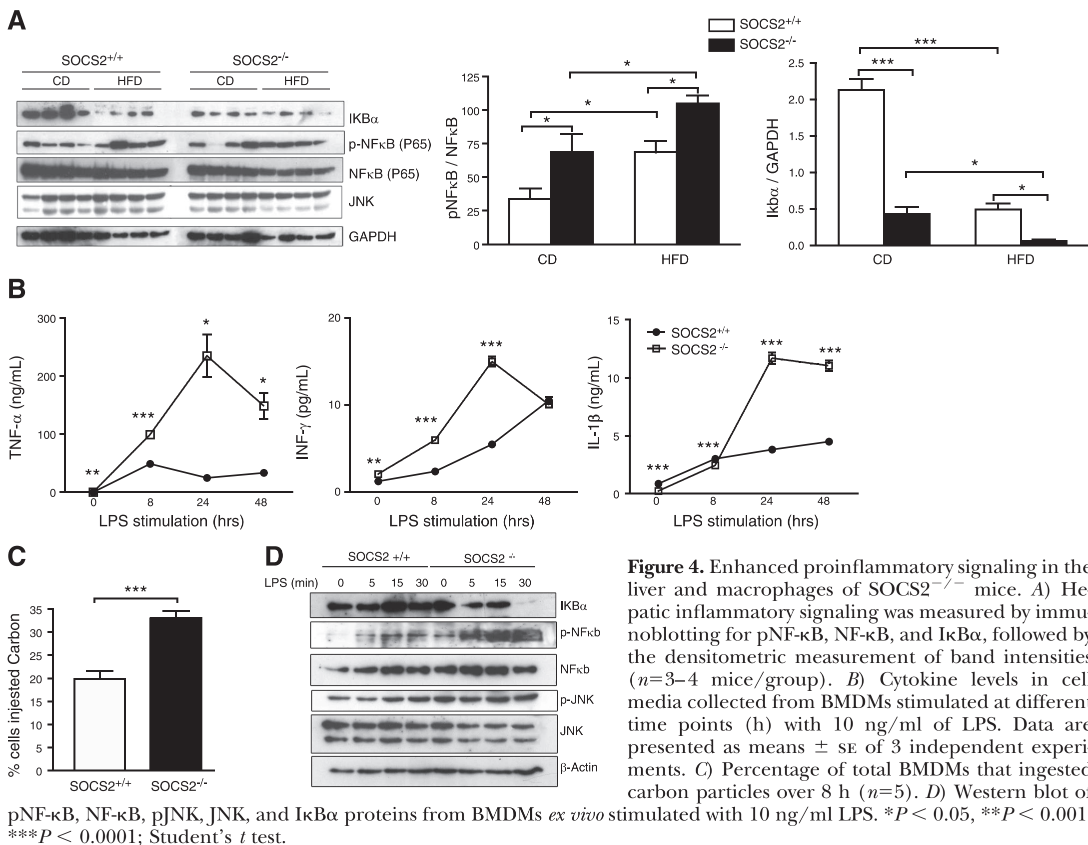


Figure 4. Enhanced proinflammatory signaling in the liver and macrophages of SOCS2$^{-/-}$ mice. *A*) Hepatic inflammatory signaling was measured by immunoblotting for pNF-κB, NF-κB, and IκBα, followed by the densitometric measurement of band intensities (n=3–4 mice/group). *B*) Cytokine levels in cell media collected from BMDMs stimulated at different time points (h) with 10 ng/ml of LPS. Data are presented as means ± SE of 3 independent experiments. *C*) Percentage of total BMDMs that ingested carbon particles over 8 h (n=5). *D*) Western blot of pNF-κB, NF-κB, pJNK, JNK, and IκBα proteins from BMDMs *ex vivo* stimulated with 10 ng/ml LPS. *$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$; Student's t test.

ments are needed to analyze the influence of GH and adipose tissue lipolysis in the SOCS2$^{-/-}$ mice liver.

Surprisingly, the SOCS2$^{-/-}$ mice showed an exacerbated response to high-fat feeding, leading to worsened insulin sensitivity, whereas the SOCS2$^{-/-}$ and WT mice showed few differences in insulin signaling when maintained on a normal CD. This finding suggests that hyperactivity of the hepatic GH receptor signaling alone, as it is observed in SOCS2$^{-/-}$ mice, is unlikely to account for the diet-dependent deterioration in glucose control observed in this study. A more likely explanation is that the anti-insulinic actions of GH are exacerbated by diet-related mechanisms under SOCS2 control. We provide evidence for the existence of three such mechanisms: hyperinsulinemia, peripheral adiposity, and excessive production of inflammatory cytokines. Toll-like receptor-driven macrophage activation in liver and adipose tissues by high-fat feeding leads to the production of inflammatory cytokines, a process that is required for diet-induced hepatic insulin resistance (3). The loss of SOCS2 leads to an altered response to HFD in mice, resulting in increased expression of inflammatory cytokines and enhanced NF-κB activation. This seems to be mediated by direct actions of SOCS2 on macrophage activation, as demonstrated in *ex vivo* experiments showing that BMDMs from the SOCS2$^{-/-}$ mice exhibit increased phagocytic activity *in vitro* and are hyperresponsive to LPS stimulation, leading to expression of *IL-6*, *iNOS*, *IL-1*β, and *IFN*-γ (Supplemental Fig. S4*A*). A previous study described an anti-inflammatory role of SOCS2, mediating the activity of aspirin-triggered lipoxins (27). Our data suggest that the anti-inflammatory actions of SOCS2 may be mediated to some extent through inhibition of the LPS response in macrophages, leading to NF-κB activation. Transgenic mice with enhanced NF-κB activity in hepatocytes exhibit insulin resistance driven by inflammation, which resembles the HFD-fed SOCS2$^{-/-}$ mice (28).

Measurements of cytokine mRNA levels in hepatic tissue provide a strong indication that liver non-parenchimal cells participate in the hepatic response to HFD in SOCS2$^{-/-}$ mice. However, whether this altered reactivity is related to GH or other signals remains unknown. In relation to GH, we showed previously that GH treatment of SOCS2$^{-/-}$ mice results in enhanced hepatic expression of cytokine-regulated genes (12, 13) and GH is known to exacerbate the inflammatory response in LPS-treated rodents and worsen the conditions of critically ill patients (29). Further work is needed to clarify the role of GH signaling in different tissues and cell types in the HFD-fed SOCS2$^{-/-}$ mice.

It is often postulated that inflammation contributes

to hepatic steatosis, although the causality of this relationship is not always supported by experimental data (30, 31). Although in certain models, such as mice fed a methionine- and choline-deficient diet or the SOCS1$^{-/-}$ mice, severe inflammation can contribute to liver steatosis (24, 32); this situation does not reflect the subacute inflammation that is observed in human nonalcoholic steatohepatitis (NASH; ref. 33). Mimicking this situation in a mouse model through low-level activation of NF-κB in hepatocytes is sufficient to trigger subacute inflammation, but this is not accompanied by steatosis (28). In another example, inhibition of 5-lipoxygenase expression in the ApoE$^{-/-}$ model of NASH can revert inflammation and insulin resistance but has little effect on hepatic lipid content (31). Therefore, inflammation *per se* does not necessarily cause hepatosteatosis. This concept is strengthened by our studies in SOCS2$^{-/-}$ mice that demonstrate that modest inflammation can be accompanied by diminished levels of steatosis, a phenotype that is also observed in mice with reduced capacity to synthesize TG due to reduced expression of *DGAT2* (34).

In summary, our results demonstrate that SOCS2 plays an important role in regulating the response to high-fat dietary stress. The SOCS2$^{-/-}$ mice provide a novel model to understand the complex relationship between inflammation, GH actions, and nutrition in the control of hepatic glucose and lipid homeostasis. Future use of this model may help to outline the contribution of different mechanisms in the development of fat-induced hepatic insulin resistance and components related to lipid overload in comparison with components related to inflammatory stress. FJ

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Review

The Influence of Estrogens on the Biological and Therapeutic Actions of Growth Hormone in the Liver

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Abstract: GH is main regulator of body growth and composition, somatic development, intermediate metabolism and gender-dependent dimorphism in mammals. The liver is a direct target of estrogens because it expresses estrogen receptors which are connected with development, lipid metabolism and insulin sensitivity, hepatic carcinogenesis, protection from drug-induced toxicity and fertility. In addition, estrogens can modulate GH actions in liver by acting centrally, regulating pituitary GH secretion, and, peripherally, by modulating GHR-JAK2-STAT5 signalling pathway. Therefore, the interactions of estrogens with GH actions in liver are biologically and clinically relevant because disruption of GH signaling may cause alterations of its endocrine, metabolic, and gender differentiated functions and it could be linked to dramatic impact in liver physiology during development as well as in adulthood. Finally, the interplay of estrogens with GH is relevant because physiological roles these hormones have in human, and the widespread exposition of estrogen or estrogen-related compounds in human. This review highlights the importance of these hormones in liver physiology as well as how estrogens modulate GH actions in liver which will help to improve the clinical use of these hormones.

Keywords: growth hormone; 17β-estradiol; liver; growth; metabolism; STAT5

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1. Introduction

The liver responds in a sex-specific manner to growth hormone (GH) and sex hormones. GH is the main regulator of body growth, somatic development, metabolism, sex-differentiated functions in the liver, and aging [1–7]. Because the liver has the highest levels of GH receptor (GHR), it is a major target for GH; however, virtually all human tissues are responsive to GH. The sex-specific GH secretion from pituitary has been shown to have a great impact on hepatic transcriptional regulation [2,4,8,9]. The Signal Transducer and Activator of Transcription (STAT)-5b is of particular importance in the regulation of the endocrine, metabolic, and sex-differentiated actions of GH in the liver. In the liver, GHR-STAT5 signaling regulates the expression of the target genes that are associated with several physiological processes, such as body growth, the cell cycle, and lipid, bile acid, steroid, and drug metabolism. Importantly, the disruption of GHR-JAK2-STAT5 signaling is associated with liver disease, which includes fatty liver, fibrosis, and hepatocellular carcinoma.

A major natural estrogen in mammals, 17β-estradiol (E2) has physiological actions that are not limited to male or female reproductive organs [10,11]. Estrogens exert their physiological influence through two estrogen receptor (ER) subtypes, ERα and ERβ. These subtypes belong to the nuclear receptor family of ligand-activated transcription factors [12]. Together with a mechanism based in ligand-activated transcription, estrogens can modulate gene expression by using a second mechanism in which the ERs interact with other transcription factors through a process referred to as transcription factor crosstalk. Estrogen may also elicit effects through non-genomic mechanisms, which involve the activation of protein kinase cascades via membrane-localized ERs. Moreover, the mechanisms involved in ER signaling are influenced by cell phenotype, the target gene, and activity or crosstalk with other signaling networks.

The potential interactions between estrogens and the GH-regulated endocrine, metabolic and sex-differentiated functions in the liver are biologically and clinically relevant. Estrogens can modulate GH actions in the liver by acting centrally to regulate pituitary GH secretion and modulating GH signaling peripherally. Most previous studies have focused on the influence of estrogens on pituitary GH secretion [13]; however, there is also strong evidence that estrogens modulate GH action at the level of GHR expression and signaling. In particular, E2 has been shown to induce suppressor of cytokine signaling (SOCS)-2 and -3, which are protein inhibitors for cytokine signaling that in turn negatively regulate the GHR-JAK2-STAT5 pathway [11,14–19]. Finally, the liver is a direct estrogen target because it expresses ERα [12], which is connected to liver development [20], the regulation of hepatic metabolic pathways [11], growth [21], protection from drug-induced toxicity [22], hepato-carcinogenesis [23], fertility [24], lipid metabolism and insulin sensitivity [11,25].

Estrogen-GH interplay is clinically relevant because of the physiological roles that these hormones have in mammals and the widespread use of estrogen and estrogen-related compounds in humans. This relevance has been supported by clinical observations in which the administration of pharmacological estrogen doses in humans impairs the GH-regulated endocrine and metabolic functions in the liver [26]. Thus, the deficiency of GH or E2 activities and the interaction of estrogen with GH biology may dramatically influence liver physiology during development and in adulthood. This review highlights the importance of these hormones in liver physiology and describes how estrogens can modulate GH

action in the liver. A better understanding of estrogen-GH interplay will lead to improved clinical management of these hormones.

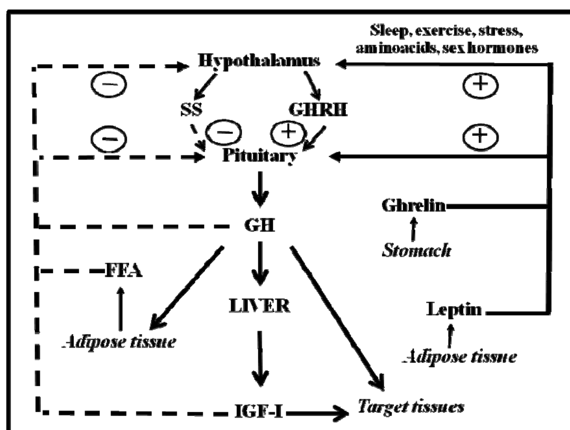
2. Physiological Basis of Pituitary GH Secretion

GH is a polypeptide that is secreted primarily from the somatotrophs within the anterior pituitary gland. In addition to the pituitary gland, GH is produced in extra-pituitary tissue, which indicates that GH has local paracrine-autocrine effects that are distinct from its classic endocrine-somatotropic effects [27]. The regulation of pituitary GH secretion involves a complex neuroendocrine control system that includes the participation of several neurotransmitters and the feedback of hormonal and peripheral (metabolic) factors [28]. Figure 1 shows that GH secretion from the pituitary gland is regulated by two major hypothalamic peptides: GH-releasing hormone (GHRH) and the inhibitory hormone somatostatin (SS). The balance of these stimulating and inhibiting peptides is indirectly affected by many physiological stimulators (e.g., nutrients, sleep, exercise, thyroid hormones and sex hormones) and inhibitors (e.g., glucocorticoids, Insulin-like Growth Factor (IGF)-I, GH). The final integration of these signals occurs in the hypothalamus. Pituitary GH secretion is reduced mainly by the negative feedback of two circulating signals: the pituitary GH itself and the liver-derived IGF-I, which is produced by the GH. In addition to hypothalamic (GHRS, SS) and endocrine (IGF-I, GH) factors, other peripheral (metabolic) factors, such as insulin, glucose, amino acids, free fatty acids (FFA), leptin, neuropeptide Y, and ghrelin, influence pituitary GH release. These factors, which appear to coordinate the metabolic status of the organism with GH secretion, are primarily related to or derived from the metabolic status of the organism; this relationship is consistent with the GH role in regulating substrate metabolism, adiposity, and growth. This role is exemplified by adiposity, which is a powerful negative regulator of GH secretion. FFA can act directly on the pituitary gland to inhibit the GH release, which is postulated to complete a feedback loop because GH stimulates lipid mobilization. In addition, adipocytes produce the hormone leptin, which, in contrast to FFA, stimulates GH secretion in rodents at the hypothalamus level [29]. Finally, ghrelin is another GH-secretory factor that is highly expressed in the endocrine cells of the stomach [30]. On the other hand, the selective lack of ghrelin receptor signaling in humans may lead to a syndrome characterized by short stature [31], and ghrelin analogs have been shown to effectively enhance serum IGF-I levels in humans [32].

Sex steroids are also physiological regulators of pituitary GH secretion and regulate sex-specific liver physiology. Both neonatal and post-pubertal sex steroids control the ability of the hypothalamus to drive the sexual dimorphism of pituitary GH secretion in adulthood [2,13]. Sexual dimorphism in rodents appears to be regulated by estrogen secretion in adult females and by neonatal and adult androgen secretion in males. In adulthood, the male characteristic liver metabolism is dependent on continuous androgen exposure. Neonatal exposure to testosterone imprints the male process of neuroendocrine control over pulsatile pituitary GH secretion, which is first seen during puberty, when the adult pattern of GH secretion becomes evident, and continues through adulthood. If androgen re-programming does not occur, the feminine secretion pattern remains (continuous GH secretion). In postpubertal rats, the male blood pattern consists of high-amplitude pulses (approximately 200 ng/mL) spaced approximately 3–4 hours apart with no measurable trough levels. In contrast, the female pattern consists of continuous low-amplitude pulses (25–50 ng/mL), and GH is always present. The sexually

dimorphic pattern of GH secretion is also seen in humans, although it is not as marked as it is in the rat. Interestingly, the depletion of liver-derived IGF-I in male mice causes the feminization of some GH-regulated sexually dimorphic liver function markers. The loss of the feedback effect exerted by IGF-I on the hypothalamic-pituitary system results in increased GH secretion and includes elevated baseline GH levels between the pulses, which resemble the female pattern of pituitary GH release.

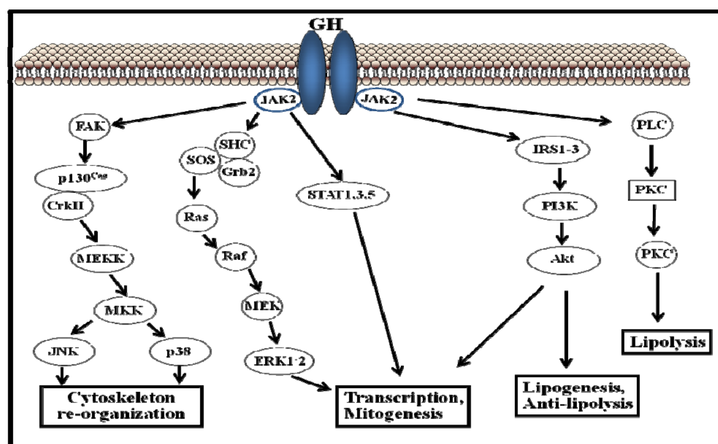
Figure 1. Schematic representation of the somatotropic axis. GHRH and SS, two hypothalamic hormones, control the synthesis and release of GH from the pituitary gland. GHRH is negatively (dashed lines) regulated by feedback from blood GH and IGF-I concentrations. FFA inhibits GH release, whereas leptin and ghrelin stimulate it. Sex hormones and other factors act centrally to stimulate the release of GH. Circulating GH directly stimulates IGF-I production in many organs. IGF-I production in the liver provides the main source of blood IGF-I. GH directly affects many target tissues, sometimes independent of the IGF-I action.



3. The Cellular Regulation of GH Signaling

The GHR belongs to the type I cytokine receptor, a family of receptors without intrinsic kinase activity [33]. Figure 2 shows the traditional view of the initiation of GH signaling: one molecule of GH binds two GHR monomers and induces their dimerization. Through trans-phosphorylation, GH binding to the GHR results in the activation of adjacent JAK2 molecules and cytoplasmic tyrosine kinases associated with the GHR. Activated JAK2 phosphorylates the GHR on tyrosine residues, which in turn recruits members of the STAT family of transcription factors. Of the various STAT proteins (STAT 1 to 6), STAT5b has been widely associated with GH biological actions; however, STAT1, 3, and 5a have also been shown to be recruited by the GHR. STAT5 phosphorylation by JAK2 results in their dissociation from the receptor, dimerization, and translocation to the nucleus, where STAT5 modulate the transcription of the target genes (e.g., IGF-I, ALS, SOCS2, SOCS3, CIS) [34,35]. The STATs represent one of five known pathways in GH-induced signaling; other pathways include the MAPK and PI3K (Figure 2).

Figure 2. Schematic representation of growth hormone-activated signalling pathways.



The duration of GH-activated signals is a critical component of this hormone's biological actions. Studies of the primary hepatocytes and several cell lines have shown that GH-induced JAK2-STAT5 activation is transient and that maximal activation is achieved within the first 30 min of stimulation, followed by a period of inactivation [36]. This process is clearly illustrated in the case of hepatic GH actions, in which the signal duration regulates gender differences in the liver gene expression [37,38]. As mentioned above, the male pattern of GH secretion in rats is episodic and peaks every 3–4 hours, with no measurable trough levels. This inactive period is characterized by an inability to achieve maximal JAK2-STAT5 activation by GH in the following 3 hours unless the GH is withdrawn from the media. Consequently, the intracellular activation of STAT5 is also episodic, and periods of low GH circulating levels are required to achieve the maximal activation of STAT5. Female rats exhibit a more continuous GH secretion pattern with higher basal levels and smaller, irregular intermittent peaks that show reduced STAT5b activation when compared with male rats. The differences in STAT5b activation are responsible for several of the gender differences in the hepatic gene expression [39]. GHR cell surface levels are the primary determinant of GH responsiveness [40]. Transcriptional, translational, and posttranslational level factors can influence GHR synthesis, thereby regulating the cell sensitivity to the GH actions. These factors include the nutritional status, the endocrine context, the developmental stage, and estrogens [36]. The removal of cell surface GHRs by endocytosis is an early step in the termination of GH-dependent signaling. By modulating both GHR internalization and proteasomal degradation, GHR ubiquitination is a key control mechanism in the down-regulation of GH signaling. In addition to GHR down-regulation, other mechanisms are required to complete the inactivation of GH signaling. Because the activation of GH-dependent signaling pathways is critically based on the protein phosphorylation of tyrosine, serine or threonine residues, the obvious mechanism to deactivate this process is the action of protein phosphatases. Recently, several studies have identified the phosphatases that are involved in the specific inactivation of GHR signaling. The signal regulatory protein (SIRP)-α, which belongs to a family of ubiquitously expressed transmembrane glycoproteins, negatively regulates GH-activated signaling by inhibiting the phosphorylation of JAK2,

STAT5b, STAT3, and ERK1-2 [41]. Finally, SOCS proteins [42] have been identified as key components of the negative regulators of the GHR-JAK-STAT signaling pathway. SOCS proteins have been shown to modify cytokine actions through a classic negative feedback loop. In general, SOCS protein levels are constitutively low; however, their expression is rapidly induced by the stimulation of different cytokines or by growth factors, such as GH. SOCS proteins bind the receptor/JAK complex and down-regulate the JAK-STAT signaling pathway. Particularly, the phenotype of SOCS2 null mice (SOCS2KO) identifies SOCS2 as a key physiological player in the negative regulation of GH signaling [43,44]. Other studies have demonstrated that SOCS2 is essential for the regulation of the GH actions that are indirectly related to somatic growth. For example, SOCS2 blocks the GH-dependent inhibition of neural stem cell differentiation. Consequently, SOCS2KO mice have fewer neurons in their developing cortexes, whereas SOCS2 overexpression results in increased neural differentiation. Recently, it has been demonstrated that SOCS2 inhibits intestinal epithelial [45] and prostate cell proliferation [46], which are induced by GH *in vivo*. Evidence also indicates that other hormones, e.g., insulin, xenobiotics, and steroid hormones (including estrogens), can induce SOCS expression [42]. Consequently, the regulation of SOCS protein expression provides a mechanism for crosstalk, and multiple factors can regulate the activity of specific cytokines. Particularly, SOCS2 may be a physiological mechanism by which estrogen can suppress GH-dependent JAK2 phosphorylation [15].

4. STAT5 Plays a Relevant Role in GH-Dependent Regulation of Body Growth and Composition, Liver Metabolism and Gender-Dependent Dimorphism

GH exerts its physiological influence through transcriptional regulation and acute changes in the catalytic activity of several enzymes [4,8,47–49]. The global expression microarray analysis of GH actions in the liver clearly indicates that most of the known physiological effects of GH can be explained through its effect on the transcription of specific genes [6,34,39,50]. To this end, GH is known to activate a network of transcription factors in the liver, including nuclear receptors/transcription factors, such as HNF (4α, 6, 3β, PPARβ, CAR, FXR, SHP, SREBP, CRBP, C/EBPβ, and STAT5b. Based on the gene ontology analysis of liver transcript profiles from the targeted disruption/mutation of GHR-signaling pathway components (or GHR itself) and GH administration to GH-deficient mice and rats, the main metabolic process affected by GH status is energy/fuel metabolism, particularly lipid/fat metabolism; the metabolism of carbohydrates, proteins, steroids, and drugs is also strongly influenced. Combined with the clinical studies of GH-insensitive mutants, these animal findings have revealed that the transcription factor STAT5b is a key GH signaling intermediary for the regulation of postnatal growth, lipid metabolism, and the sexual dimorphism of hepatic gene expression. In addition, many transcripts are regulated independently of STAT5b, presumably as a result of the GHR-dependent activation of the ERK, Src, and PI3K signaling pathways.

4.1. Body Growth

GH is predominantly linked with linear growth during childhood. The liver is a major GH target tissue and the principal source of circulating IGF-I. The GH-dependent transcription of IGF-I is regulated by STAT5 binding sites in the IGF-I gene [51]. Thus, both IGF-I and its transcriptional regulator STAT5 have key roles in mediating the actions of GH in body growth [28,51]. Importantly,

intermittent (male pattern) GH administration in rodents is a more potent stimulus of body growth rate, IGF-I expression, and STAT5b nuclear translocation in the liver than continuous (female pattern) GH administration. This difference supports the notion that the larger body growth in male rodents compared to female rodents could be caused by more effective stimulation of IGF-I and STAT5b mediated transcription. IGF-I proteins are also induced by GH in many tissues, and the local induction of IGF-I in chondrocytes plays an important role in longitudinal growth. However, GH is more effective than IGF-I because GH exerts additional growth-promoting actions independently of IGF-I [52].

Global disruption of STAT5b in mice causes the loss of sexual dimorphic growth characteristics, which reduces the affected males to the size of females; the female mice appeared unaffected [50]. Parallel observations were made with serum IGF-I concentrations, which were reduced by 30–50% in the affected male mice but not in the females. However, the combined disruption of STAT5a/b significantly reduced weight gain in the female mice and suppressed their body growth more than in the STAT5b null mice alone; the results approached the levels that were observed in either the GH- or GHR-deficient mice [35]. These studies demonstrated that STAT5b is important for male-specific body growth, whereas STAT5a regulates body growth in both sexes. Experiments on SOCS2KO mice also support the notion that STAT5b is critical for GH-regulated growth in mammals [42]. Importantly, SOCS2KO mice have enhanced growth, whereas combined STAT5bKO and SOCS2KO mice do not, demonstrating the necessity of STAT5b for the excess body growth observed in the SOCS2KO mice. In addition to the endocrine actions, the paracrine involvement of STAT5a/b in the interaction between GH and muscle is evidenced by the loss of muscle and mass in the IGF-I transcripts, which is observed with the muscle-specific deletion of STAT5a/b [53]. As mentioned above, the growth of female STAT5bKO mice is normal, whereas postnatal growth in GHR-deleted female mice is profoundly retarded. These data suggest that in addition to STAT5b, other transcription factors are related to growth. This relationship is exemplified by the glucocorticoid receptor (GR), which is a critical co-activator of STAT5b in the liver [54]. Importantly, the STAT5b and GR co-regulated transcripts were preferentially enriched in the functional groups related to growth and maturation (*i.e.*, IGF-I). Moreover, both direct and indirect interactions between ERs and STAT5 [55] should be added to the list of mechanisms that are regulated by the nuclear receptors that modulate GH-dependent transcription.

4.2. Metabolism

GH exerts important metabolic actions throughout life. The metabolic effects of GH predominantly involve the stimulation of lipolysis in the adipose tissue, which results in an increased flux of free fatty acids (FFAs) into the circulation. In the muscle and liver, GH stimulates triglyceride (TG) uptake by enhancing the expression of lipoprotein lipase (LPL), and subsequent TG storage. The effects of GH on carbohydrate metabolism are more complicated and may be mediated indirectly via the antagonism of insulin action. Furthermore, GH has a net anabolic effect on protein metabolism because it stimulates protein synthesis while repressing proteolysis. GH has anabolic effects and increases muscle size in GH-deficient individuals [3–5].

The mechanisms of GH actions on lipid metabolism are complex and involve transcriptional and acute changes in catalytic enzyme activities [4,8,47,48]. It is well established that human GH is a lipolytic hormone. The long-term administration of GH includes a decrease in fat deposition and an increase in fat mobilization, thereby increasing circulating FFA and glycerol levels. GH reduces fat mass, particularly in individuals who have accumulated excess fat during periods of GH deficiency (GHD). Obesity is clinically evident in GHD patients, and a decline in GH levels correlates with age-related obesity. The lack of GH or GH signaling induces early obesity in mice [56,57]. Furthermore, GHD in adulthood causes a syndrome that is characterized by increased visceral adiposity, decreased muscle mass, metabolic disturbances, and increased mortality associated with cancer or vascular complications. This syndrome closely resembles metabolic syndrome and can be ameliorated by GH replacement [3,4]. Interestingly, the GH treatment of both healthy and GHD individuals decreases whole-body carbohydrate oxidation and concomitantly increases whole-body lipid oxidation. This process opens the possibility that the GH-induced increases in FFA efflux from adipose tissue could, via the provision of substrates for gluconeogenesis, abrogate the need for amino acids and proteolysis. The increased expression of β3-adrenergic receptor in adipocytes followed by the activation of hormone-sensitive lipase (HSL) is one of the GH mechanisms that lead to lipolytic effects. Additional effects include the uncoupling of the electron transport chain, which enhances mitochondrial heat generation at the expense of energy production from ATP. In the muscle and liver, GH stimulates the uptake and subsequent storage of TG by enhancing lipoprotein lipase (LPL) expression, contrary to the effect of GH on adipose tissue. GH stimulates TG uptake in the skeletal muscle primarily by increasing LPL expression, thereby promoting lipid utilization. However, several factors, such as nutrition, exercise, and sex steroid hormone status, could modify the GH-induced TG storage and lipid oxidation in the skeletal muscle. In the liver, GH treatment can induce a state of TG storage. Three possible mechanisms may be involved in this process: (a) the inhibition of intrahepatic TG (IHTG) lipolysis, (b) the inhibition of lipid oxidation, and (c) enhanced lipogenesis. There are data supporting all three hypotheses. In bGH-transgenic mice, there is a significantly reduced expression of hepatic HSL, suggesting that GH inhibits the lipolysis of IHTG. In addition, studies on bGH-transgenic, GHRKO, PPARαKO, and GH-treated rats (hypophysectomized or hypothyroid) have revealed that GH serves to down-regulate the genes involved in lipid oxidation (e.g., PPAR-α, acyl CoA oxidase, and CPT-1) and increases the expression of the genes promoting lipid synthesis (acetyl CoA carboxylase) in the liver [17,58–60]. Interestingly, the deletion of the STAT5 gene in the liver resulted in hepatic steatosis and increased phosphorylation of STAT1 and STAT3 under basal and GH-induced conditions, suggesting that GH may stimulate IHTG storage in a STAT5-independent manner. However, the deletion of the hepatic GHR gene in mice also resulted in hepatic steatosis because of enhanced lipogenesis and reduced TG secretion from the liver. However, these effects cannot be completely attributed to GH action on the liver because these mice had decreased levels of circulating IGF-I and hyperinsulinemia [17,59]. GHR-JAK2-STAT5 signaling deficiency has also been studied by the mutagenesis of GHR in mice, a model that causes severe obesity in mature mice in proportion to the loss of STAT5b activity [4]. Collectively, these experiments have shown that STAT5 regulates several key enzymes or genes that are otherwise involved in lipid and energy balance. Genetically modified animals and microarray analyses have provided new insights into the long-known anti-adiposity actions of GH and highlighted a key role for STAT5 in these actions. This role is supported by the

original findings that STAT5b-deleted male mice become obese in later life [50] and that STAT5b deletion in a mature human was associated with obesity [61]. Based on altered transcript expression, several processes have been implicated. For example, the up-regulation of some lipogenic genes (e.g., CD36, PPARγ, PGC1α/β, FAS, SCD1, LPL, and VLDLR) may contribute to increased hepatic steatosis and adiposity in deficient GHR-JAK2-STAT5 signaling models, whereas the expression of antilipogenic genes, such as FGF21 and INSIG2, are decreased. The anti-obesity actions of GH are enhanced by the pulsatility of GH secretion, which is evident in males because pulsatile STAT5 activation, as mentioned above, is important for sexual dimorphism in hepatic gene expression (including IGF-1). Despite normal plasma FFA and minimal adiposity, the absent GHR activation could lead to hepatic steatosis because the activated STAT5 prevents this pathology [3].

4.3. Insulin Sensitivity

The effects of GH on both glucose and lipid metabolism are key components in the GH-dependent induction of insulin resistance. In the liver, GH has a stimulatory effect on glucose production, which may be a result of its antagonism of insulin action leading to hepatic/systemic insulin resistance. GH increases glucose production by increasing glycogenolysis; however, GH has either a stimulatory effect or no effect on gluconeogenesis. Moreover, over-expressing the human GH gene in rats increases the basal hepatic glucose uptake and glycogen content [62]. In contrast, the GHD (Ames) and GHRKO mice have improved insulin sensitivity and an up-regulation of hepatic insulin signaling, suggesting that GH antagonizes insulin signaling in the liver [63]. As mentioned above, GH-induced insulin resistance may develop from the increased FFA mobilization in the adipose tissue, which can affect liver insulin sensitivity and lead to insulin resistance and the up-regulation of PEPCK and G6Pase. However, the LID (IGF-I-specific liver deficient) mice show a 75% reduction in circulating IGF-I levels and a 3- to 4-fold increase in the circulating GH level and insulin resistance without a significant increase in the circulating FFA levels. Paradoxically, while crossing LID mice with GH transgenic mice, the serum FFA levels significantly increased, and there was an improvement in insulin sensitivity during a hyperinsulinemic-euglycemic clamp due to higher hepatic, adipose tissue and skeletal muscle glucose uptake [64]. This result suggests that in addition to FFA, other factors may also contribute to GH-induced insulin resistance. One candidate is the SOCS family of proteins, whose expression is induced by GH [42]. Another mechanism by which GH may induce insulin resistance is by increasing the expression of p85, a regulatory subunit of PI3K [3]. Finally, given the large homologies between the insulin and IGF-I systems, it is not surprising that IGF-I profoundly affects carbohydrate metabolism. Alternatively, IGF-I may enhance insulin sensitivity by suppressing GH release via negative feedback. Therefore, the activation of IGF-I signaling adds more complexity to the understanding of the molecular mechanisms involved in GH-induced insulin resistance *in vivo*.

4.4. Gender Dimorphism in the Liver

Sex-dependent gene expression and GH regulation characterizes several of the hepatic gene families involved in endobiotic and xenobiotic metabolism and relevant metabolic functions (e.g., lipid metabolism); 20–30% of all hepatic genes in rodents have a sex-specific expression pattern [39,47–50,65]. Most of these hepatic sex differences are explained by the female-specific secretion of GH through the

induction of female-predominant transcripts and the suppression of male-predominant transcripts. STAT5b is a key player in this scenario. Results from experiments with STAT5b null mice have shown that STAT5b is responsible for the masculinization of the male liver. STAT5b binding sites have been found in the promoters of several sex-differentiated CYP rat genes (e.g., Cyp2c12, Cyp2c11, Cyp2a2). Conversely, other transcription factors (e.g., HNF6 and HNF3b) are more efficiently activated in the female liver or by the continuous GH secretion pattern. Sex differences are found in the hepatic genes involved in endobiotic and xenobiotic metabolism as well as in GH-regulated lipid metabolism. HNF4 and HNF3b are relevant transcription factors for regulating the genes involved in glucose and lipid metabolism [66,67], and they likely also contribute to sexual dimorphism. The continuous administration of GH has been shown to increase the hepatic expression of transcription factor SREBP-1c and its downstream target genes [68], as well as hepatic TG synthesis and VLDL secretion [69]. As mentioned above, GH actions in the liver lead to increased lipogenesis (*i.e.*, SREBP1c induction) and decreased lipid oxidation (*i.e.*, inhibition of PPARα) and promote anabolic growth in peripheral tissues (e.g., muscle and bone) [47–49]. In contrast, estrogens can cause the opposite effect (in comparison with GH) on hepatic lipid metabolism and insulin sensitivity, which represents a relevant point of regulatory interactions between estrogens and GH (see below).

5. The Liver Is a Target for Estrogen

The liver is a direct target of estrogens because it expresses ERα [12], which is connected with liver development [20], the regulation of hepatic metabolic pathways [11,25], growth [21], protection from drug-induced toxicity [22], hepatocarcinogenesis [23], fertility [24], lipid metabolism, and insulin sensitivity [11,25]. In addition, as mentioned above, estrogens can modulate the effects of GH on the liver by acting centrally, regulating pituitary GH secretion, and peripherally modulating GH signaling. Therefore, the liver represents a site where critical interactions can be developed between estrogens and GH.

5.1. Estrogen Receptor Signaling

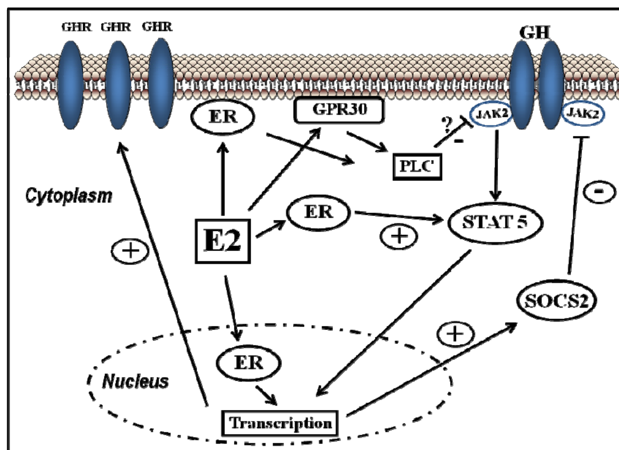
Estrogens exert their physiological effects through two ER subtypes, ERα and ERβ, which belong to the nuclear receptor family of ligand-activated transcription factors [12]. Structurally, ERs share a common framework with the other members of the nuclear receptor family. The *N*-terminal A/B domain is the most variable region, with less that 20% amino acid identity between the two ERs, and confers specific subtype actions on targeted genes. This region harbors the activation function-1 (AF-1), which is ligand independent, and demonstrates promoter and cell-specific activity. The centrally located C domain harbors the DNA binding domain (DBD), which is involved in DNA binding and receptor dimerization. This domain is highly conserved between ERα and ERβ with 95% amino acid identity. The D domain is referred to as the hinge domain and shows low conservation between ERα and ERβ (30%). This domain has been shown to contain a nuclear localization signal. The *C*-terminal E domain is the ligand-binding domain (LBD), and the two subtypes display 59% conservation in this region. The LBD contains a hormone-dependent activation function (AF-2) and is responsible for ligand binding and receptor dimerization. The F domain has less than 20% amino acid identity between the two ER subtypes, and the functions of this domain remain undefined. Full transcriptional

activity of the ERs is mediated through a synergistic action between the two activation domains: AF-1 and AF-2. Both ERα and ERβ contain a potent AF-2 function; however, unlike ERα, ERβ appears to have a weaker corresponding AF-1 function and depends more on the ligand-dependent AF-2 for its transcriptional activation function. The activities of the two ER subtypes are controlled by the binding of the endogenous hormone E2 or by synthetic non-hormonal compounds in the LBD. This binding triggers several events, such as overall conformational changes of the ERs, receptor dimerization and DNA binding to specific estrogen response elements, and interaction with coregulators (chromatin remodelers, coactivators, and corepressors), which are essential effectors in the biological activities of ligand-activated ERs. Each class of ER ligands induces a unique ER conformation that promotes specific coregulator protein interactions and associations between the ER *N*- and *C*-terminal transcription activation functions, AF-1 and AF-2, respectively [70]. E2, a nonselective agonist, has a similar affinity (Kd = 0.05–0.1 nM) for ERα and ERβ̃ ER-subtype-selective agonists have been developed; PPT and DPN are ERα- and ERβ-selective agonists, respectively. In addition, the ERs bind a wide range of synthetic compounds with strikingly diverse structures, including selective estrogen receptor modulators (SERMs) (e.g., raloxifene). The SERMs are synthetic ER ligands that display tissue-selective pharmacology; as anti-estrogens (antagonists), they oppose the action of estrogens in certain tissues while mimicking the action of endogenous estrogens (agonists) in other tissues [70]. Environmental contaminants (e.g., polycyclic aromatic hydrocarbons, phthalates, pesticides), a class of estrogens termed xenoestrogens, and phytoestrogens also have estrogenic actions. Although their affinity for ERs is mostly 100 to 10,000 times lower than that of E2 [71], it is not questionable whether xeno- and phytoestrogens are biologically relevant in humans and farm animals. The tissue-selective expression of ERs can also determine estrogen physiology. ERα is mainly expressed in reproductive tissues, the kidney, bones, white adipose tissue, and the liver, while ERβ is expressed in the ovary, the prostate, the lungs, the gastrointestinal tract, the bladder, hematopoietic cells, and the central nervous system. Therefore, specific therapeutic actions of estrogens on tissues (e.g., the liver) may be obtained through selective ERα agonists (e.g., PPT) [72].

Classical estrogen signaling occurs through the direct binding of ER dimers to estrogen-responsive elements in the regulatory regions of estrogen-targeted genes followed by the activation of the transcriptional machinery at the transcription start site [12]. Estrogen also modulates gene expression by a second mechanism in which ERs interact with other transcription factors (e.g., STAT5) through a process referred to as transcription factor crosstalk. Estrogen may also elicit effects through non-genomic mechanisms, which involve the activation of downstream kinases pathways, such as PKA, PKC, and MAPK, via membrane-localized ERs. An orphan G protein-coupled receptor (GPR)-30 in the cell membrane has also been reported to mediate non-genomic and rapid estrogen signaling. In summary, the mechanisms involved in ER signaling are influenced by cell phenotype, the target gene, and the activity or crosstalk with other signaling networks. Figure 3 shows how E2 can interact with the GHR-JAK2-STAT5 signaling pathway. E2 can modulate GH actions through changes in GHR expression or through crosstalk with the GH-activated JAK2-STAT5 signaling pathway. The ERα-dependent induction of SOCS-2 followed by the inhibition of JAK2-STAT5 signaling is a relevant mechanism that could explain how estrogens directly inhibit GH-regulated actions in the liver [15,16]. We have observed that the long-term administration of physiological doses of E2 to GH-deficient male rats (hypothyroid) can induce the mRNA expression of SOCS-2 in the liver [16].

Hypothetically, other members of the STAT family of negative regulators may contribute to estrogen interaction by GH signaling in the liver [15]. The E2 activation of ERα or ERβ followed by direct interaction between ERs and STAT5 may also regulate STAT5-dependent transcriptional activity. Paradoxically, estrogens can also activate STAT5 signaling in a pituitary manner and even in a JAK2-independent manner [55,73–75].

Figure 3. Signalling pathways activated by E2 and its crosstalk with growth hormone.



5.2. E2 Modulates the GH Promoting of Skeletal Growth

It is well known that sex steroids and GH interact closely to regulate pubertal growth [13]. In addition to the well-established role of the GH-IGF-I axis, estrogens are also known to play an essential role in the pubertal growth spurt and bone mineral accrual. Key findings in the context of estrogen resistance or deficiency include low bone mineral density and the failure to establish peak bone mass. Estrogen-deficient men experience no pubertal growth spurts and have a sustained linear growth without epiphyseal fusion. Estrogens induce the closure of the epiphyseal growth plate during late puberty, thereby limiting longitudinal growth and final bone size. During early puberty, estrogens stimulate longitudinal growth, a stimulatory effect that is considered to primarily reflect interaction with the GH-IGF-I axis. Estrogens stimulate GH secretion and the GH-induced hepatic synthesis of IGF-I [13]. Experiments with ER null mice have shown that ERα mediates important estrogen effects in the skeleton during growth and maturation [21]. Similar phenotypes can be found for aromatase-deficient rats, which cannot produce estrogens. Reports of natural mutations in the ERs and the aromatase gene in men, along with evidence from estrogen-resistant male mice and the administration of aromatase inhibitors in male rats, have also called attention to the physiological role of estrogens in skeletal growth. In addition to their interactions with the GH-IGF-I axis, estrogens can regulate pubertal skeletal growth and bone mineral acquisition independently of GH or GHR [76], suggesting that estrogens could rescue pubertal growth during GH resistance through a novel mechanism of independent GHR stimulation of IGF-I production in the liver (e.g., after activation through phosphorylation, STAT5 can stimulate IGF-I transcription). In contrast to physiological levels,

the administration of pharmacological doses of estrogens results in the drastic reduction of circulating IGF-I, which most likely reflects the inhibitory effect of estrogens on the GH-JAK2-STAT5 signaling pathway in the liver (see below).

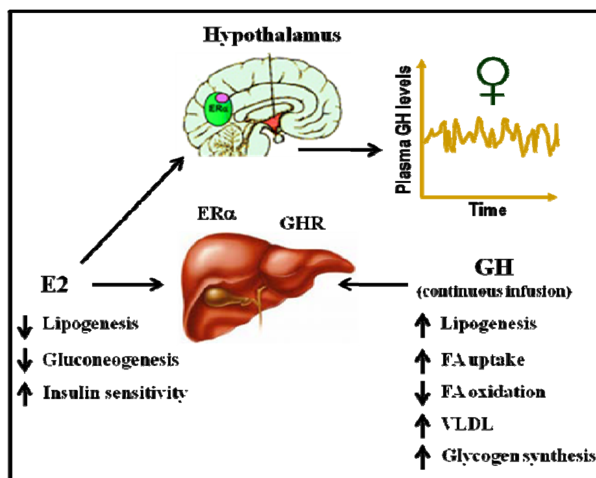
5.3. Gender Dimorphism in the Liver Is Regulated by the Pattern of GH Secretion and Sex Steroids

Genome-wide screens of gene expression have shown that the GH- and sex-dependent regulation of hepatic gene expression are not confined to steroid or drug metabolism, and a number of other hepatic genes have been found to be up- and/or down-regulated by the different patterns of GH or sex-steroid exposure [8,39,47–50,65]. GH- and sex-dependent hepatic transcripts encoding plasma proteins, enzymes, transcription factors and receptors involved in the metabolism of proteins, carbohydrates, lipids, or signaling regulation have been identified. There is a consensus that the response to different sex GH patterns is the major cause of gender dimorphism in the liver. However, it is also likely that factors other than the sexually dimorphic pattern of GH secretion are behind some sex differences in rat livers. Potential mechanisms that could contribute to "liver sexuality" are the pituitary-independent effects of estrogens through an interaction with ERα or the GH-JAK2-STAT5 signaling pathway in the liver.

5.4. E2 Regulates Lipid Metabolism and Insulin Sensitivity: Potential Crosstalk with GH

Acting on both ERα and ERβ, estrogens are recognized as important regulators of glucose homeostasis and lipid metabolism [75]. Several studies have shown that ERα controls inflammation, lipid, glucose, protein, and cholesterol homeostasis in the liver, leading to the conclusion that E2 via ERα is antidiabetogenic. In contrast, ERβ might be diabetogenic. Both male and female ERαKO mice develop insulin resistance and impaired glucose tolerance, similar to humans who lack ERα or aromatase. ERα mainly mediates the beneficial metabolic effects of estrogens, such as anti-lipogenesis, improved insulin sensitivity and glucose tolerance, and reduced body weight/fat mass. In contrast, ERβ activation appears to be detrimental for the maintenance of regular glucose and lipid homeostasis. The insulin resistance in ERαKO mice is largely localized to the liver, including increased lipid content and hepatic glucose production. Interestingly, the expression of liver lipogenic genes can be decreased after the administration of E2 to diabetic Ob/Ob or female mice fed high-fat diets. Similarly, the aromatase knockout mouse, which cannot produce E2, has increased intra-abdominal adiposity and develops steatosis and an impairment of lipid oxidation in the liver. As mentioned above, GH-GHR-JAK2-STAT5 deficiency in adults causes adiposity and hepatic steatosis. Therefore, E2 and GH regulate a common cellular network related to the physiological control of lipid metabolism (Figure 4). In our lab, we have shown that the subcutaneous administration of near-physiological doses of E2 to male rats with GH deficiencies (hypothyroid rats) dramatically influenced the hepatic transcriptional response to pulsatile GH administration (male pattern). Particularly, the expression of several genes related to the endocrine, metabolic, and sex-differentiated functions of GH were drastically inhibited by E2 [77].

Figure 4. Physiological control of hepatic lipid metabolism by E2 and GH.



6. The Modulation of GH Actions by Estrogens Is Clinically Relevant

Multiple regulatory interactions between estrogen and GH can be achieved in the liver. Observational studies in children have reported that puberty is significantly associated with increases in mean sex steroids, GH and IGF-I concentrations and the IGF-I response to an injection of GH. These observations suggest that the endogenous gonadal steroid milieu increases GH sensitivity in girls and boys during puberty. However, whether exogenous estrogen alters GH sensitivity during childhood must still be clarified. E2 can reduce the level of circulating IGF-I when used to prime GH stimulation tests in prepubertal children or when taken as an oral contraceptive or as hormone replacement therapy in menopausal or hypopituitary women. In adulthood, exposition to estrogens is associated with GH resistance. It is well accepted that oral estrogens can impair the metabolic and endocrine function of the liver in adulthood. Studies in women indicate that oral estrogens, particularly higher doses, impair the IGF-I response to GH [78–80]. GH (but not IGF-I) levels are higher in young women than in age-matched men. In GH-deficient males (compared to female patients), GH treatment induces a greater increase in lean mass and a decrease in fat mass or a greater increase in the indices of bone turnover and bone mass. IGF-I levels are lower in GH-deficient women, and the IGF-I increase in response to GH treatment is approximately half that of their male counterparts, which results in women requiring a higher replacement dose of GH than men. Studies of GH-deficient and postmenopausal women provide compelling evidence that estrogen levels achieved in the portal circulation after ingestion of therapeutic doses of oral estrogen impair GH-regulated liver functions. The oral administration of therapeutic doses of estrogen to hypopituitary patients can inhibit the endocrine and metabolic effects of GH; circulating IGF-I levels, lipid oxidation, and protein synthesis are suppressed, with a reciprocal elevation in carbohydrate oxidation [81]. The oral administration of estrogen led to a significant increase in fat mass and a loss of lean body mass compared to that observed during transdermal estrogen therapy. Interestingly, the effects on fat oxidation and IGF-I induced by the oral route of estrogen administration contrast the effects of GH and are consistent with an antagonistic

effect on GH actions. In summary, therapeutic doses of estrogen may affect the endocrine and metabolic actions of GH in the liver and are somehow different from the physiological doses of E2. Estrogen inhibits hepatic IGF-I production in a concentration-dependent manner regardless whether this inhibition is achieved through the portal or systemic circulation [82–84]. As mentioned above, estrogen can inhibit the effects of GH in the liver by inducing the negative regulators of GH signaling [15,85].

7. Conclusions

Estrogen interactions with GH can be executed indirectly at the level of pituitary GH secretion and directly at the cellular level. The impact of estrogens on GH-regulated endocrine (e.g., IGF-I), metabolic (e.g., lipid metabolism, insulin sensitivity), and sex-differentiated (e.g., lipid, endobiotic and xenobiotic metabolisms) functions in the liver are physiologically and therapeutically relevant. The detrimental impact of oral estrogens on the metabolic actions of GH is clinically relevant. Furthermore, SERMs and phytoestrogens are gaining widespread use. The endocrine and metabolic consequences of long-term exposition to novel estrogen-related compounds are still largely unknown. These complex interactions deserve further research because they can potentially impact human health.

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Conflict of Interest

The authors declare no conflict of interest.

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Influence of Neonatal Hypothyroidism on Hepatic Gene Expression and Lipid Metabolism in Adulthood

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Abstract

Thyroid hormones are required for normal growth and development in mammals. Congenital-neonatal hypothyroidism (CH) has a profound impact on physiology, but its specific influence in liver is less understood. Here, we studied how CH influences the liver gene expression program in adulthood. Pregnant rats were given the antithyroid drug methimazole (MMI) from GD12 until PND30 to induce CH in male offspring. Growth defects due to CH were evident as reductions in body weight and tail length from the second week of life. Once the MMI treatment was discontinued, the feed efficiency increased in CH, and this was accompanied by significant catch-up growth. On PND80, significant reductions in body mass, tail length, and circulating IGF-I levels remained in CH rats. Conversely, the mRNA levels of known GH target genes were significantly upregulated. The serum levels of thyroid hormones, cholesterol, and triglycerides showed no significant differences. In contrast, CH rats showed significant changes in the expression of hepatic genes involved in lipid metabolism, including an increased transcription of PPARα and a reduced expression of genes involved in fatty acid and cholesterol uptake, cellular sterol efflux, triglyceride assembly, bile acid synthesis, and lipogenesis. These changes were associated with a decrease of intrahepatic lipids. Finally, CH rats responded to the onset of hypothyroidism in adulthood with a reduction of serum fatty acids and hepatic cholesteryl esters and to T3 replacement with an enhanced activation of malic enzyme. In summary, we provide *in vivo* evidence that neonatal hypothyroidism influences the hepatic transcriptional program and tissue sensitivity to hormone treatment in adulthood. This highlights the critical role that a euthyroid state during development plays on normal liver physiology in adulthood.

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Introduction

The thyroid hormones (THs) are essential for development, growth, and metabolism [1,2,3]. The physiological importance of the THs becomes evident under the condition of congenital-neonatal hypothyroidism (CH) [2]. If not treated immediately, CH has a profound impact on physiology and can permanently imprint neurological and endocrine systems, which, in turn, leads to mental retardation, growth arrest, and metabolic disturbances. During ontogeny, there exists a critical period when normal TH levels are required, and TH replacement after that period cannot correct the changes in gene expression that was caused by CH. This is exemplified by abnormal insulin-like growth factor binding protein (IGBFP)-2 expression in neonatal hypothyroid rats that can be corrected by TH replacement if started during the first week of life but not later [4].

Most of the effects of the THs result from their interaction with TH receptors (TR), which can bind to the TH response element (TRE) located in several target genes [1]. A TRE is present upstream of the rat GH gene [5], the product of which has a significant role in body growth and metabolism in postnatal life [3]. GH expression is induced by T3 and reduced in hypothyroid states [6,7,8]. In fact, the growth-promoting effects of T3 can be largely explained by its ability to induce normal GH secretion and to regulate the expression patterns of the GH receptor in the liver and the IGF-I receptor in peripheral tissues [9]. Therefore, the THs might exert physiological actions on the liver through a direct transcriptional regulation of several target genes [1] and indirect mechanisms (e.g., modulating actions of GH and metabolic sensors such as LXR) [6,7,8,9,10,11]. In addition, it has been shown that the effects of the THs on glucose metabolism might also be dependent on their actions in the brain, which affect the

autonomic control of peripheral metabolic tissues such as the adipose tissue in addition to the liver [12,13,14]. These findings suggest that CH could cause permanent alterations in peripheral metabolic tissues through irreversible developmental alterations in the brain. The THs clearly have some lipid-lowering actions, as demonstrated by recent human studies [15]. For example, the ability of THs to reduce plasma LDL-c levels has been explained by its capacity to increase the expression of LDL receptors and the activities of lipid-lowering enzymes in the liver. THs increase the number of LDL receptors without necessarily modifying their transcription, which supports a post-transcriptional regulation of these proteins [16]. Some lipid-lowering enzymes are direct transcriptional targets. These are exemplified by the CYP7A1 gene [17,18], the product of which has a major cholesterol-lowering activity. Malic enzyme (ME) and fatty acid synthase (FAS), two critical regulators of lipogenesis, are also directly regulated by the THs [19,20,21]. In contrast, the effects of THs on other lipogenic genes such as sterol regulatory element-binding protein (SREBP)-1c are more controversial: THs induced SREBP1c expression in the livers of rats [22,23], whereas they strongly downregulated SREBP1c in the livers of mice [24].

In rats, the serum concentrations of the THs are low at birth and increase progressively, reaching adult levels at approximately the third week of life when the maturation of the hypothalamus-pituitary-thyroid axis is achieved [25]. Administration of the antithyroid drug methimazole (MMI) to pregnant rats before the onset of fetal thyroid function is known to abolish both the maternal and fetal thyroid function [26]. In this context, exposure to MMI during the fetal-neonatal period of life is an attractive model to study the influence of CH on the adult pattern of genes that are under the control of the THs during rat development [4]. In addition, the disturbances in endocrine status during the neonatal period of life may affect the susceptibility to chronic diseases or biological insults in adulthood [14,27,28,29,30]. However, the precise mechanism whereby CH influences liver physiology in adulthood remains poorly understood. We hypothesized that CH might lead to changes in TH and/or GH action during development that have physiological repercussions in adulthood. Therefore, there were two aims of this study. First, we assessed the influence of CH on the somatotropic axis, liver gene expression, and serum and hepatic lipid biomarkers in adulthood. Second, we investigated how rats, which were transiently exposed to CH, adapted to the biological insult of the onset of hypothyroidism in adulthood. In the present study, we provide *in vivo* evidence that exposure to CH alters postnatal development, which influences the liver transcriptional program that is associated with altered lipid homeostasis and tissue sensitivity to hormones.

Materials and Methods

Materials

Recombinant human GH was kindly donated by Pfizer Laboratories (Spain). Tri-Reagent and the rest of the products cited in this work were purchased from the Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated.

Animal study design

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the University of Las Palmas de G.C. and conducted in accordance with European and Spanish laws and regulations. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Las Palmas de G.C. (permit number: 2006-07824). All efforts were made to minimize suffering. Male Sprague-Dawley rats (n = 6 per group) were used throughout these experiments. Animals were kept under a constant dark/light cycle in a controlled temperature (21–23°C) environment and had free access to a standard diet (A04 SAFE; Panlab, Barcelona, Spain) and tap water throughout the experiment. For mating purposes, four females were housed overnight with two males starting at 21:00 h. Females were checked by 7:00 h the next morning, and the presence of a vaginal plug was designated as gestational day (GD) 0. Within 24 h after birth, excess pups were removed so that 8 pups were kept per dam. The indirect MMI exposure method (i.e., through maternal milk) utilized in this experiment has been extensively employed to induce a transitory congenital-neonatal hypothyroidism (CH) for the determination of short- and long-term physiological effects [4,26,29,30]. Briefly, 0.02% MMI was administered in the drinking water for pregnant rats from GD12 until weaning at post-natal day (PND) 30, which is when pups were mature enough to support their own nutritional needs. The MMI-containing water was changed twice per week. Untreated rats served as concurrent euthyroid and age-matched controls (INTACT). Rats that were weight paired with the CH groups were also included to control for the effects of reduced body weight on hepatic gene expression as follows: 1) WP30, weight-paired rats with CH group on PND30, corresponds to animals that were sacrificed on PND24 and 2) WP, weight-paired rats with CH group on PND80, which were sacrificed on PND53. For the generation of adult hypothyroid rats (TX), 0.05% MMI was added to the drinking water for 3 weeks [23] starting at PND60 until sacrifice at PND80. Thus, two additional groups were created as follows: 1) adult TX rats without CH (TX/−CH) and 2) adult TX rats with CH (TX/+CH). A schematic diagram of this experimental model is shown in Supplementary File S1. The presence of hypothyroidism was corroborated by monitoring the body weight and serum levels of T3 and T4. During the last week of life, TX rats were either injected with T3 (20 µg/kg b.w.) as a single daily ip injection or with GH (0.4 mg/kg/day) divided into two daily sb injections that were carried out at 08:00 h and 20:00 h. T3 was dissolved in a minimum volume of 0.01 N NaOH and was brought up to the appropriate concentration with sterile saline. In parallel, TX animals received equivalent amounts (0.20 ml) of the vehicle alone (VEH). Twenty-four hours (in the case of T3) or twelve hours (in the case of GH) after the last injection, animals were sacrificed by exsanguination after pentothal anesthesia. Twelve hours before the rats were sacrificed, the diet was removed from the cages to minimize the effect of food. Serum samples were collected and stored at −80°C until analysis. Portions of the liver were snap frozen in liquid nitrogen and stored at −80°C until being processed for mRNA or biochemical analysis.

Growth and food intake analysis

Body weights, tail lengths, and food intake were measured once a week for all animals. The measurement of tail length was used for monitoring growth [31,32,33,34]. The percentages of weight rate (WR) or tail rate (TR) were calculated by the following formulas: WR = [(W (g) (new) – W (g) (old)/W (g) (old)]*100 or TR = [(T (cm) (new) – T (cm) (old)/T (cm) (old)]*100, respectively. Food consumption was estimated by subtracting the amount of food left on the grid from initial food weight. Food spilled on the floor of the cage was not weighed, but spillage was minimal because the diet was supplied as pellets. Mother and pups were housed in the same cage until weaning on PND30. Then, weaned pups were housed in pairs to control for food efficiency. The weekly caloric intake was calculated on the basis of food

consumption x caloric value of the diet (2900 kcal/g). Feed efficiency (FE), which denotes the body weight increase per gram of food consumed or the ability to transform calories consumed into body weight [35], was calculated by the following formula: mean body weight gain (g)/total caloric intake. The weights of the liver, heart, and kidneys as well as the organ weight/total body weight ratios were also calculated on PND30 and PND80.

Serum IGF-I, TSH, T3, and T4 analysis

The **s**erum levels of IGF-I (Quantikine; R&D Systems) and TSH (Gentaur Molecular Products, Belgium) were determined by using rat immunoassays and following the manufacturers' recommendations. All of the samples were assayed together, and each sample was assayed in duplicate. The serum free T4 and T3 concentrations (ng/dl) were measured in duplicate by an enzyme immunoassay (Access Immunoassay Systems, Beckman Coulter, Inc., USA) with detection limits of 0.60 ng/dl and 88 ng/dl, respectively.

Serum lipid analysis

Lipoproteins were separated essentially as previously described [36] using a Superose®-12 PC 3.2/30 column (Pharmacia Biotech, Uppsala, Sweden). Serum from each animal (2.5 µL) was separated for cholesterol and triglycerides, which were subsequently assayed on-line. Total cholesterol and triglycerides were assayed using cholesterol and triglyceride colorimetric enzymatic kits (Roche/Hitachi Diagnostic GmbH, Mannheim, Germany). The serum triglyceride value for each animal was normalized to the respective glycerol content. Absorbance was continuously measured at 500 nm, and data were collected every 10 s using EZ Chrom™ software (Scientific Software, San Ramon, CA).

Hepatic lipid analysis

Frozen liver aliquots were used for lipid extraction [37], and aliquots of lipid extracts were quantified after separation by one-dimensional TLC [38] and image analysis using the G5-700 Bioimage TLC scanner (Bio-Rad, CA). The spots were quantified as integrated optical density against an internal standard of cholesteryl formate and against calibration curves of the different lipid standards.

RNA isolation, cDNA microarray, probe preparation, and hybridization

Total RNA was isolated by the homogenization of frozen liver as previously described [23]. All samples were treated with RNase-free DNase (Promega, Madison, WI). The RNA yields were measured by UV absorbance, and the quality of total RNA was analyzed with a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). A microarray containing 27,000 rat 70-mer oligo probe sets, which was produced at the KTH Microarray Center (www.biotech.kth.se), was used to evaluate the effects of CH on liver gene expression in adulthood. Five micrograms of high-quality total RNA from liver was reversed-transcribed, labeled, and hybridized according to the manufacturer's protocol (Pronto™ Plus System; Promega). After 16 h of hybridization, the slides were scanned using a GenePix Microarray Scanner (Axon Instruments, CA). Four independent hybridizations were performed to compare individual animals from the CH group (n = 4) with those from the INTACT group (n = 4) on PND80 for a total of four analyses.

Data processing and analysis

Image analysis was performed using GenePix Pro 6.0 software (Axon Instruments, Union City, CA) as previously described [23]. The LOWESS (Locally Weighted Scatter Plot Smoother) method in the SMA (Statistics for Microarray) [39] package (www.bioconductor.org) was used to normalize the raw data. The probe sets not present in at least three of the four chips were considered as meaningless and therefore were eliminated to reduce data complexity. Identification of differentially expressed genes was performed using the SAM (Significance Analysis for Microarrays) statistical technique [40]. A *q* value was assigned to each of the detectable genes in the array. This value is similar to a *P*-value and measure the lowest false discovery rate (FDR) at which the differential expression of a gene is considered significant. A minimal FDR of 0.05 was assigned for each gene. An additional selection requirement was added to this statistically based criterion, which was based on the absolute changes in the gene expression ratios. A value of 1.5 (50%) ($\log_2$ ratio CH/INTACT≥|0.58|) was chosen to describe ratios as up- or downregulated. Functional classifications (Gene Ontology) and pathway analysis (KEGG) of differentially expressed genes that were affected by CH were performed by using the web-based tool DAVID [41]. All microarray data are MIAME compliant, and the raw data have been deposited in Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo).

Gene expression analysis by real-time quantitative PCR (qPCR)

The mRNA expression levels of genes were measured using qPCR as previously described [23]. Briefly, 2 µg of total RNA was treated with RNase-free DNase I (Promega) and reverse transcribed by using an iScriptTM kit (Bio-Rad Laboratories). Two microliters of cDNA served as a template in a 20-µl qPCR reaction mix containing the primers and SYBR Green PCR Master mix (Diagenode, Belgium). Quantification of the gene expression was performed with an ABI PRISM® 7000 SD PCR System. A dissociation protocol was performed to assess the specificity of the primers and the uniformity of the PCR generated products. Exon-specific primers were designed by the Primer 3 program [42] and are listed in Supplementary File S2. The level of individual mRNAs measured by qPCR was normalized to the level of the housekeeping genes cyclophilin and ribosomal 28S by using the Pfaffl method [43]. For graphing purposes, the relative expression levels were scaled such that the expression of the INTACT control group equalled one.

Statistical analysis

The data are expressed as the means ± SD. The significance of differences between the groups was tested by either a two-tailed Student's *t* test or a one-way ANOVA, which was followed by post hoc comparisons of the group means according to the GraphPad Prism 5 program (GraphPad Software, San Diego, CA). A two-tailed Student's *t* test was performed on PND80 for body weight and tail length to assess the effect of CH and the completeness of recovery. Statistical significance was reported if $P<0.05$ was achieved.

Results

Neonatal hypothyroidism delays body growth development and is followed by catch-up growth

There were no significant MMI treatment-related differences in dam's body weights and intake of feed and drinking water throughout gestation (data not shown). There were no apparent MMI treatment-related effects on the offspring's body weights over the 3–4 PND. As expected [4,26], the INTACT pups on the tap water recorded normal size and growth during development, while

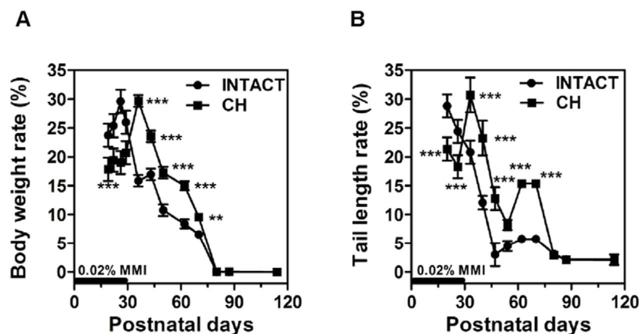

Figure 1. Effects of neonatal hypothyroidism on body growth development. Percentages of body weight rate (A) or tail length rate (B) were measured at 7-d intervals as described in Material and Methods. Results are expressed as mean ± SD from six individual animals in each group. **, $P<0.01$, ***, $P<0.001$ for comparison with INTACT group.
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defects due to the MMI treatment in CH rats were evident by a reduction in body weight (Supplementary File S3; panel A) and tail length (Supplementary File S3; panel B) from the second week of life. Upon weaning on PND30, biochemical hypothyroidism was shown, and significantly low circulating T3 (pg/ml) (9.93±0.66 *vs.* 7.80±0.83; $P=0.0008$) and elevated TSH (ng/ml) (2.73±2.04 *vs.* 6.72±2.5; $P=0.012$) levels were found in CH rats in comparison with the age-matched INTACT group. In addition, circulating IGF-I (ng/ml) levels were reduced by 55% in the CH group (716.33±132.62 vs. 323.25±45.97; $P=0.0001$). Furthermore, the mRNA levels of ME (0.15±0.10 vs. 1±0.04; $P=0.0001$) and Spot14 (0.30±0.10 vs. 1±0.02; $P=0.0001$), two genes positively regulated by TH [1], were also markedly reduced in the CH group. In liver, the fetal expression pattern of high IGFBP-2 levels was replaced by the adult pattern of low levels of IGFBP-2 only in the presence of normal levels of TH during rat development [4]. CH rats expressed higher levels of IGFBP2 mRNA in comparison with the INTACT or WP control rats (Supplementary File S3; panel C). Collectively, these data support the presence of neonatal hypothyroidism on PND30.

Once the MMI treatment was discontinued on PND30, the animals gradually recovered and significant catch-up (defined as growth rate that is greater than normal for age after a period of growth inhibition) [44] occurred in terms of body weight (Supplementary File S3; panel D) and tail length (Supplementary File S3; panel E). It was evident in all hypothyroid rats that, **w**hen MMI was withdrawn, they rapidly began to increase their growth rates in a compensatory manner to make up for their deficits and to catch up with the normal age-matched control rats. These results suggest that, in agreement with what has been previously shown [45,46,47,48], the plasma levels of THs were rapidly recovered (i.e., within 2–4 days after discontinuation of MMI treatment). Interestingly, the feed efficiency was significantly increased in CH rats (Supplementary File S3; panel F). On PND80, a statistically significant difference remained in body mass (g) (430±29.69 vs. 333±29.72; $P=0.002$) (Supplementary File S3; panel A), tail length (cm) (21.9±0.62 vs. 20.3±0.73; $P=0.002$) (Supplementary File S3; panel B), and circulating IGF-I levels (ng/ml) (1541.24±76.74 vs. 1305.15±126.27; $P=0.003$) between INTACT and CH groups, respectively, indicating that growth was incomplete. Importantly, at the time of sacrifice, the growth rate, measured as a percentage of body weight gain (Fig. 1A) or tail length gain (Fig. 1B), was similar between the two groups, which suggests that the CH animals may have finished a period of rapid catch-up growth. The weights of the liver and kidneys at the end of the study (i.e., PND80), corrected by total body weight, were similar in the CH rats in comparison with age-matched INTACT rats (data not shown), which also suggests that the compensatory growth of organs in relation to body weight was achieved. Furthermore, on PND80, the serum levels of T3 (ng/dl) (45.15±5.77 vs. 47.52±3.17), T4 (ng/dl) (1.94±0.27 vs. 1.52±0.18), total cholesterol (mM) (1.54±0.15 vs. 1.65±0.21), and triglycerides (mM) (1.20±0.23 vs. 1.18±0.28) in the CH group did not differ from the INTACT animals. Subtle changes in hepatic IGF-I (Fig. 2A) and IGFBP-3 (Fig. 2B) mRNA levels together with increased levels of IGFBP-2 (Fig. 2C), a gene that is a marker of delayed development or hypothyroidism in rats [4,49], were also shown on PND80. Because the expression of IGFBP-2 mRNA levels was similar in the CH and WP groups, this result indicates that the overexpression is a consequence of delayed growth and low body weight rather than a long-lasting consequence of CH. However, in comparison with age-matched INTACT rats, several genes remained unaltered or were upregulated in CH rats whereas they were significantly downregulated in the WP control group (Fig. 3–5). Collectively, these results suggest that the induction of neonatal hypothyroidism alters the normal development of the liver gene expression program, which may impact GH-related liver functions.

Influence of neonatal hypothyroidism on Growth Hormone-regulated genes

An altered growth rate or a long-lasting influence on somatotropic axis (i.e., GH, liver and IGF-I) could be related to the effects of CH on the hepatic GH signaling system [50,51]. To test this hypothesis, we next analyzed the mRNA expression levels of the GH-regulated genes CIS and SOCS2, which act as negative regulators of GH signaling. On PND30, the CIS mRNA expression level was significantly upregulated ($P<0.001$) in CH rats (9.93±4.45) in comparison with the INTACT (1±0.03) and WP30 (0.87±0.67) groups, whereas SOCS2 remained unchanged. However, on PND80, SOCS2 (Fig. 3A) and CIS (Fig. 3B) were upregulated whereas SOCS3 (Fig. 3C) and additional GH-independent negative regulators of STAT-mediated signaling such as PIAS3 (Fig. 3D) and SOCS5 (Fig. 3E) were downregulated. We

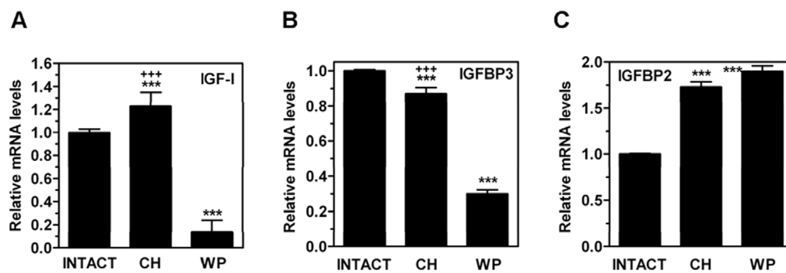

Figure 2. Effects of neonatal hypothyroidism on mRNA expression levels of IGF-I and IGFBP genes in adult rat liver. On PND80, the hepatic mRNA levels of IGF-I (A), IGFBP3 (B), and IGFBP2 (C) were measured by qPCR in rats exposed to neonatal hypothyroidism (CH), age-matched (INTACT) or weight-paired (WP) control groups. The mean mRNA expression level of each gene in the INTACT group is defined as 1, with all other expression values reported relative to this level. Bars represent mean ± SD from at least six individual animals. ***, $P<0.001$ for comparison with INTACT group. +++, $P<0.001$ for comparison with WP group.
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next explored whether the increased hepatic expression of SOCS2 and CIS mRNA levels in adult rats exposed to CH was associated with a negative regulation of GH target genes or instead reflected enhanced GH signaling. CYP2C11 (Fig. 3F) and CYP2C13 (Fig. 3G), two biomarkers of the male gene expression pattern in rats that are under GH control [52], were overexpressed in CH in comparison with the INTACT or WP groups, whereas the female-predominant CYP2C7 gene was downregulated (Fig. 3H). These results show that CH influences the mRNA regulation of GH target genes in the adult liver. The absence of major effects on IGF-I expression suggests the presence of complex transcriptional mechanisms *in vivo* that deserve further research.

Neonatal hypothyroidism influences the liver gene expression program in adulthood, and it is associated with decreased levels of intrahepatic lipids

To better understand the influence of transient neonatal hypothyroidism on adult liver physiology, we next carried out an exploratory analysis of the global gene expression changes in the CH group on PND80 compared to INTACT rats by using DNA microarrays. We identified the set of genes that were differentially regulated in the CH group and analyzed the biological processes represented based on gene annotations in the Gene Ontology (www.ncbi.nlm.nih.gov/geo) and the KEGG pathway databases [41]. These exploratory analyses indicated that genes with altered expression in the CH group were functionally related to lipid metabolism ($P=0.0001$). In particular, "PPARα" was the signaling pathway (KEGG) most represented among the CH regulated genes, suggesting alterations in hepatic lipid metabolism even in the absence of overt changes in circulating cholesterol and triglyceride levels (see below). Accordingly, our study showed that the hepatic content (mg/g of tissue; n = 6) of triglycerides (3.91±0.6 vs. 1.49±0.18; $P<0.001$), cholesteryl esters (0.30±0.02 vs. 0.21±0.04; $P<0.001$), free fatty acids (FFA) (0.18±0.03 vs. 0.13±0.004; $P<0.05$), and phospholipids (57.8±4.1 vs. 48.7±8.8; $P<0.05$) were significantly reduced in CH in comparison with INTACT rats. However, no changes in the free cholesterol (2.4±0.2 vs. 2.1±0.3) content (mg/g of tissue) were observed.

In order to determine whether the decreased hepatic content of lipids in CH rats was due to increased lipid catabolism, decreased lipid synthesis, increased lipid excretion, or some combination of these mechanisms, we analyzed the hepatic mRNA levels for proteins mediating these processes by using qPCR. First, Figure 4A shows that the mRNA levels of PPARα, a master regulator of β-oxidation, were upregulated in the CH group. Similar results were observed when measuring the mRNA expression of carnitine palmitoyl transferase-1 (CPT1) (Fig. 4B), a PPARα target gene coding for a protein that is important for transferring fatty acyl-CoAs into the mitochondria for β-oxidation [53]. In contrast, PPARα (Fig. 4A) and CPT1 (Fig. 4B) were downregulated in the WP group. Phosphoenolpyruvate carboxykinase (PEPCK), a rate-limiting gene in gluconeogenesis, remained unaltered in the CH group, whereas it was significantly reduced in the WP control group (data not shown).

ME is a TH-regulated enzyme that converts malate into pyruvate to generate NADPH which is destined for lipogenesis and other biosynthetic processes [54]. We found that the ME mRNA levels (Fig. 4C) were upregulated in the CH rats on PND80, but the levels of SREBP1c (Fig. 4D), a master regulator of lipogenesis, were significantly downregulated. The SREBP1c target genes, acetyl-CoA carboxylase-1 (ACC1) (Fig. 4E), the product of which is the rate-limiting metabolite for fatty acid biosynthesis and inhibits CPT-1 [55], and fatty acid synthase (FAS) (Fig. 4F), as well as several genes known to be regulated by PPARα such as CYP4F1 (Fig. 4G), palmitoyl-CoA oxidase (AOX) (Fig. 4H) and liver fatty acid-binding protein (L-FABP) (Fig. 4I), were not affected in the CH group whereas they were significantly reduced in the WP control group. The steady-state mRNA level for insulin-induced gene 1 (INSIG-1) (Fig. 5A), an SREBP target, was also reduced in the CH rats. Overall, our data do not support the activation of a lipogenic program in the CH rats. Interestingly, the hepatic mRNA expression levels of SREBP2 (Fig. 5B) and its target gene LDLR (Fig. 5C), the product of which is involved in hepatic VLDL uptake, were 2–3-fold lower in the CH rats compared with the INTACT rats. Similar results were obtained for the gene expression levels of CD36 (Fig. 5D), which is involved in the uptake of FFA and microsomal triglyceride transfer protein (MTTP) (data not shown), which is involved in triglycerides assembly, indicating that diminished uptake of fatty acids may contribute to the reduced hepatic TG levels observed in the CH rats [56]. ABCA1 (Fig. 5E), a transporter responsible for cholesterol efflux, was also reduced whereas LXR (Fig. 5F), hepatic lipase (HL) (Fig. 5G), and HMGCoA-S (Fig. 5H) remained unchanged. HMGCoA-R (Fig. 5I) was upregulated in CH group. In contrast, the mRNA expression levels of LXR, HL, HMGCoA-R, and HMGCoA-S were significantly downregulated in the WP

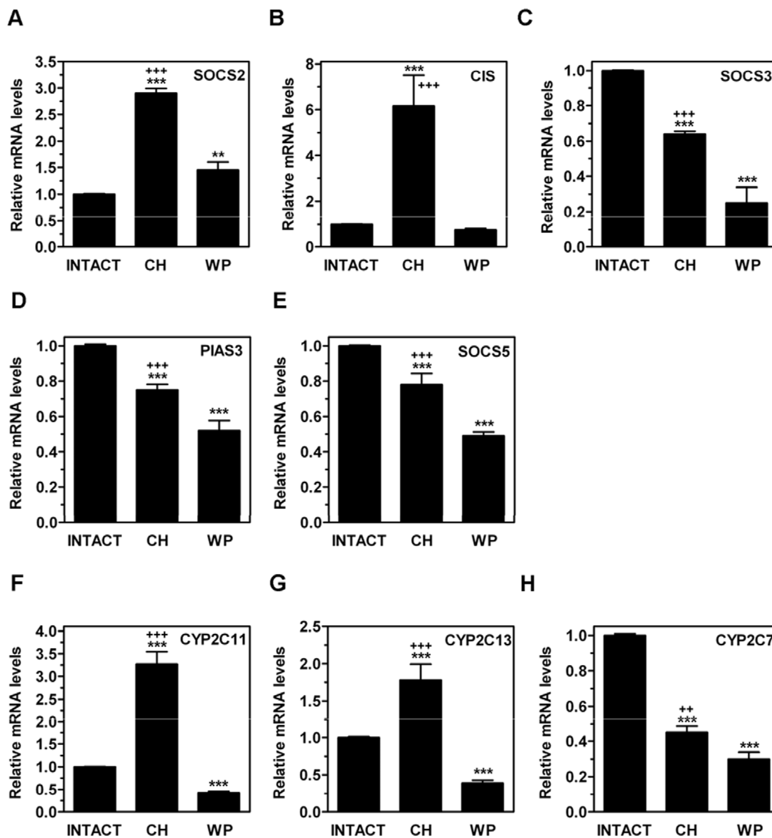

Figure 3. Effects of neonatal hypothyroidism on mRNA expression levels of SOCS/CIS and male predominant genes in adult rat liver. On PND80, the hepatic mRNA levels of SOCS2 (A), CIS (B), SOCS3 (C), PIAS3 (D), SOCS5 (E), CYP2C11 (F), CYP2C13 (G) and CYP2C7 (H) were measured by qPCR in rats exposed to neonatal hypothyroidism (CH), age-matched (INTACT) or weight-paired (WP) control groups. The mean mRNA expression level of each gene in the INTACT group is defined as 1, with all other expression values reported relative to this level. Bars represent mean ± SD from at least six individual animals. ***, $P<0.001$ for comparison with INTACT group. ++, $P<0.01$; +++, $P<0.001$ for comparison with WP group.
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group. In addition, the mRNA expression levels of enzymes responsible for the catabolism of cholesterol such as 7α-hydroxylase (CYP7A1) (Fig. 6A), which is the rate-limiting enzyme in hepatic bile acid synthesis, sterol 27 hydroxylase (CYP27A1) (Fig. 6B), and 12α-hydroxylase (CYP8B1) (Fig. 6C), which is an enzyme involved in cholic acid synthesis [57,58], were downregulated in the CH and in the WP groups in comparison with the INTACT rats. Farnesoid X receptor (FXR) (Fig. 6D), a receptor for bile acids [57], was also downregulated in the CH rats. In contrast, small heterodimer partner (SHP) (Fig. 6E), a gene induced by FXR, remained unchanged, which suggests that there is low activity of the bile acid-FXR signaling pathway. Collectively, these data indicate that a transient neonatal exposure to hypothyroidism causes changes in lipid metabolism in the liver and, particularly, in the cholesterol metabolism pathway that persist long after removal of the original insult, in animals that are euthyroid and exhibit similar growth rate as intact rats.

Neonatal hypothyroidism influences the hepatic response to hormone replacement in adulthood

Although CH rats were euthyroid on PND80 (i.e., the time when the comparative gene expression was performed), they showed changes in liver gene expression in comparison with age-matched INTACT rats (see above) that involved previously characterized T3 (e.g., ME) or GH (e.g.SOCS2, CIS, IGF-I, CYP2C11, INSIG-1, and IGFBP-3) regulated genes. Thus, we hypothesized that the different transcriptional profile in the CH group could be explained by altered tissue responsiveness to T3 and/or GH, two hormones that are drastically reduced by hypothyroidism [6,7,8]. To test this hypothesis, we developed a

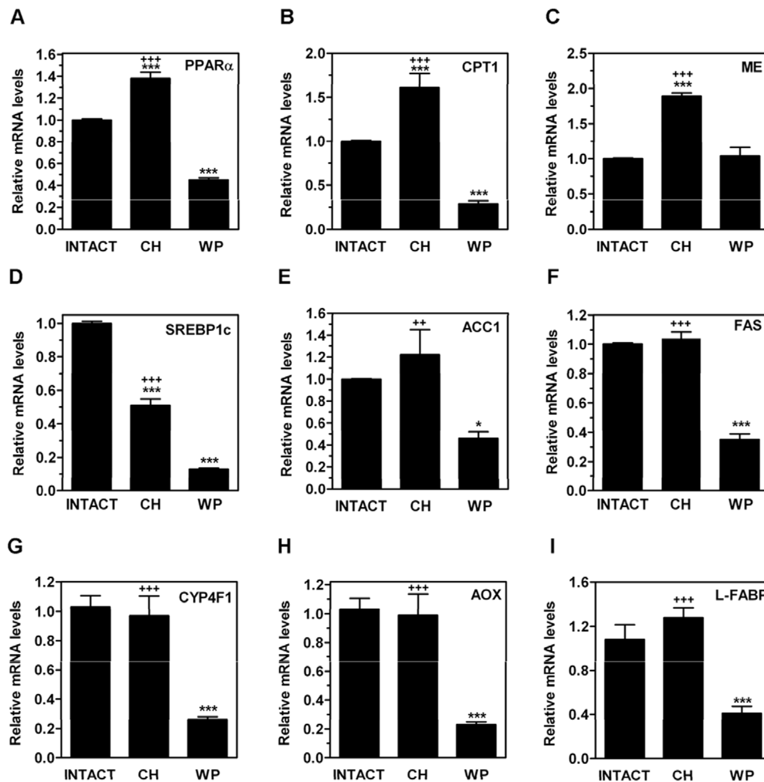

Figure 4. Effects of neonatal hypothyroidism on mRNA expression levels of genes related with lipid metabolism in adult rat liver. On PND80, the hepatic mRNA levels of PPARα (A), CPT1 (B), ME (C), SREBP1c (D), ACC1 (E), FAS (F), CYP4FI (G), AOX (H), and L-FABP (I) were measured by qPCR in CH, age-matched INTACT, or weight-paired (WP) control groups. The mean mRNA expression level of each gene in the INTACT group is defined as 1, with all other expression values reported relative to this level. Bars represent mean ± SD from at least five individual animals. *, $P<0.05$; ***, $P<0.001$ for comparison with INTACT group. ++, $P<0.01$; +++, $P<0.001$ for comparison with WP group.
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second burst of hypothyroidism (TX) in adulthood, which was followed by hormone replacement as described in "Materials and Methods". As expected, in vehicle or GH-treated TX rats, the serum levels of T3 were significantly reduced whereas the levels of T3-replaced animals did not differ from the INTACT control rats (data not shown). We showed a decrease in the total body weights of TX rats ($P<0.001$) and little subsequent weight gain in the TX/−CH (Fig. 7A) and TX/+CH rats (Fig. 7B). Independent of CH status, we showed the following: 1) the development of TX increased the circulating cholesterol levels and decreased the triglyceride serum levels (Table 1), which were mainly due to an increase of LDL and HDL cholesterol and a decrease of VLDL (data not shown), respectively, while the T3 hormone replacement restored the circulating cholesterol; 2) T3 and GH treatments increased the body weight gain in TX rats but it was unable to normalize it completely (Fig. 7A,B); and 3) T3 and GH treatments increased the hepatic level of IGF-I mRNA and, unlike T3, GH was capable of fully restoring the level to normal (Fig. 7C). However, the development of TX in the CH group (i.e., TX/+CH) resulted in a greater than 3-fold reduction of circulating FFAs, which was not observed in the TX/−CH group, and the T3 replacement restored it (Table 1). Next, we evaluated the effects of hormone replacement on the hepatic lipid content (Table 2). Independent of the CH status, T3 treatment restored hepatic cholesterol levels. However, in comparison with INTACT, GH increased the hepatic cholesteryl esters in the TH/−CH but not in the TH/+CH group. Additionally, to evaluate whether an altered response to the T3 treatment was associated with an altered transcriptional response, we measured the changes in gene expression of T3-regulated genes such as ME and FAS. The effect of GH was also measured. As expected, the development of TX in TX/−CH rats significantly reduced the mRNA levels of ME (Fig. 7D) and FAS (Fig. 7E). However, the development of TX in the TX/+CH group did not decrease ME (Fig. 7D) and, surprisingly, increased the mRNA levels of FAS up to 5-fold (Fig. 7E). Furthermore, T3 replacement in the TX/−CH rats increased the mRNA expression levels of ME and FAS by 12- and 3-fold, respectively. However, T3 replacement in the TX/+CH

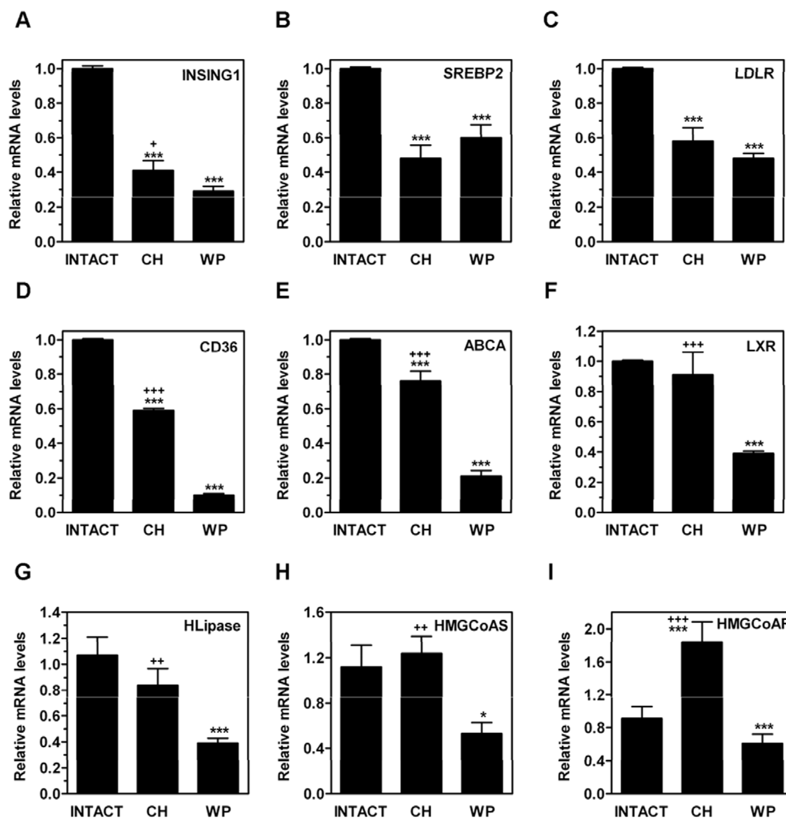

Figure 5. Effects of congenital hypothyroidism on mRNA expression levels of INSIG-1, SREBP2, LXR, and genes involved in lipid transport in adult rat liver. On PND80, the hepatic mRNA levels of INSIG1 (A), SREBP2 (B), LDLR (C), CD36 (D), ABCA (E), LXR (F), HLipase (G), HMGCoAS (H), and HMHCoAR (I) were measured by qPCR in CH, age-matched INTACT or weight-paired (WP) control groups. The mean mRNA expression level of each gene in the INTACT group is defined as 1, with all other expression values reported relative to this level. Bars represent mean ± SD from at least five individual animals. *, $P<0.05$; ***, $P<0.001$ for comparison with INTACT group. +, $P<0.05$; ++, $P<0.01$; +++, $P<0.001$ for comparison with WP group.
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rats increased the expression of ME up to 30-fold which suggested an altered tissue sensitivity to T3 replacement. Taken together, these findings suggest that the tissue responsiveness to TH was altered in rats previously exposed to CH. At this point, we can only speculate about the molecular mechanisms that could support our hypothesis. However, because altered hepatic levels of TR might support a different metabolic response to TH in the liver [12,14,59], we made an exploratory analysis of the expression of TRβ/TRα mRNA in the CH rats. The hepatic mRNA level of TRα (Fig. 7F) was significantly reduced in the CH group whereas the TRβ mRNA (Fig. 7G) remained unaltered. However, the mRNA expression levels of TRα and TRβ were downregulated in the WP group, which suggests that TRα, unlike TRβ was influenced by CH.

Discussion

The THs are essential for development, growth, and metabolism [1,2,3]. The present study shows that transient neonatal hypothyroidism in male rats gave rise to endocrine alterations that not only affected postnatal growth but also influenced hepatic physiology and responsiveness to THs replacement in adulthood.

Growth-inhibiting conditions exist during development in association with malnutrition, glucocorticoid excess, systemic diseases, GH-IGF-I deficiency, or hypothyroidism [44,60,61]. In this work, several biomarkers of neonatal hypothyroidism (i.e., decreased circulating THs and hepatic mRNA expression levels of ME and Spot14) and high expression of IGFBP-2 were associated with decreased circulating IGF-I and a delayed somatic growth rate on PND30. Furthermore, when the growth-inhibiting condition (i.e., MMI) was removed, somatic growth rate (weight

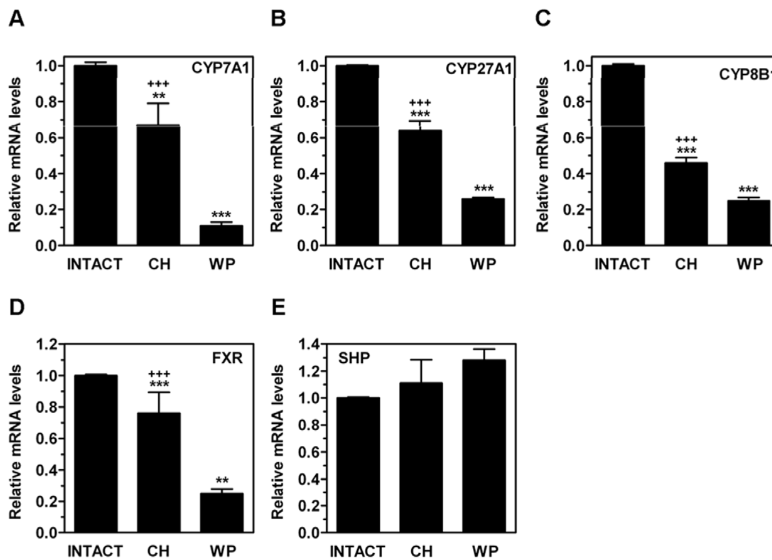

Figure 6. Effects of congenital hypothyroidism on mRNA expression levels of genes involved in bile acid synthesis in adult rat liver. On PND80, the hepatic mRNA levels of CYP7A1 (A), CYP27A1 (B), CYP8B1 (C), FXR (D), and SHP (E) were measured by qPCR in CH, age-matched INTACT or weight-paired (WP) control groups. The mean mRNA expression level of each gene in the INTACT group is defined as 1, with all other expression values reported relative to this level. Bars represent mean ± SD from at least five individual animals. **, $P<0.01$; ***, $P<0.001$ for comparison with INTACT group. +++, $P<0.001$ for comparison with WP group.
doi:10.1371/journal.pone.0037386.g006

and tail lenght gain) and food efficiency increased in CH rats, which is a phenomenon known as catch-up growth [44,60]. By PND80, however, this effect had subsided, and somatic growth rate (see body weight gain and tail length gain in Supplementary File S3) in CH animals was similar to age-matched INTACT controls. Alternatively, higher food efficiency remained in euthyroid CH group on PND80 along with significance differences in total body weight and size in comparison with INTACT group, which suggests, in agreement with previously reported data [62], a higher but less efficient rate of metabolism (i.e., a reduced ability to transform calories consumed into total body weight and size) in rats previously exposed to CH. The CH rats on PND80 also showed increased mRNA levels of several GH target genes (i.e., IGF-I, SOCS-2, CIS, CYP2C11, CYP2C13) suggesting that the increased hepatic GH activity observed in these animals was possibly associated with catch-up growth. In contrast, other well-known GH target genes in female rats such as CYP2C7 and CD36 were downregulated in CH group. This apparent paradox could be explained by sexually dimorphic pattern of gene expression in rat liver [63,64]. The downregulation of female-predominant genes (e.g., CYP2C7 and CD36) concomitant with the induction of male-predominant genes (e.g., CYP2C11 and CYP2C13) suggests that a male pattern of gene expression was enhanced in CH rat liver.

In the current study, we show that transient CH is associated with changes in SOCS-2 and CIS expression, which are key negative regulators of GH-dependent somatic growth *in vivo* [51,65]. GH resistance can be shown in rat models of sepsis and uremia and in small rats for gestational age (SGA) without catch-up growth. This was associated with an increased expression of SOCS-2 and CIS and impaired JAK/STAT signaling [66,67,68]. In our model, however, catch-up growth was associated with the overexpression of SOCS-2 and CIS in adult CH rats. Whether the overexpression of SOCS and CIS is associated with delayed growth development and catch-up growth in CH rats requires further research.

Growth-inhibiting conditions during fetal-neonatal period of life may influence lipid metabolism in adulthood [69,70,71,72]. Human and rats, who do show catch-up of somatic growth and increased feed efficiency after withdrawal of growth-inhibiting condition (e.g., SGA or caloric restriction), have higher risk of fat in the liver and increased adiposity in adulthood [69,70,71,72]. Now, we show that somatic growth inhibition by neonatal hypothyroidism influences hepatic lipid metabolism in adulthood. CH rats showed a concomitant upregulation of PPARα and CPT1, a gene related to fatty acid catabolism. Furthermore, adult CH rats showed a downregulation of CD36, which is involved in fatty acid uptake and a well-known PPARα target gene, along with the reduced transcription of genes involved in cholesterol uptake (LDLR), cellular sterol efflux (ABCA), triglyceride assembly (MTTP), bile acid synthesis (CYP8B1, CYP7A1 and CYP27A1), and lipogenesis (SREBP1c) [73]. These data indicate that CH significantly influenced lipid metabolism in adulthood and, most likely, contributed to the diminished hepatic levels of triglycerides, cholesteryl esters, and FFA. Conversely, because expression levels of several lipid genes in CH adult group were altered in similar direction to that detected in WP group, our data could be explained, in part, as a consequence of delayed growth [33,34].

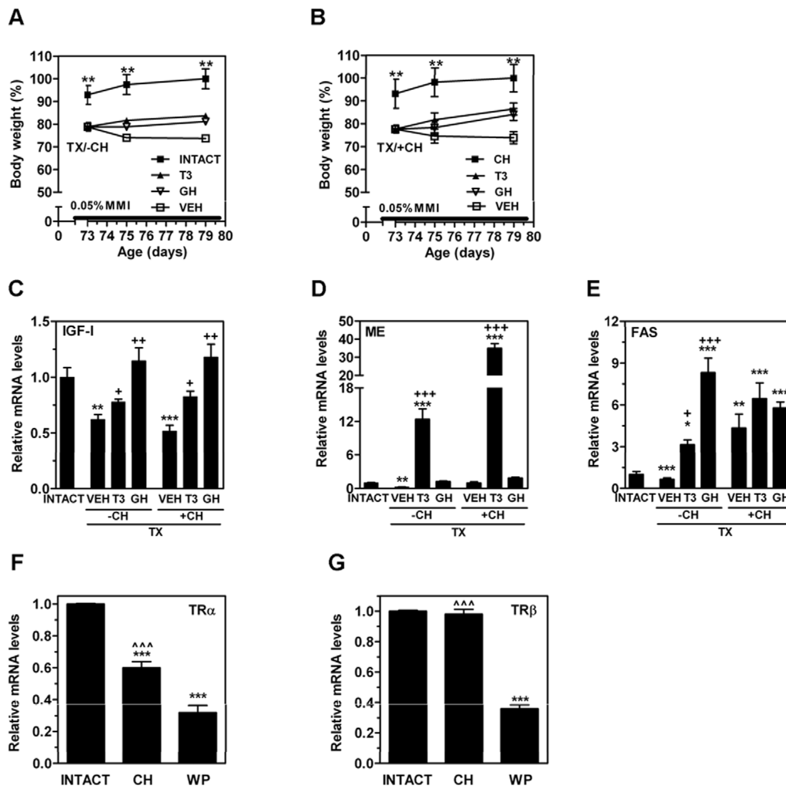

Figure 7. Effects of hormonal replacement on body weight and hepatic mRNA expression levels of IGF-I, ME, and FAS in adult hypothyroid rats without or with transient exposure to neonatal hypothyroidism. Four groups were studied: 1) age-matched rats (INTACT); 2) adult rats with neonatal hypothyroidism (CH); 3) hypothyroid adult rats without CH (TX/−CH); and 4) hypothyroid adult rats with CH (TX/+CH). During the last week of life, TX/−CH and TX/+CH groups were treated with either T3 or GH daily. Control animals were injected with saline (VEH). Body weight (A and B) as well as hepatic mRNA levels of IGF-I (C), ME (D) and FAS (E) were measured. The hepatic mRNA levels of TRα (F) and TRβ (G) were also measured by qPCR in CH, age-matched INTACT or weight-paired (WP) control groups. The mean mRNA expression level of each gene in the INTACT group is defined as 1, with all other expression values reported relative to this level. Results represent mean ± SD from at least six individual rats *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ for comparison with INTACT group (panel A) and for comparison with CH group (panel B); +, $P<0.05$; ++, $P<0.01$; +++, $P<0.001$ for comparison with vehicle-treated TX group.^^^, $P<0.001$ for comparison with WP group.
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Furthermore, a reduced content of hepatic lipids in CH group could be caused by prolonged catch-up growth which might cause an increased lipid catabolism in growing animals (i.e., CH group) in comparison with those that have completed their body growth (i.e., INTACT). To determine whether some of these changes caused by CH are life-long adaptations, similar analysis would need to be performed in older animals [33,34]. Furthermore, the reduced content of hepatic lipids in CH group could be caused by prolonged catch-up growth which might cause increased lipid catabolism in growing animals (i.e., CH group) in comparison with those that have completed their body growth (i.e., INTACT). However, several reports have suggested that catch-up growth is likely associated with increased level of hepatic lipids and adiposity [72], which would not be in agreement with this explanation. Additionally, despite all of the changes observed in liver, the levels of circulating lipids (triglycerides and cholesterol) and lipoproteins (data not shown) were similar to those in the INTACT aged-matched littermates, which suggest that CH rats were able to maintain lipid homeostasis and support the increased energy demands imposed by an accelerated growth rate. This is apparently achieved by redistributing lipids from the liver towards peripheral tissues rather than through active hepatic lipogenesis, which is an energy-consuming process that would compete with peripheral energy needs.

In this study, we show that transient neonatal hypothyroidism influences transcriptional program in adult liver. Despite being euthyroid, adult CH animal showed a modified transcriptional profile in liver in comparison with age-matched INTACT rats, which might be explained by altered tissue responsiveness to T3 and/or GH, two hormones that are drastically reduced by

Table 1. Serum lipids from PND80 male rats at baseline (INTACT), without (−CH) or with (+CH) transient neonatal exposure to MMI, during thyroid hormone deprivation (vehicle) and hormonal replacement.

	Cholesterol (mM)	Triglycerides (mM)	FFA (mM)
INTACT	1.38±0.13	1.59±0.65	0.69±0.17
TX/−CH			
Vehicle	3.01±0.13***	0.62±0.17**	0.58±0.17
T3	1.69±0.16+++	0.67±0.20**	0.75±0.30
GH	2.77±0.39***	0.44±0.13***	0.28±0.04***; ++
TX/+CH			
Vehicle	3.15±0.31***	0.72±0.05**	0.24±0.07***
T3	1.52±0.18+++	0.61±0.17**	0.59±0.13+++
GH	2.70±0.57***	0.66±0.20**	0.25±0.06***

INTACT and CH animals were exposed to MMI at PND60 as described in Materials and Methods. From day 73, animals were injected daily with vehicle, T3 or GH for 7 d. The animals were sacrificed on PND80 and serum lipids were measured. Results are expressed as mean ± SD (n = 6). Statistical comparison was performed for treated animals using INTACT animals or vehicle as controls.
****, *P*<0.01;**
*****, *P*<0.001 for comparison with INTACT rats;**
++, *P*<0.01;
+++, *P*<0.001 for comparison with vehicle.
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hypothyroidism [6,7,8]. However, independent of CH status, several of the responses to hypothyroidism and hormone replacement were similar. As expected, development of TX increased circulating cholesterol levels and decreased the serum triglyceride levels, while T3 hormone replacement restored circulating cholesterol level. Additionally, T3 and GH treatments increased the body weight gain and hepatic levels of IGF-I mRNA. At first glance, these results suggested that the CH rats, after suffering a biological insult (i.e., a second burst of hypothyroidism in adulthood), showed a biological response similar to age-matched INTACT rats. However, several of the responses to TX or hormone replacement suggested an altered lipid metabolism in the CH rats. First, a significant reduction of circulating FFAs by TX in TX/+CH group but not in TX/−CH group, an effect that was restored by T3 replacement. Second, GH-increased hepatic cholesterol esterification occurred in the TH/−CH animals but not in the TH/+CH animals. Third, GH treatment reduced serum VLDL fraction in the TX/−CH but not in the TX/+CH rats (data not shown). We did not observe major alterations in hepatic reactivity to GH in terms of lipid changes which suggests that the capacity of GH treatment to reduce serum triglyceride levels in TX/−CH rats, but not in TX/+CH rats, is most likely due to altered GH activity in extrahepatic tissues, such as fat and muscle [3]. Finally, hepatic concentrations of lipids in T3-treated TX/+CH rats did not differ significantly from TX/−CH group, which suggested that the homeostatic capacity of CH tissue in response to T3 was not dramatically affected. However, this is in contrast to the enhanced ME expression in the TX/+CH group in response to the T3 treatment. Increased ME mRNA expression did not seem to be a general response to T3 replacement because SREBP1c and 2 showed a less pronounced change (data not shown). ME is directly regulated by the binding of TR to a TRE in the promoter of the ME gene [74]. Our measurement of mRNA levels for TR receptors in liver showed unaltered expression of the major isoform TRβ and reduced levels of TRα, making it unlikely that changes in ME expression can be attributed to altered TR content. Noticeably, the ME regulatory region also contains binding sites for PBX1 and 2 [75], CEBPα [76], and an E-box [77] that can modulate the response to T3. At this point, we cannot exclude the possibility that CH effects on these transcription factors and other nuclear co-regulators influence ME expression but additional experiments are needed to test this hypothesis. Likewise, a clearer mechanistic explanation for the metabolic changes observed in the CH rats would require, among others measures, the analysis of fat and muscle metabolism.

Table 2. Hepatic lipids in liver from PND80 male rats at baseline (INTACT), without (−CH) or with (+CH) transient neonatal exposure to MMI, during thyroid hormone deprivation (vehicle) and hormonal replacement.

	Free cholesterol (mg/g of tissue)	Triglycerides (mg/g of tissue)	Cholesteryl esters (mg/g of tissue)	Fatty acids (mg/g of tissue)	Phospholipids (mg/g of tissue)
INTACT	2.4±0.2	3.9±0.6	0.30±0.02	0.18±0.03	57.8±4.1
TX/−CH					
Vehicle	1.9±0.1**	2.1±0.2***	0.37±0.04*	0.15±0.01*	51.6±3.4*
T3	2.3±0.3++	1.5±0.2***;+	0.33±0.05	0.14±0.01**	58.2±5.9
GH	2.1±0.2**	1.4±0.1***;++	0.54±0.15 ***;++	0.12±0.01***;+	50.7±4.9*
TX/+CH					
Vehicle	1.9±0.2**	2.1±0.3***	0.21±0.02**	0.13±0.01**	48.9±4.8*
T3	2.0±0.3*	1.7±0.4***	0.34±0.09++	0.13±0.01**	54.6±5.5
GH	1.7±0.2***	1.6±0.3***	0.31±0.07++	0.12±0.01**	43.8±2.3***;+

INTACT and CH animals were exposed to MMI at PND60 as described in Materials and Methods. From day 73, animals were injected daily with vehicle, T3 or GH for 7 d. The animals were sacrificed on PND80 and hepatic lipids were measured. Results are expressed as mean ± SD (n = 6). Statistical comparison was performed for treated animals using INTACT animals or vehicle as controls.
***, *P*<0.05;**
****, *P*<0.01;**
*****, *P*<0.001 for comparison with INTACT rats;**
+, *P*<0.05;
++, *P*<0.01 for comparison with vehicle.
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Androgens may influence the hepatic response to CH. It is well known that neonatal hypothyroidism results in increased circulating levels of testosterone in male rats, which is secondary to increased testis size [29]. We also observed two-fold higher serum testosterone levels in the CH rats compared to the age-matched adult INTACT rats (data not shown). Although the liver is not considered to be a primary target of testosterone action, androgens maintain specific male pattern of pituitary GH secretion and actions on liver [78], and it has been shown that specific deletion of androgen receptor (AR) in liver of male animals causes hepatic insulin resistance with decreased fatty acid β-oxidation and steatosis [79], which implicates the hepatic AR as a positive factor in maintaining physiological control of glucose and lipid homeostasis. Alternatively, in prostate, a well-known target tissue of androgen action, testosterone has lipogenic effects, such as inducing the expression of FAS [80]. Therefore, we cannot exclude the possibility that some of the transcriptional effects detected in the liver of the CH rats (e.g., increased CYP2C11, CYP2C13, and PPARα) were secondary to increased levels of circulating testosterone.

In summary, our findings support the hypothesis that TH deprivation during neonatal period of life causes long-lasting influence on the liver transcriptome and provokes an altered responsiveness to biological insult in adulthood. Several findings that cannot be explained by the lower body weight in CH rats, compared to WP, include genes regulated by GH (e.g., IGF-I, CIS, CYP2C11, and CYP2C13) and genes involved in hepatic lipid metabolism (e.g., PPARα, CPT1, ME, ACC1, FAS, CYP4F1, AOX, L-FABP, LXR, HMGCoA-S, and HMGCoA-R). Being clinically relevant, the changes observed in the transcriptional responses to T3 highlight the possibility that CH influences tissue reactivity to thyromimetic drugs in adulthood [81]. Interestingly, thyroid-disrupting compounds, which can cause neonatal hypothyroidism, include a wide range of chemicals from naturally occurring compounds, pharmaceuticals, and a number of xenobiotics [82]. The long-lasting influence of growth-inhibiting conditions on hepatic metabolism is intriguing and warrants further study to explore whether the alterations observed in this study cause metabolic disruptions or chronic diseases.

Supporting Information

File S1 Schematic diagram of rat model used to study the effects of congenital hypothyroidism on adult rat liver. Congenital-neonatal hypothyroid male rats (CH) were produced by 0.02%-MMI administration in the drinking water of pregnant rats (GD12) until weaning at PND30. For generation of adult hypothyroid rats (TX), 0.05% MMI was added to the drinking water for 3 weeks starting at PND58. Four groups were studied: 1) euthyroid age-matched rats (INTACT); 2) CH; 3) TX rats without CH (TX/−CH); and 4) TX rats with CH (TX/+CH). During the last week of life, TX/−CH and TX/+CH groups were treated with either T3 or GH daily for 7 days as described in Materials and Methods. Control animals were injected with saline. Each group included six individual animals.
(TIF)

File S2 Gene names and primer sequences (5′- 3′) used for real-time PCR.
(TIF)

File S3 Effects of neonatal hypothyroidism on body growth development. Body weight (A) and tail length (B) were measured at 7-d intervals. On PND30, the hepatic mRNA levels of IGFBP2 (C) were measured by qPCR in rats exposed to neonatal hypothyroidism (CH), age-matched (INTACT) or weight-paired (WP30) control groups. Body weight gain (D), tail growth gain (E) and food efficiency (F) were measured at 7-d intervals as described in Material and Methods. Results are expressed as mean ± SD from six individual animals in each group. **, $P<0.01$, ***, $P<0.001$ for comparison with INTACT group. +++, $P<0.001$ for comparison with WP group.
(TIF)

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Author Contributions

Conceived and designed the experiments: LF-P AF-M CB GN EH. Performed the experiments: RS-F MM-G CB NK LF-P PP LH-H. Analyzed the data: LF-P AF-M PP GN CB. Wrote the paper: LF-P AF-M CB.

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Liver X receptor agonist downregulates growth hormone signaling in the liver

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Abstract

Liver X receptor (LXR) agonists have been shown to influence the development of hyperlipidemia and atherosclerosis in mouse models. It has also been demonstrated that some LXR agonists can cause hepatic steatosis in experimental animals. Growth hormone (GH) is known to regulate hepatic metabolism and the absence of hepatic GH receptors (GHR) leads to hepatic steatosis. In this study, we analyzed whether the actions of LXR agonists could involve interference with GH signaling. We showed that LXR agonists impair GH signaling in hepatocytes. LXR agonist treatment attenuated GH induction of suppressor of cytokine signaling 2 (*SOCS2*), *SOCS3*, and *CIS* mRNA levels in BRL-4 cells. Likewise, the activity of a luciferase reporter vector driven by the GH response element (GHRE) of the *SOCS2* gene was inhibited by simultaneous treatment with an LXR agonist. The inhibitory effect of LXR agonists on GH signals can be mimicked by overexpression of the LXR regulated factors, sterol regulatory element binding protein 1 (SREBP1) and SREBP2, in hepatic cells. In both cases total and phosphorylated signal transducers and activators of transcription 5b (STAT5b) protein levels were significantly reduced. DNA binding assays demonstrated that SREBP1 binds to an E-box within a previously defined GHRE in the *SOCS2* gene promoter, but does not compete with STAT5b binding to a nearby site in the same promoter construct. Taken together, our findings indicate that the inhibitory effects of LXR agonists on GH signaling are mediated by SREBP1, through the downregulation of STAT5b gene transcription and stimulation of STAT5b protein degradation. The findings provide a new insight into the understanding of the molecular actions of LXR agonists, which may be of relevance to their pharmacological actions.

Keywords: 9-cis retinoic acid; growth hormone; lipid; LXR; RXR; SOCS2; T0901317.

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Introduction

The liver X receptor-α (LXRα) and LXRβ (also known as NR1H3 and NR1H2, respectively), belong to the nuclear receptor superfamily of ligand activated transcription factors [1]. LXRs heterodimerize with the retinoic acid receptor (RXR), to regulate transcription of target genes, upon activation by oxidized derivatives of cholesterol, also known as oxysterols [1]. In the liver, LXRα activation induces a complex transcriptional network involved in the control of intracellular non-esterified cholesterol levels. Experimental studies with synthetic ligands, T0901317 and GW3965, indicate that the pharmacological activation of LXR protects against atherosclerosis, reduces plasma cholesterol levels and improves glucose tolerance in models of type 2 diabetes [2]. Unfortunately, these positive effects are accompanied by hypertriglyceridemia and hepatic steatosis, which presents risks for the development of cardiovascular disease [3]. This raises concerns regarding the application of LXR agonists in the management of common metabolic disorders linked to obesity and type 2 diabetes. A better understanding of the molecular basis for the pleiotropic effects of LXR in the liver, may lead to an improved safety profile for future LXR modulators.

Growth hormone (GH) actions are triggered by its binding to the GH receptor (GHR) and the activation of its associated kinase, JAK2. This, in turn, results in the tyrosine phosphorylation of the transcription factor signal transducers and activators of transcription 5b (STAT5b), which translocates to the nucleus to modulate expression of GH target genes, such as insulin growth factor-1 (*IGF-1*) and the suppressor of cytokine signaling 2 (*SOCS2*) [4]. The main metabolic actions of GH are anabolic, promoting protein synthesis in the muscle through the catabolism of fatty acids as the energy source. Because of GH effects on lipid mobilization from the liver, GH deficient patients and mice where GH signaling is specifically inactivated in the liver [5], are characterized by hepatic steatosis [6]. It is interesting to note that treatment of animals with the LXR agonist T0901317 also causes severe hepatic steatosis [3] and LXRα/β

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knockout mice ($LXR^{-/-}$) are resistant to high fat diet induced hepatic steatosis [7, 8]. Interestingly, in this model, the levels of hepatic type 2 deiodinase mRNA, which is under GH control, were elevated [7]. LXR and GHR activations in the liver also lead to antagonizing effects on glucose metabolism. LXR agonists show an antidiabetic effect by inhibition of gluconeogenesis and reduction of the expression of phosphoenolpyruvate carboxykinase (*PEPCK*) and glucose-6-phosphatase [2], while GHR activation induces hepatic gluconeogenesis and increases the mRNA levels of gluconeogenic enzymes [9].

The key regulatory role of GH on liver lipid metabolism and insulin sensitivity opens the question of whether some of the physiological actions that are attributed to the LXR ligands could be explained by interference with GH signaling. We performed this study to provide new insights into the relationship between LXR and GHR signaling in hepatocytes.

Materials and methods

Materials

Recombinant rat GH was from the National Hormone and Pituitary Program, National Institutes of Health (NIH, Bethesda, MD, USA) and recombinant human GH was from Novo Nordisk A/S (Gentofte, Denmark). Oligonucleotides were obtained from ThermoElectron (Bremen, Germany). T0901317 and GW3965 (LXR agonists), 9-cis retinoic acid (RXR agonist) and cycloheximide (CHX) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Plasmid cloning and site-directed mutagenesis

The luciferase reporter vector containing the GHRE (696bp) in the first intron of *SOCS2* human gene, named P5, has been previously described [10]. The fatty acid synthase (*FAS*) promoter constructs (pFAS) were a generous gift of Dr. T. Osborne [11] and the promoter construct of ATP-binding cassette (pABCA1) was a gift from Dr. Castrillo A [12]. Myc-SREBP1a and myc-SREBP2 (sterol regulatory element binding protein) expressing vectors were a generous gift from Dr. J. Ericsson [13].

Cell culture and transfection assays

Buffalo rat liver (BRL) cells stably transfected with the rat GHR, designated BRL-4 cells, were cultured as described [10]. Primary human hepatocytes were isolated from discarded organ-donor tissue. Isolated hepatocytes were obtained from the liver cell laboratory, KI-CLINTEC, Huddinge University Hospital. Ethics approval to use hepatocytes was given by the local Ethical Committee, Stockholm (DNr: 2010/678-31/3). Cells were maintained in Williams E medium as described previously [14]. DNA plasmid transfections were carried as recommended by the manufacturer's instructions on 70%–80% confluent cells using lipofectamine with Plus reagent (Invitrogen, Carlsbad, CA, USA). Cells were treated with 1 μM LXR agonists, 10 μM RXR agonist or 0.1% DMSO as vehicle control and with GH 50 nM.

Reporter gene assay

pSOCS2-TK-LUC (1 ug) was co-transfected with 10 or 50 ng of the nuclear form of SREBP1a, SREBP2 or pCDNA3 (Invitrogen), and 50 ng β-galactosidase reporter plasmid. Medium was replaced with serum-free DMEM 16–18 h posttransfection, and cells were treated with rGH and harvested 36 h after transfection in reporter lysis buffer for luciferase assays (Promega, Madison, WI, USA). Transfection experiments were performed at least three times.

Analysis of gene transcription

Total RNA was isolated from treated cells using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). cDNA synthesis and quantitative real-time PCR were performed as described previously [10]. Expression levels of genes were normalized to those of ribosomal RNA S18 or cyclophilin genes.

Cell lysis and immunoblotting

Cells were treated as described in the figure legends. Protein extraction and SDS-PAGE were performed as described previously [10]. Antibodies against STAT5, pSTAT5 (Tyr694) and SOCS2 were purchased from Cell Signaling Technology (Frankfurt A. M., Germany) and, SREBP1, SREBP2, β-actin, myc and GHR antibodies were purchased from Santa Cruz Biotechnology (Beverly, MA, USA).

CHX chase experiments

BRL-4 cells were transfected with myc-SREBP1a, myc-SREBP2 or control plasmid. Cells were split into different plates for treatment with 100 μg/mL CHX (Sigma-Aldrich) 24 h post-transfection and then lysed at different time points for protein extraction.

ABCD assay (avidin, biotin, complex, DNA)

In this assay, protein-DNA complexes were immobilized by biotinylated oligonucleotides to a streptavidin matrix. BRL-4 cells were treated with LXR/RXR agonists for 48 h and then stimulated with GH for 10 min, before lysis in 50 mM HEPES pH 7.5, 150 mM KCl, 1 mM EGTA, 1% NP-40, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM Na_3VO_4, 1X cocktail inhibitor, 50 mM NaF and 1 mM PMSF. BRL4 cell extracts (250 μg) were incubated with 2 μg biotinylated oligonucleotide and 5 μg salmon sperm DNA (Stratagene, La Jolla, CA, USA). Mixtures were incubated overnight at 4°C under constant rotation. Then 40 μL equilibrated streptavidin agarose beads (Amersham, Arlington Heights, IL, USA) were added to the mix and further incubated for 2 h at 4°C on a rotator. After centrifugation, beads were washed repeatedly with lysis buffer containing 50 mM KCl, then boiled in sample buffer and separated by SDS-PAGE. SREBP1 and STAT5b bindings were detected by Western blot. We also repeated the ABCD assay using extracts from separately treated cells after 48 h treatment with LXR/RXR agonist or after 10 min treatment with GH. Equal amounts of total protein from each cell lysate were used for the assay. As control oligonucleotides for SREBP1 and STAT5b binding, we used sterol response element (SRE) element in the acetyl-CoA carboxylase (*ACC1*) genes and GHRE, containing two STAT5b sites, in *SPI-2.1* gene. 3′ Biotinylated oligonucleotides were ordered from ThermoElectron (Bremen, Germany) with the following forward oligonucleotides sequences: WT: 5′-cccgcg-gtcacgtgaggccgattcctggaaagttcctggaaagcc g-3′, mut-Ebox: 5′-cccgcggt**gt**cgtgaggccgatt-cctggaaagttcctggaaagcc g-3′, mut-STAT5b: 5′-cccgcggtcacgtgaggccga**aa**cctggaaag**aa**cc tg-gaaagcc g-3′, mut-Ebox/STAT5b: 5′-ccc cggt**gt**cgtgaggccga**aa**cctg-gaaag**aa**cctggaaa gccg-3′, SRE: 5′-tcgcatcacaccaccgcg g-3′, GHRE: 5′-tcgacgcttctactaatcccatgttctgag aaatcatccag-3′. The forward and complementary oligonucleotides were heated for 10 min at 95°C then cooled down to anneal at room temperature.

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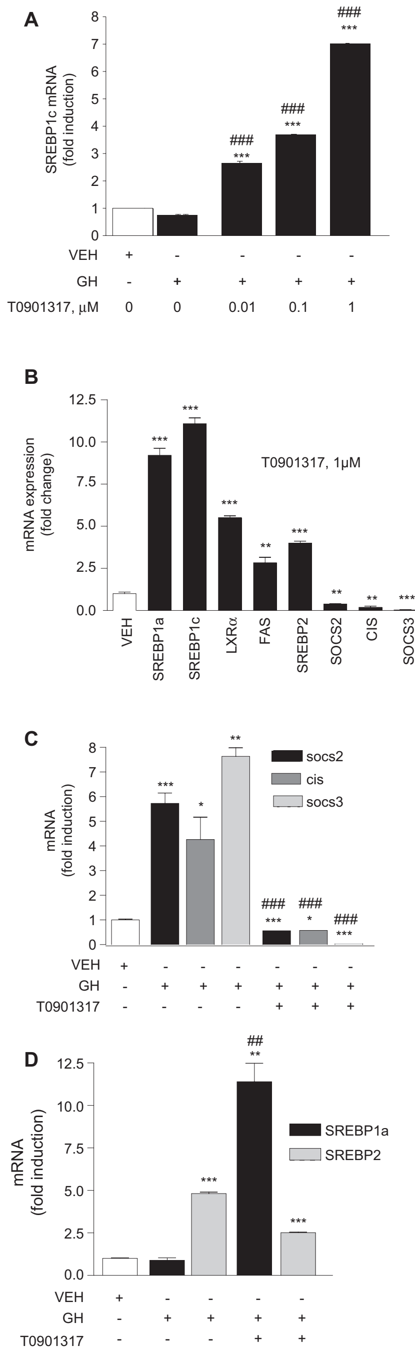

Figure 1 Effects of T0901317 on GH-dependent expression of SOCS2 mRNA in BRL-4 cells. (A) BRL-4 cells were treated for 4 h with different doses of LXR agonist (T0901317) or vehicle (VEH) then followed with 2 h rGH stimulation before quantification of gene expression, normalized to cyclophiline gene expression. (B) BRL-4 cells were treated for 40 h with 1 μM LXR agonist (T0901317) followed by quantification of gene expression. (C)+(D) BRL-4 cells were treated similarly as (B) then followed with rGH (50 nM) stimulation for 2 h before quantification of gene expression. VEH=0.1% DMSO, Me_2SO. Values are expressed as means±SEM.
*Indicates significant differences compared to GH-unstimulated cells, # indicates significant differences to cells treated with GH alone. **$p<0.01$, ***$p<0.001$.

Statistical analysis

Statistically significant differences were assessed with the two-tailed Student's t-test. All statistical analysis was performed using SPSS for Windows (version 10.0, Chicago, IL, USA). Statistical significance was reported for $p<0.05$.

Results

GH induced gene expression is inhibited by LXR agonist

We used BRL cells stably transfected with the rGHR (BRL-4) [15] to analyze GH signals. These cells express endogenous JAK2 and STAT5b and respond to GH with the transcriptional induction of STAT5b-regulated genes, such as *CIS* and *SOCS2*, although they express little *IGF-1* [15]. We first performed a dose-response experiment in BRL-4 cells to assess their response to T0901317, by measuring changes in *SREBP1c* expression (Figure 1A). Maximal activity was observed at 1 μM, thus this was chosen as the working concentration for the following experiments. In order to understand the interaction between LXR and GH signaling, we studied the effect of the LXR synthetic ligand T0901317 on the mRNA levels of selected genes. As shown in Figure 1B, 40 h treatment of BRL-4 cells with the LXR agonist resulted in significantly increased mRNA levels of *LXRα*, *SREBP1a* and *SREBP2*, as well as its downstream target fatty acid synthase (*FAS*) gene. In addition, we observed a marked downregulation of GH-regulated genes, *SOCS2*, *SOCS3* and *CIS*. We further studied the LXR agonist effect after 2 h stimulation in the presence or absence of GH. As expected, GH has a significant stimulatory activity on the expression of *SOCS2*, *SOCS3* and cytokine-induced SH2 protein (*CIS*) (Figure 1C). It also increased *SREBP2* expression but failed to stimulate the expression of *SREBP1a* (Figure 1D). In a combined treatment with the LXR agonist, significant inhibition of GH induced gene expression was detected (Figure 1C).

LXRs form heterodimeric complexes with RXRs [1]. Hence, we examined the effect of short-term treatment (4 h) with the RXR ligand, 9-cis retinoic acid, alone or in combination with the LXR agonist, in the regulation of GH induction of SOCS2 gene expression. Treatment of BRL-4 cells with GH resulted in a 14-fold increase in the levels of *SOCS2* mRNA while 4 h pretreatment with the RXR and

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LXR agonist alone or in combination resulted in a moderate induction of *SOCS2* mRNA levels (Figure 2A). Individual pretreatment with each of the agonists did not have an effect on GH-stimulated *SOCS2* expression. In contrast, the combined pretreatment resulted in a significant inhibition of the GH effect parallel to maximal induction of *SREBP1c* (Figure 2B). Together, these data demonstrate that GH-dependent gene expression is negatively regulated by LXR activation in hepatic cells.

LXR agonist inhibits STAT5b activation by GH

We have previously identified two evolutionary conserved *cis* GAS-like elements (TTCNNNAGG) in the *SOCS2* gene that bind to STAT5b and mediate GH transcriptional activity [10]. In order to better understand the effects of the LXR ligand T0901317 on GH signaling, we analyzed the activity of a luciferase reporter gene driven by the GHRE identified in the *SOCS2* promoter [10]. As controls, we used promoters of LXR responsive genes: *ABCA1* transporter (pABCA1) and *FAS* (pFAS). The latter contains an SREBP1 binding site that indirectly mediates LXR actions. As shown in Figure 2C, GH stimulates the luciferase activity of pSOCS2 while 6 and 24 h treatment with the LXR agonist resulted in inhibition of this effect, while stimulating its targets pABCA1 and pFAS. This finding suggests that the mechanisms of LXR mediated inhibition of GH signaling involve the regulation of STAT5b activity.

Having demonstrated the antagonistic effect of the LXR ligand T0901317 on STAT5b mediated transcription, we next analyzed its effects on GHR signaling. Firstly, we analyzed GH activation of STAT5b in BRL-4 cells treated with the LXR and RXR agonists, alone or in combination. As observed in Figure 3A, GH treatment increased STAT5b phosphorylation, but had minor effects on SREBP1 protein precursor levels. On the other hand, treatment with either the LXR or RXR agonist did not alter basal STAT5b phosphorylation levels, but led to an increased level of SREBP1, an effect that was further enhanced by the combined treatment. In this setup, pretreatment with either the RXR or LXR agonist, or the combination of both, significantly decreased the levels of STAT5b in the presence of GH, while the amount of active (phosphorylated) STAT5b was only significantly affected by the combined treatment. In order to explore the wider relevance of these findings, we analyzed the effects of LXR and RXR agonists in human primary hepatocytes, which express high GH receptor levels endogenously. As shown in Figure 3B, treatment with LXR and RXR agonists enhanced the expression of premature and mature forms of SREBP1 protein. After 30 min of GH treatment, we observed reduced levels of total and phosphorylated STAT5b as well as GHR in cells treated with the LXR and RXR agonists, as compared with cells treated with vehicle control. To confirm our findings, we analyzed the effects of another LXR-specific, but weaker, agonist GW3965 [16]. As can be seen in Figure 3C, GW3965 treatment of BRL-4 cells increased SREBP1 expression and decreased STAT5b protein levels, similar to the effects observed for T0901317.

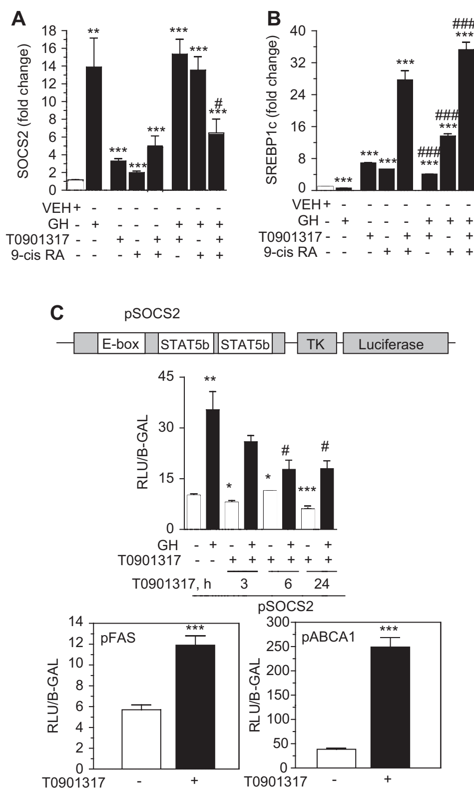

Figure 2 LXR and RXR agonist represses GH-dependent transcriptional activation of SOCS-2. (A, B) Expression of SOCS2 and SREBP1c was measured after 4 h treatment with LXR (T0901317), RXR (9-cis RA) agonists or a combination of both. The relative expression levels were scaled to the vehicle-matched control group. (C) Luciferase activity of SOCS2 promoter (pSOCS2) from BRL-4 cells after treatment with LXR agonist (T0901317) for 3, 6, and 24 h with or without 12 h stimulation with rGH. Transfection of plasmids pFAS (marker of SREBP1 activity) and pABCA1 (marker of SREBP2 activity) were used as positive controls for LXR agonist treatment. Data shown are means±SD.
*Indicates significant differences compared to GH-unstimulated cells, # indicates significant differences to cells treated with GH alone.
*$p<0.05$, **$p<0.01$, ***$p<0.001$.

SREBP-1 downregulates SOCS2 promoter activity

The effects of the LXR ligand T0901317 on GH induction of *SOCS2* expression, coincide with the elevated expression of SREBP1a, suggesting the involvement of this transcription factor in the negative regulation of *SOCS2* gene transcription at the promoter level. Therefore, we tested the influence of SREBP1a overexpression on the activity of the *SOCS2* promoter. The luciferase-driven *SOCS2* promoter was transiently co-transfected in BRL-4 cells, with different concentrations

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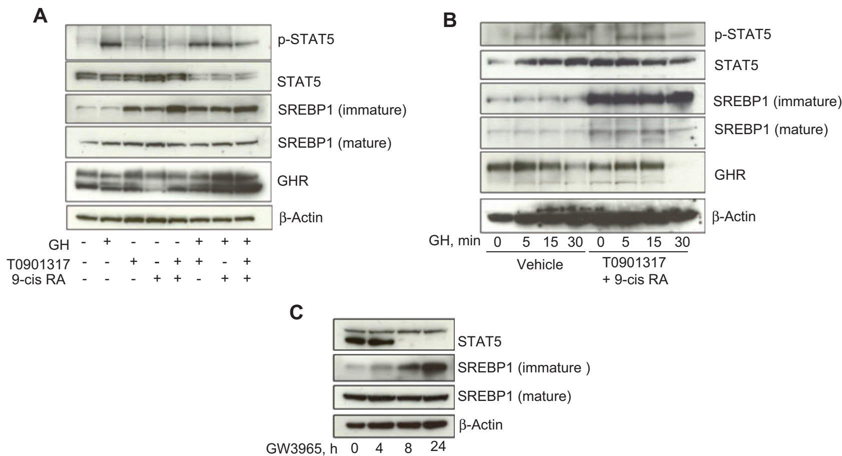

Figure 3 LXR activation downregulates STAT5b in primary hepatocytes and in cultured liver cells. (A) Western blot of protein extracts from BRL-4 cells treated for 8 h with 1 μM LXR agonist (T0901317) and 10 μM RXR, with or without 4 h stimulation with rGH. (B) Primary human hepatocytes were treated for 16 h with LXR (T0901317) and RXR (9-cis RA) agonists followed by GH stimulation as indicated. (C) BRL-4 cells were treated with 1 μM LXR agonist, GW3965, at different time points.

of a plasmid encoding the active nuclear form of SREBP1a. As a control experiment, transfection with SREBP1a plasmid induced the promoter activity of *FAS* gene (pFAS) (Figure 4A). We observed a significant downregulation of *SOCS2* promoter activity after co-transfection with 10 and 50 ng of SREBP-1a (90% and 92% downregulation, respectively), (Figure 4B). These data mimic the findings observed in Figure 1A, where LXR agonist treatment of BRL-4 cells resulted in reduced *SOCS2* expression, coinciding with enhanced expression of *SREBP1a*.

A highly conserved putative SREBP1 binding site, E-box, CANNTG, exists 13 bp upstream of the STAT5b binding site in the GHRE found in the *SOCS2* gene promoter [10], Figure 4C. Thus, the inhibitory effect of SREBP1 on GH-induced *SOCS2* expression, could potentially be explained by a sterical hindrance of STAT5b binding to the promoter. To analyze this possibility, we performed protein-DNA binding assays using biotinylated DNA fragments of the *SOCS2* promoter carrying the E-box and the STAT5b binding sites (Wild type, WT), or the mutant version of E-box and STAT5b. We used two oligonucleotide fragments as positive controls for SREBP1 and STAT5b DNA binding activity (Figures 4C , D). The first consists of a DNA fragment containing an SREBP binding site (SRE) found in the *ACC1* gene promoter [17]. The second control consists of a DNA fragment containing two STAT5b binding sites (GHRE) found in the *SPI-2.1* rat gene promoter [18]. Treatment with LXR and RXR agonists increased the SREBP1 protein levels in the total cell lysates and accordingly enhanced the binding to the WT and STAT5b mutated fragments, but not to the E-box mutated construct. This finding demonstrates that SREBP1 can bind to the *SOCS2* promoter. On the other hand, STAT5b binding to the WT and E-box mutated DNA fragment was enhanced by GH stimulation. However, the binding was attenuated when cells were co-treated with LXR and RXR agonists, coinciding with the reduction in the total STAT5b levels detected in the total cell lysates. In an additional analysis, we treated BRL-4 cells with the LXR/RXR agonist and GH separately, to avoid the LXR mediated downregulation of STAT5b protein levels. Lysates from independently treated cells were mixed in a 1:1 ratio and incubated with DNA fragments as shown in Figure 4C. The mixed lysates of cells independently treated with GH or T0901317 exhibited similar STAT5b protein levels, Figure 4D. The binding of STAT5b to the WT construct was enhanced in the samples stimulated with GH and the binding was not affected by co-incubation with LXR/RXR agonist treated lysates, which exhibited high levels of SRBEP1 binding activity. This finding does not support the model where *SOCS2* downregulation by LXR agonists is due to a competition between STAT5 and SREBP1 for their respective binding to the *SOCS2* promoter. It adds support to the hypothesis that inhibition of *SOCS2* promoter activity upon LXR activation is due to downregulation of STAT5b protein levels.

SREBP1 downregulates STAT5b protein levels

To further elucidate the possible mechanisms whereby an LXR agonist mediated reduction of STAT5b, we analyzed STAT5b protein levels in BRL-4 cells overexpressing SREBP1a or SREBP2. In Figure 5A, we showed that these cells exhibit a significant reduction in both total and active phosphorylated STAT5 protein levels as compared to controls. To investigate if the reduction of STAT5b levels by LXR results from post-translational instability, we treated BRL-4 cells with CHX and followed the degradation of STAT5b. As shown in Figure 5B, a faster decrease of STAT5b proteins levels after CHX

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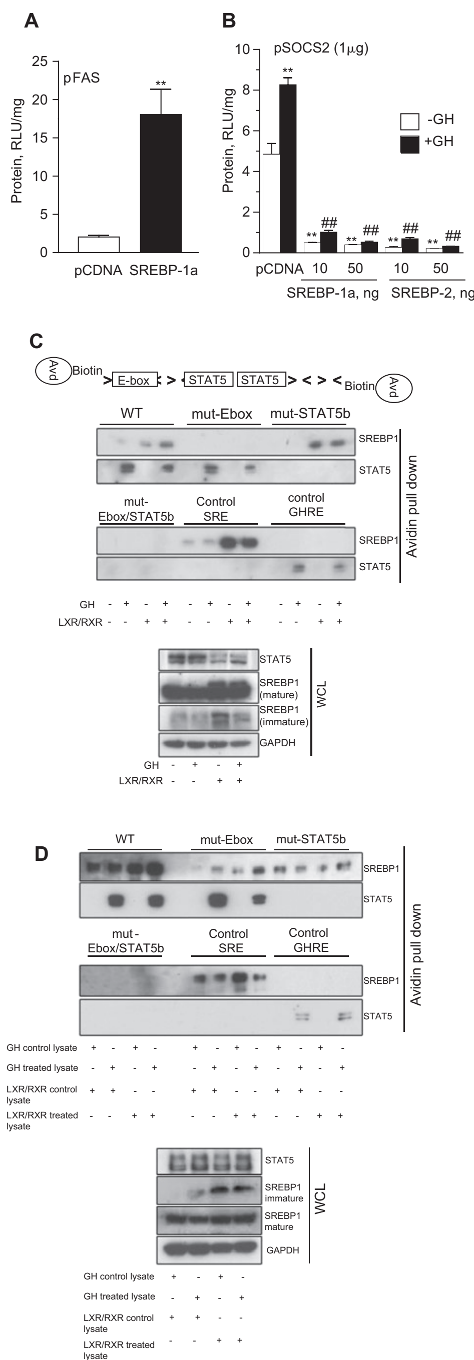

Figure 4 SREBP1 downregulates GH-induced SOCS2 promoter activity and does not compete with STAT5b binding to the promoter. (A, B) BRL-4 cells were co-transfected with the pFAS luciferase reporter plasmid as a control of SREBP1a effect, and with SOCS2 promoter-driven luciferase reporter plasmid (pSOCS2) and SREBP-1a, SREBP-2, or the control vector pCDNA3 at the indicated concentrations. Luciferase activity was measured accordingly. Values are expressed as means±SD.
*Indicates significant differences to non-stimulated control transfected cells, # indicates significant differences to control transfected cells treated with GH.
**p<0.01. (C, D) BRL-4 cells were treated for 16 h with LXR and RXR agonists followed by 10 min rGH stimulation before lysis. Lysates were used for avidin-Biotin DNA complex assay using DNA fragments of wild type or mutation of response elements for SREBP1 (E-box) or STAT5b as described in the method section. SRE is a control DNA fragment for SREBP1 binding and GHRE is a control for STAT5b binding. A blot for whole cell lysate (WCL) is shown in the bottom panel. (C) GH stimulation was carried out on cells treated with LXR-RXR. (D) GH and LXR-RXR treatments were carried on cells separately and lysates were mixed together accordingly.

treatment was observed in cells overexpressing SREBP1a and SREBP2. We then measured STAT5b mRNA levels after 40 h of treatment with LXR/RXR combination or after SREBP1a and SREBP2 overexpression. We found a modest decrease in the STAT5b expression in cells treated with LXR/RXR, or cells overexpressing SREBP1a and SREBP2 (Figure 5C). These findings indicate that elevation of SREBP1 and SREBP2 levels may mediate the inhibitory effects of the LXR ligand T0901317 on GH stimulated transcription through inhibition of *STAT5b* gene expression and by increasing the degradation of STAT5b protein.

Discussion

In the present study, we demonstrated that LXR activation with the synthetic ligand T0901317 attenuates hepatic GH signaling by reducing STAT5b activity and thereby inhibiting GH induced gene transcription. We also showed that the effects of LXR agonists on GH signaling are mimicked by overexpression of two LXR downstream target genes, *SREBP1* and *SREBP2*. The inhibitory effects of these lipogenic factors on STAT5b protein levels can partly be explained by their inhibitory effects on *STAT5b* gene transcription and the enhancement of STAT5b protein degradation.

In this study, we provide several lines of evidence to support the inhibitory activity of the LXR agonist T0901317 on STAT5b activity in hepatocytes. T0901317 treatment significantly reduced GH induction of mRNA levels of STAT5b regulated gene, *SOCS2*. Likewise, the reporter luciferase activity, driven by the STAT5b response element in the *SOCS2* gene promoter, was inhibited by the LXR agonist. Direct measurements of T0901317 effects on STAT5b protein levels, and its DNA binding activity, also show the inhibitory effects. Finally, overexpression of a well-known LXR target gene, SREBP1a, can mimic the effects of LXR ligands in reducing GH activated transcription and STAT5b levels. The

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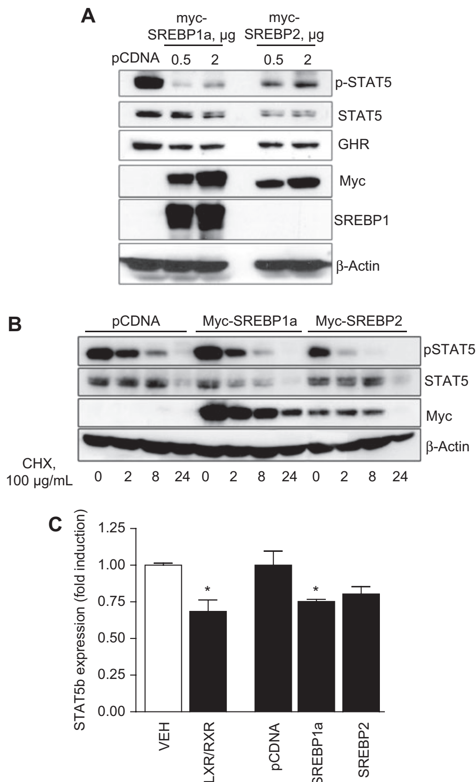

Figure 5 SREBPs downregulate STAT5b activity in liver cells. Myc tagged SREBP1a and SREBP2 proteins were overexpressed in BRL-4 cells followed by measurement of STAT5b protein and mRNA levels. (A) Western blot for phosphorylated and total STAT5b in cells transfected with myc-SRBEP1a, myc-SREBP2 or pCDNA3 control vector. (B) Cycloheximide (CHX) chase experiments were performed after overexpression of SREBP1a and SREBP2 in BRL-4cells. Then stability of STAT5b was measured by following the protein levels by Western blot. (C) Fold change in STAT5b mRNA expression after 48 h treatment of LXR and LXR agonist and in cells overexpressing SREBP1a and SREBP2. The ribosomal S18 mRNA levels were used as controls and used to normalize the data. Data shown are means±SD.
*Indicates a significant difference to control cells. *$p<0.05$.

CHX chase experiments demonstrated that SREBP1a promotes the degradation of STAT5b. Little is known about the mechanisms that regulate STAT5b turnover. Previous work has implicated proteasomal degradation in the downregulation of activated STAT5a and demonstrated its ubiquitination in the nucleus [19]. Furthermore, a recent in depth proteome screening of ubiquitylation sites, has identified the ubiquitin modification of lysine 567 of the human STAT5b [20]. Proteasomal degradation of STAT5a is modulated by a short motif comprising amino acids 751–762 of the C-terminal domain [19]. This region is highly conserved in STAT5b, corresponding to amino acids 757–768 [21]. The E3 ubiquitin ligase complex responsible for the ubiquitination of STAT5 in the nucleus remains to be identified. Our data would suggest that the activity of this E3 ligase could be modulated by SREBP1a and SREBP2 activation.

Most of the mechanistic studies presented in this work were performed in rat BRL cells stably transfected with the rat GHR, with the key finding also confirmed in primary human hepatocytes. In primary hepatocytes, we observed that LXR stimulation results in severe downregulation of GH receptor levels parallel to downregulation of STAT5b, suggesting that additional mechanisms exist whereby LXR agonists inhibit GH actions. Liver microarray analysis of human, mice, and rat livers have demonstrated that LXR activation leads to decreased GH receptor mRNA levels, providing the most likely explanation for these effects [2, 22, 23]. We have also made an interesting observation that the GHR protein levels in rat BRL-4 cells, in which its expression is driven by the CMV promoter, were not changed by the LXR agonist treatment, but yet we observed a significant reduction of STAT5b levels. Therefore, the LXR driven mechanisms that target GH signals seem to operate on both the GHR as well as STAT5b. This level of redundancy suggests that GH antagonism may be an important aspect of LXR physiological actions. All data presented in this study concern experiments performed in cultured cells. Therefore, further studies are warranted to evaluate the contribution of GH on the pharmacological actions of LXR agonist in vivo. Additional studies are also needed to verify whether additional tissue specific actions of LXR involve STAT5b downregulation. Tissue variations in the expression of different LXR isoforms or SREBPs content, as well as differences in the treatment modalities, may influence LXR ligand actions on STAT5b levels. For example, it has been reported that T0901317 treatment in lymphocytes did not affect the induction of STAT5b serine and tyrosine phosphorylation by IL-2 [24].

The demonstration that SREBP1 activation leads to STAT5b downregulation in hepatic cells may have wider implications, as several physiological situations are associated with increased hepatic expression of this factor. Notably, SREBP1 is stimulated by insulin after feeding, which may serve as a mechanism to control GH sensitivity postprandially [25]. Our findings may also have important consequences towards the goal of designing selective LXR agonists with better activity profiles. If indeed, GH antagonism is a prominent feature of LXR activation, it may be very difficult to separate LXR driven hepatic steatosis from its beneficial actions on insulin sensitivity, as they may be commonly regulated through the inhibition of STAT5b activity. In contrast, LXR agonists with reduced activity towards SREBPs, may still be of use in the treatment of hypercholesterolemia, which may rely on direct regulation of transporters, such as *ABCDA1* and *ABCDG1*, and cholesterol metabolizing genes, such as 7α-hydroxylase (*CYP7A1*) by LXR [1].

In conclusion, the results presented in this paper indicate that LXR agonists downregulate STAT5b protein levels and

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suppresses GHR activity in hepatocytes. Further investigation of the physiological consequences of the LXR/GHR interaction should provide evidence for a better informed use of LXR agonist and may explain some of its effect across a range of metabolic phenotypes.

Acknowledgments

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