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Intratesticular Delivery of Tumor Necrosis Factor-α and Ceramide Directly Abrogates Steroidogenic Acute Regulatory Protein Expression and Leydig Cell Steroidogenesis in Adult Rats

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Systemic or intratesticular release of TNFα and IL1β have been implicated in the reduced testosterone biosynthesis and impaired production of competent spermatozoa found in human patients suffering from sepsis or chronic inflammation. Although *in vitro* and *in vivo* studies have demonstrated that TNFα and IL1β intercept the hypothalamic-pituitary testis axis at different levels, the site(s) of action and relative contribution of each cytokine to the overall testicular failure associated to systemic inflammatory processes remains poorly defined. In this study we show that intratesticular delivery of TNFα induced a rapid (4 h) and sustained (up to 24 h) reduction in steroidogenic acute regulatory (StAR) protein expression and testosterone biosynthesis in nonstimulated or human chorionic gonadotropin-treated intact or hypophysectomized rats. Bilateral treatment with cell-permeant short-chain ceramides (C2-cer or C6-cer) reproduced the early (4 h) inhibitory action of TNFα on testosterone biosynthesis and testicular StAR expression. The inhibitory action of C2-cer or C6-cer was not observed in animals treated with inactive analogs (dihydroceramide), phosphorylcholine, sphingosine, or sphoingosine-1P. In sharp contrast to the previously described ability of IL1β to prevent human chorionic gonadotropin-stimulated Leydig cell steroidogenesis *in vitro*, serum testosterone and testicular StAR protein expression remained unchanged in animals bilaterally injected with this cytokine. These data support the concept that TNFα triggers different effector mechanisms to directly inhibit Leydig cell StAR expression and steroidogenesis, which ultimately contribute to the global reproductive failure associated with chronic inflammation and sepsis. (*Endocrinology* 144: 4763–4772, 2003)

THE HYPOTHALAMIC-PITUITARY-TESTIS axis ensures that the interstitial Leydig cells produce the appropriate amounts of testosterone required to maintain spermatogenesis and male secondary sex characteristics (reviewed in Refs. 1–3). Biosynthesis of all types of steroid hormones is initiated in the inner mitochondrial membrane in which cholesterol is converted into the common precursor steroid pregnenolone by the P450 cholesterol side chain cleavage (P450scc) enzyme (reviewed in Refs. 4–7). Pregnenolone formation is restricted by the aqueous intermembrane space of the mitochondria, which prevents the access of the hydrophobic cholesterol substrate to the P450scc site of action (4–7). To circumvent this limitation, steroid biosynthesis relies on a complex cAMP-dependent two-step process: mobilization of cholesterol from intracellular stores to the outer mitochondrial membrane and its subsequent translocation to the mitochondrial matrix (4–7). This later event resulting in the accumulation of a metabolizable cholesterol pool at the P450scc site of action requires *de novo* synthesis of the steroidogenic acute regulatory (StAR) protein and represents the true rate-limiting step of the steroidogenic pathway (5–7). Pregnenolone is converted into progesterone by the 3β-hydroxysteroid dehydrogenase enzyme and subsequently metabolized in testicular Leydig cells to androstenedione and testosterone by the sequential action the of P450c17–20 lyase (*Cyp*17) and 17β-hydroxysteroid dehydrogenase enzymes (for a review see Ref. 8).

Despite the well-established fact that testosterone biosynthesis is primarily regulated by the pulsatile release of pituitary LH, there are compelling evidences that Leydig cell steroidogenesis is additionally modulated by circulating and/or locally produced hormones, growth factors, and cytokines (1–3). In this setting, the severe hypogonadism and reduced plasma testosterone levels of males suffering from critical illness, burns, and sepsis (9–11) have been related to the elevated concentrations of macrophage-derived cytokines such as TNFα and IL1β present in the serum of these patients (12–14). Such causal relationship is supported by the reduced steroidogenic activity observed after systemic treatment with TNFα (15, 16), when IL1β is directly delivered into the cerebral ventricles (17, 18) or in lipopolysaccharide (LPS)-challenged animals (19–23).

Although cessation of steroidogenesis in LPS-treated an-

Abbreviations: AS-1, Anti-StAR polyclonal antibody; BW, body weight; C2-cer, *N*-acetyl sphingosine; c6-cer, *N*-hexanoyl ceramide; DHC, *N*-acetyl sphinganine; DIG, digoxigenin; dNTP, deoxynucleotide-triphosphate; dUTP, 11–2′-deoxy-uridine-5′-trisphosphate; EtOH, ethanol; G3PDH, glycerol-3-phosphate dehydrogenase; 25HC, 25-hydroxycholesterol; hCG, human chorionic gonadotropin; HPA, hypothalamic-pituitary-adrenal; LPS, lipopolysaccharide; P450scc, P450 cholesterol side chain cleavage; SDS, sodium dodecyl sulfate; SM, sphingomyelin; SMase, sphingomyelinase; StAR, steroidogenic acute regulatory protein.

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imals (19–23) may be causally related to the high circulating levels of TNFα and IL1β (12–14), the testis is a highly integrated cellular system (1–3) in which Leydig cells are in close contact with tubular Sertoli cells and resident testicular macrophages, which account for as much as 20% of the total cell population of the interstitial space (reviewed in Refs. 24 and 25). In this scenario, because TNFα (26–28) and IL1β (28, 29) are released by LPS-activated testicular macrophages and both agents share the ability to abrogate LH-induced and cAMP-mediated steroidogenesis in cultured Leydig cells (30–39), it has been proposed that locally produced cytokines may contribute to the overall steroidogenic failure observed in animal models of inflammation (24, 25).

In addition to stimulating a common set of signaling events involved in multiple redundant cellular responses, the TNFα receptors (p55TNFR and p75TNFR) and IL1β receptors (p60IL1R and p80IL1R) are structurally unrelated transmembrane proteins that activate different effector mechanisms that ultimately mediate the wide array of specific cellular and systemic responses characteristic of each cytokine (reviewed in Refs. 40–42).

Many overlapping cellular responses induced by TNFα and IL1β are initiated by a receptor-mediated activation of sphingomyelinase (SMase) that in turn promotes the hydrolysis of sphingomyelin to generate the inactive by-product phosphocholine and a regulatory ceramide moiety (reviewed in Ref. 43). We have described that the rapid activation of sphingomyelin (SM) hydrolysis triggers a relevant mechanism whereby TNFα and IL1β prevent FSH- and cAMP-stimulated steroidogenesis in cultured granulosa cells (44, 45). In contrast, data on this ceramide-mediated effector mechanism have not been reported in normal Leydig cells and so far activation of the SM pathway appears to be restricted to the inhibitory action of TNFα on hCG-induced StAR expression in the *Cyp*17-defficient MA-10 Leydig tumor cell line (38).

Moreover, although the direct activation of the SM pathway with exogenous SMase or cell-permeant ceramides reproduces the inhibitory actions of both cytokines on human chorionic gonadotropin (hCG)-stimulated P450scc and *Cyp*17 gene expression (37–39), these treatments also abrogate gonadotropin-induced StAR expression, which is a TNFα-specific response to Leydig cells (36–38).

In addition to these direct inhibitory actions of TNFα and IL1β on Leydig cell steroidogenesis *in vitro* (30–39), the testicular failure observed in immune-challenged animals might also be causally related to alterations in serum gonadotropin levels (21, 46) and/or activation of the hypothalamic-pituitary-adrenal (HPA) axis (reviewed in Refs. 47–49). Concerning this later situation, immune insults such as infection and chronic inflammation stimulate the pituitary release of ACTH and subsequently the biosynthesis and secretion of adrenal steroids (47–49). Although the release of adrenal steroids constitutes a defensive and ultimately protective mechanism against tissue damage during inflammation and sepsis, these antiinflammatory hormones also exert direct inhibitory actions on Leydig cell steroidogenesis (21, 22, 50, 51) and therefore may contribute to the reduced testicular function associated to these pathological situations (47–49).

Even though the above-mentioned *in vivo* and *in vitro* studies demonstrate that TNFα and IL1β inhibit the hypothalamic-pituitary-testis axis at multiple levels, the precise mechanism(s), site(s) of action, and relative contribution of each cytokine to the overall testicular failure associated to local or systemic inflammation are poorly defined.

In this study we used intact and hypophysectomized rats to investigate whether direct intratesticular delivery of TNFα and IL1β exerts similar inhibitory actions *in vivo* as those described in cultured hCG-stimulated Leydig cells (30–39). In a more precise manner, we evaluated whether intratesticular treatment with TNFα and IL1β abrogates StAR expression, which represents the true rate-limiting step in steroid biosynthesis (5–7). In addition, we also considered whether the direct activation of the SM pathway with short-chain ceramide analogs reproduce in intact animals the inhibitory responses on hCG-stimulated Leydig cell steroidogenesis reported in *vitro* (37–39).

Materials and Methods

Hormones and reagents

Human recombinant TNFα and IL1β were from Calbiochem (Barcelona, Spain). Human chorionic gonadotropin was purchased from Farma-Lepori (Barcelona, Spain). Phosphorylcholine, phenylmethylsulfonyl fluoride, metal-free protease inhibitor cocktail (P8340), and LPS (from *Escherichia coli*, serotype 0127:B8) were purchased from Sigma Chemical Co. (St. Louis MO). Sphingosine, sphingosine-1P, and the membrane-permeable analogs of ceramide *N*-hexanoylsphingosine (C6-cer), *N*-acetylsphingosine (C2-cer), and *N*-acetylsphinganine [C2-dihydroceramide (DHC)] were obtained from Biomol Research Laboratories (Plymouth Meeting, PA). Ribonuclease inhibitor (n° N211), AMV reverse transcriptase (no. M 519), *Thermus aquaticus* (*Taq*) DNA polymerase (no. M166), and Sau3A I (R619) were obtained from Promega (Madison, WI). The polydeoxy-thymidine primers ($pdT_{12–15}$), deoxynucleotide-triphosphates (dNTPs), the 1-kb molecular weight standard and ΦX174/*Hae*III digestion product were from Pharmacia (Barcelona, Spain). Digoxigenin-11–2′-deoxy-uridine-5′-trisphosphate (DIG-dUTP) and the positively charged nylon membranes were obtained from Roche Molecular Biochemicals (Madrid, Spain).

Animals and treatments

Adult (300–350 g) Sprague Dawley rats were obtained from Pan Lab (Barcelona, Spain). The same source provided the age-mated (2–3 months old) hypophysectomized animals (270–290 g), which were delivered 2 wk after surgery. Animals were housed in groups of three rats per cage and maintained at least 1 wk before experiments under controlled temperature (22–24 C) and light/dark cycle (14 h light; lights switched on at 0700 h) with free access to tap water or 0.9% saline (hypophysectomized animals) and a standard pelleted diet (Purina, Barcelona, Spain). All experimental procedures were approved by the University of Las Palmas Committee on Animal Care and were conducted according to the European Union normative 86/609 concerning the handling and use of experimental animals. Intact and hypophysectomized animals were randomly assigned to experimental groups, lightly anesthetized with ether, and injected intratesticularly through the scrotal wall with cytokines [200 ng/testes·kg body weight (BW)] or identically treated with cell-permeant ceramides (C2-cer and C6-cer), DHC, phosphorylcholine, sphingosine, and sphingosine-1P (all used at the dose of 30 μg/testes·kg BW). Depending on the experiment, control animals received equivalent volumes (25 μl) of ethanol (EtOH) or pyrogen-free saline or were just subjected to the stress associated to the needle (27-gauge) penetration (52). In other experiments, intratesticular delivery of cytokines or other agents was followed (30 min later) by the ip administration of hCG (100 IU) or an equivalent volume (0.5 ml) of pyrogen-free saline.

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Tissue collection and testosterone determination

Sequential blood samples from lightly anesthetized animals were obtained from the retroorbital plexus 2 h before treatment, immediately after treatment (time 0), and at the time points indicated in each experiment. This sequential blood sampling approach minimizes the number of animals per group because it circumvents the large variations in serum testosterone levels derived from the pulsatile release of LH and the circadian pattern of Leydig cell testosterone secretion (53, 54). In other experiments, animals were killed by CO_2 asphyxiation, blood collected by heart puncture, and testes immediately removed for RT-PCR and Western blot analysis of StAR (see below). Blood samples were left for 1 h at room temperature and serum separated from the clots, transferred to new tubes, centrifuged (1000 × *g* at 4 C for 20 min) and the clean supernatants stored frozen (−20 C). Serum testosterone content was determined by RIA using commercially available reagents and following the instructions of the manufacturer (Amersham Corp., Arlington Heights, IL). The sensitivity of the assay was 10 pg/tube and the intraassay and interassay coefficients of variation of 4% and 9.5%, respectively.

RT-PCR of StAR

Total RNA was isolated using Ultraspec RNA (Biotecx, Houston, TX) and isopropyl alcohol precipitation. Equal amounts of RNA (1 μg) were incubated for 75 min at 42 C in 20 μl (final volume) of 1× PCR buffer containing 500 ng oligo $(dT)_{12–15}$ primer, 1 mM dNTP, 5 U AMV reverse transcriptase, and 20 U RNAsin ribonuclease inhibitor, and reactions terminated by heating at 95 C for 5 min and cooling the tubes on ice. After an initial denaturation step (94 C for 3 min) each cDNA sample was amplified in a Perkin-Elmer/Cetus thermocycler in 25 μl PCR buffer containing (final concentration) 2.5 μM DIG-dUTP, 100 μM of dNTP, 1.5 mM Cl_2Mg, 0.625 U *Taq* DNA polymerase, and 125 ng (10–15 pmol each) and the gene-specific oligonucleotides (forward: 5′-ACAACCAG-GAAGGCTGGAAG-3′; reverse 5′-CCTCTGCGCTTGGTACAGC-3′) synthesized and HPLC purified by DNA-International (Lake Oswego, OR). The primers complementary to nucleotides 341–360 and 632–650 of the known sequence of StAR (55–57) were designed to generate an amplification signal 309 bp of the region common to the three alternative spliced products of StAR cDNA containing an unique Sau3A I restriction site (GATC: 528–531) between bases 527 and 528 of the gene (35, 36). After an overnight digestion at 37 C in 20 μl buffer B (Promega) containing 3 U Sau3A, restriction products were resolved on 4.5% high-resolution MS-8 agarose (Hispanlab, Madrid, Spain) gels and produced identifiable bands of the predicted 186- and 123-bp size.

Oligonucleotides designated to generate a PCR product of 983 bp of glycerol-3-phosphate dehydrogenase (G3PDH) were obtained from Clontech (Palo Alto, CA) and used as amplification control. To analyze PCR products from each target gene, 10 μl of the amplified fragments were separated by electrophoresis on 1.8% agarose gels in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). The gels were incubated for 15 min in transference buffer (0.4 M NaOH, 1.5 M NaCl) and transferred to positively charged nylon membranes by positive pressure blotting (Posiblotter, Stratagene, La Jolla, CA). The membranes were cross-linked (1200 kJ/30 sec) on a Stratalinker UV cross-linker (Stratagene) and chemiluminescent detection performed with a commercially available DIG luminescent detection kit following the instructions of the manufacturer (Roche Molecular Biochemicals, no. 1363514). The quantitative data were obtained using a DIANA III densitometric scanning instrument (RAYTEST, Straubenhardt, Germany) with AIDA image analyzer software.

Immunoblot analysis of StAR

The tissues were mechanically homogenized (100 rpm, 25 passes) with a Potter Elvejelm homogenizer in TSE buffer (0.25 M sucrose, 10 mM Tris, 0.1 mM EDTA, pH 7.4) and mitochondria isolated by differential centrifugation as described (55–57). Mitochondrial proteins were extracted with 50 μl of 20 mM HEPES buffer (pH 7.4) containing (final concentration) 0.15 M NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and the recommended final concentration of protease inhibitor cocktail (100 μl/10^7 cells). Equal amounts of mitochondrial proteins (100 μg) were boiled 5 min in 2× sodium dodecyl sulfate (SDS) sample buffer [25 mM Tris-HCl (pH 6.8), 1% SDS, 5% β-mercaptoethanol, 1 mM EDTA, 4% glycerol, and 0.001% bromophenol blue] separated on SDS-7.5% polyacrylamide gels and then electrophoretically transferred to polivinyldifluoride membranes (Bio-Rad Laboratories, Madrid, Spain). After incubation (1 h at room temperature) in PBS supplemented with 4% nonfat dry milk and 0.5% Tween 20 to block nonspecific binding sites, membranes were reincubated (12 h) in the same buffer containing anticytochrome C antibody used at the final dilution (1:1000) recommended by the manufacturer (Santa Cruz Biotechnology, Santa Cruz, CA) or a polyclonal anti-StAR antibody (final dilution 1:2000) raised against a peptide corresponding to residues 88–98 of the proposed N terminus of the published sequence of the mature (30 kDa) StAR protein (55, 56). The peptide was synthesized and conjugated to keyhole limpet hemocyanin by the water-soluble carbodiimide method and used to produce antibodies in rabbits on a fee-for-service basis by Genosys Biotechnologies Inc. (Cambridge, UK). Two rabbits developed antibodies with a high titer, and only one of them (designated AntiS-1) was used in all experiments described here. The membranes were washed (three times for 10 min each wash) and incubated (1 h at room temperature) in fresh blocking buffer containing an 1:4000 dilution of horseradish peroxidase-conjugated antirabbit IgG (Amersham Corp., Barcelona, Spain) and the specific StAR or cytochrome C signals detected using an ECL detection kit (American Life Science, Buckinghamshire, UK). Analysis of the integrated ODs was performed by scanning densitometry.

Statistical analysis

Results for serum testosterone levels are pooled data (mean ± SEM) derived from at least two experiments performed on different days using a minimum of five animals per experimental group. Comparisons were performed using ANOVA and as indicated the *t* test for comparison of the means. Differences with $P > 0.05$ were not considered significant.

Results

Time-dependent inhibition of serum testosterone levels after intratesticular treatment with TNFα and C2-cer

As assessed in preliminary experiments, no significant differences in serum testosterone concentrations were observed in untreated rats as compared with animals subjected to needle penetration in both testes or bilaterally injected with equivalent volumes (25 μl) of saline or EtOH vehicles (results not shown). Hence, to reduce the number of rats, we used animals subjected to needle stress as single control group for the different vehicles.

Results from the first series of experiments illustrate that basal serum testosterone levels were similar 2 h before (6.5 ± 0.7 ng/ml) and immediately after (6.9 ± 0.9 ng/ml) intratesticular treatment with cytokines and C2-cer (Fig. 1A). Treatment with TNFα significantly reduced (30%) testosterone concentration 2 h later ($P < 0.05$), being a more pronounced inhibition (circa 70%) observed in animals bilaterally treated with the cell-permeant C2-cer analog. Testosterone concentrations were dramatically reduced by 4 h in animals treated with TNFα or C2-cer, whereas no significant differences were observed after intratesticular delivery of IL1β or needle penetration (for clarity only IL1β data are shown in Fig. 1A). Testosterone levels remained low throughout the entire experimental period in animals injected with TNFα but significantly recovered by 6 and 8 h in animals treated with cell-permeant ceramide. In additional experiments (Fig. 1B), we document that treatment with C6-cer reduced serum testosterone concentrations with a similar potency as C2-cer. In contrast, serum testosterone levels remained unchanged in rats bilaterally injected with the

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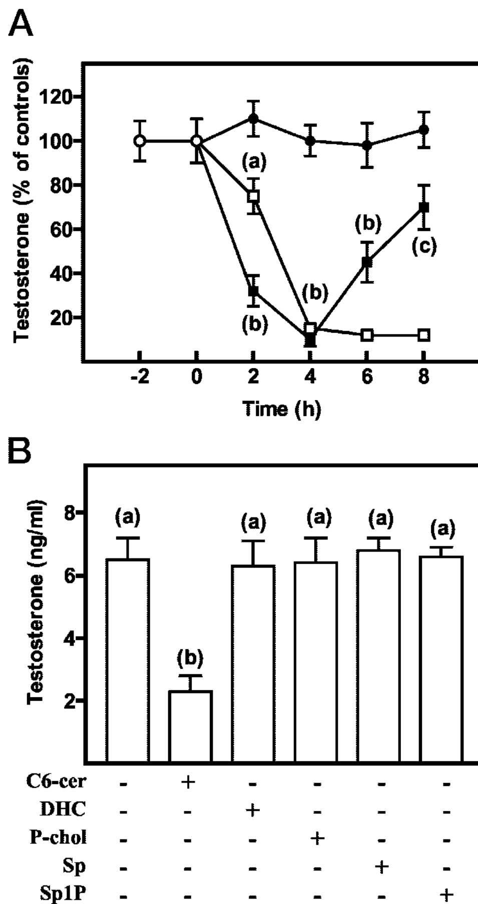

FIG. 1. Effect of intratesticular treatment with cytokines and short-chain ceramide (C2-cer) on serum testosterone levels in male rats. A, Blood samples from anesthetized rats were obtained 2 h before and immediately after intratesticular treatment (*open circles*). Animals (n = 8 per group) were injected in each testes with the same doses (200 ng/kg BW) of IL1β (*closed circles*) and TNFα (*open squares*). Groups of animals were treated in a similar manner with 30 μg/kg BW of cell-permeant C2-cer (*closed squares*) or subjected to needle penetration in each testes (for clarity this latter group is not represented because the results overlapped those of animals treated with IL1β). Sequential blood samples were obtained from the retroorbital plexus immediately after treatment (0 time) and at the time points indicated (2–8 h). In additional experiments (B), animals were subjected to needle stress or injected in each testis with equivalent concentrations (30 μg/kg BW) of another cell-permeant ceramide (C6-cer), the inactive analog DHC, phosphorylcholine, sphingosine (SP), or sphingosine-1P (SP1P). Results for serum testosterone concentrations are expressed as percent concentrations (mean ± SEM) of control values (*upper panel*) or in ng/ml (*lower panel*) and represent pooled data of two experiments performed on different days with the same number of animals per treatment. In several cases SEs are less than data point drawn, and groups with *different letters* are significantly different ($P < 0.05$).

inactive ceramide analog DHC, the by-product of SMase action phosphorylcholine (43), sphingosine, or its phosphorylated metabolite sphingosine-1P.

Intratesticular treatment with TNFα and C2-cer abrogate StAR expression in unstimulated and hCG-treated rats

To determine the site(s) and mechanism(s) whereby TNFα and C2-cer abrogate testicular steroidogenesis *in vivo*, we used a sensitive RT-PCR procedure to detect StAR transcription (Fig. 2, A–C) and developed a specific anti-StAR polyclonal antibody (AS-1), which recognizes the mature (30 kDa) mitochondrial form of the protein (Fig. 2, D and E). An amplification product for StAR of the predicted 309-bp size was detected in steroidogenic glands (adrenals and testis), whereas the 983-bp signal of the constitutive G3PDH used as amplification control was also present in brain, liver, and kidney (Fig. 2A). Verification that the amplified product represents StAR transcripts was confirmed by the presence of the predicted 186- and 123-bp fragments after overnight digestion hydrolysis with Sau3A (Fig. 2B). Because of the high sensitivity of the chemiluminescence detection method, initial experimental conditions were established for optimal reverse transcription and amplification of these products in a range sensitive to the amount of RNA input and number of cycles (Fig. 2C). Using immunoblot analysis, we detected cytochrome c in mitochondrial proteins extracted from all tissues tested. In contrast, the 30-kDa band of StAR was apparent only in adrenals and testis (Fig. 2D) and specifically abrogated when membranes containing the mitochondrial proteins were probed with AS-1 antibody previously preadsorbed (5 h) with immunogen (Fig. 2E).

With these approaches, we show that intratesticular delivery of TNFα significantly reduced 4 and 24 h later the specific mRNA signal of StAR and mitochondrial accumulation of the 30-kDa form of the protein, with no such actions observed in animals treated with IL1β (Fig. 3). Likewise, bilateral treatment with equivalent volumes (25 μl) of saline or EtOh vehicle did not affect testosterone biosynthesis and StAR expression, compared with animals subjected to needle stress (results not shown). As expected from previous experiments (Fig. 1), intratesticular treatment with C2-cer-abrogated testosterone biosynthesis and StAR expression at the early time point studied (4 h) and no differences with control or IL1β-treated animals were observed 24 h later.

Inhibitory action of TNFα and C2-cer on hCG-stimulated steroidogenesis and StAR expression in intact and hypophysectomized rats

To determine that the rapid and sustained inhibition of testicular steroidogenesis induced by TNFα is not a secondary response related to altered gonadotropin release (21, 46), in the next experiments we used hCG-stimulated animals (Fig. 4) pretreated (30 min) with cytokines or cell-permeant ceramides. As compared with unstimulated rats, testosterone concentrations dramatically augmented in hCG-treated animals 4 h later and remained elevated 24 h after gonadotropin stimulation ($P < 0.05$). The stimulatory action of hCG on testosterone biosynthesis paralleled the significant increase in mRNA levels of StAR and mitochondrial accumulation of the 30-kDa form of the protein. Although IL1β had no apparent effect on gonadotropin-induced steroidogenesis, intratesticular delivery of TNFα and C2-cer prevented the large increase in testosterone concentrations and StAR expression observed 4 h after hCG administration. No differences in testosterone levels and StAR expression were observed 24 h later in animals injected with C2-cer as compared with hCG-stimulated controls. Of interest to note, although

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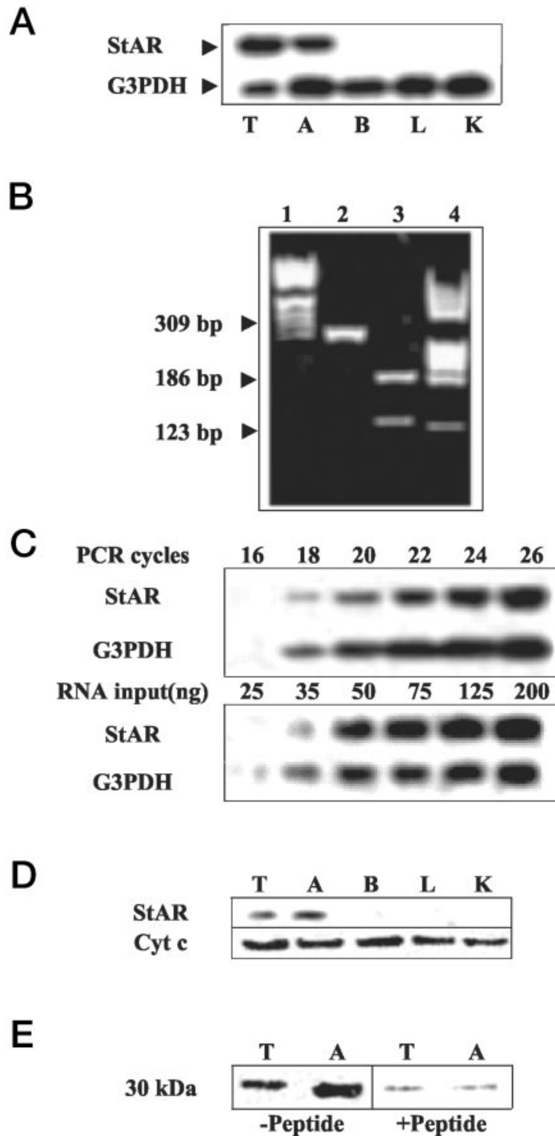

FIG. 2. Characterization of StAR transcripts and specificity of the polyclonal anti-StAR antibody. A, Total RNA was extracted from rat tissues (T, testis, A, adrenals; B, brain; L, liver; K, kidney), reverse transcribed into cDNA, and amplified in the presence of DIG-dUTP for 20 or 18 cycles (for StAR or the internal amplification control G3PDH, respectively). Chemiluminescent detection of the specific 309 bp (StAR) and 983 bp (G3PDH) was performed as described in *Materials and Methods*. B, The 309-bp product was digested overnight with Sau3A I (3 U/20 μl) and fragments separated by electrophoresis on 2% agarose gels (B). Lanes 1 and 4 represent a 1-kb ladder and ΦX174/Hae III digestion products, respectively. After digestion hydrolysis with Sau3A I, the 306-bp signal of StAR (lane2) generated two fragments of the predicted size (186 and 123 bp) shown in lane 3. C, RT-PCR amplification of StAR and G3PDH using different template amounts (25–200 ng) obtained from LH-treated male rats amplified for 20 or 18 cycles (for StAR and G3PDH, respectively) or the detailed number of cycles for each gene product using 125 ng RNA input. Proteins were extracted from mitochondrial pellets isolated by differential centrifugation in TSE buffer from the same rat tissues (T, testis, Ad, adrenal; B, brain; L, liver; K, kidney) and separated by SDS-PAGE. Proteins were electrophoretically transferred to polivinyldifluoride membranes and probed with anti-StAR AS-1 or Cyt-c antibody (D). The specific 30-kDa signal of StAR was not apparent when the AS-1 antibody was preadsorbed (5 h) with 10 μg/ml of the immunogen (E).

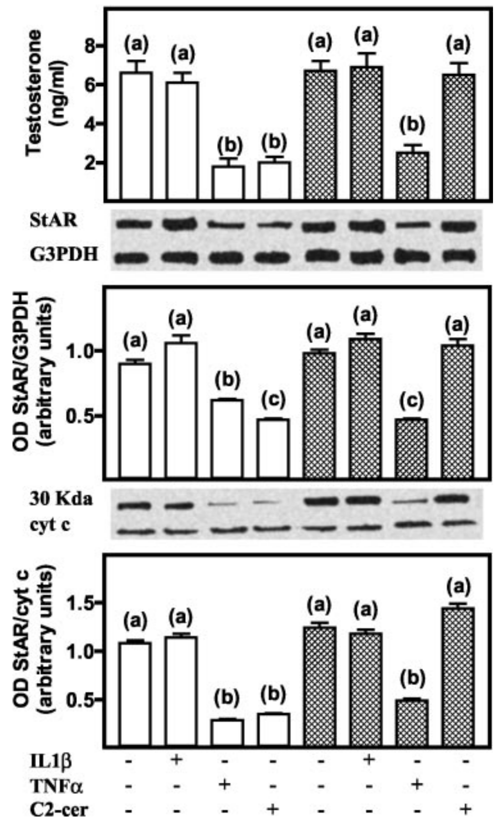

FIG. 3. Intratesticular treatment with TNFα and ceramide abrogates StAR expression and steroidogenesis in intact animals. Rats (n = 8/group) were subjected to needle stress (C) or injected in each testis with the same doses of cytokines or C2-cer used before. Animals were killed 4 h (*open bars*) or 24 h (*dashed bars*) later, blood samples obtained by heart puncture, and the testes rapidly removed and used for RT-PCR and Western blot analysis. Serum testosterone levels (mean ± SEM) represent pooled data of two experiments, using the same number of animals per group. Densitometric analysis of five different experiments and a representative RT-PCR (*middle panel*) and Western blot (*lower panel*) are depicted. *Different letters* denote significant differences between groups ($P < 0.05$).

testicular steroidogenesis still remained low 24 h after TNFα treatment, testosterone concentration and StAR expression were higher in these animals than in unstimulated control rats.

Animals receiving the doses of cytokines or ceramide analogs used in these experiments exhibited no signs of local or general inflammation observed after systemic treatment with 5 mg/kg BW of bacterial LPS (results not shown). Nevertheless, these data may simply reflect that a single cytokine is unable to fully reproduce the general condition of illness (lethargy, piloerection, shivering, and increased rectal temperature) observed in LPS-treated rats (42) and do not exclude that, once delivered, these agents are cleared from the testis via the spermatic vein to the general circulation (52).

In this regard, although the release of ACTH and the subsequent secretion of antiinflammatory adrenal steroids (47–49) constitutes a defense mechanism against immune insults, it is also well established that glucocorticoids exert direct inhibitory actions on Leydig cell steroidogenesis (21, 22, 50, 51) and therefore may contribute to the reduced testicular function associated with chronic inflammation and

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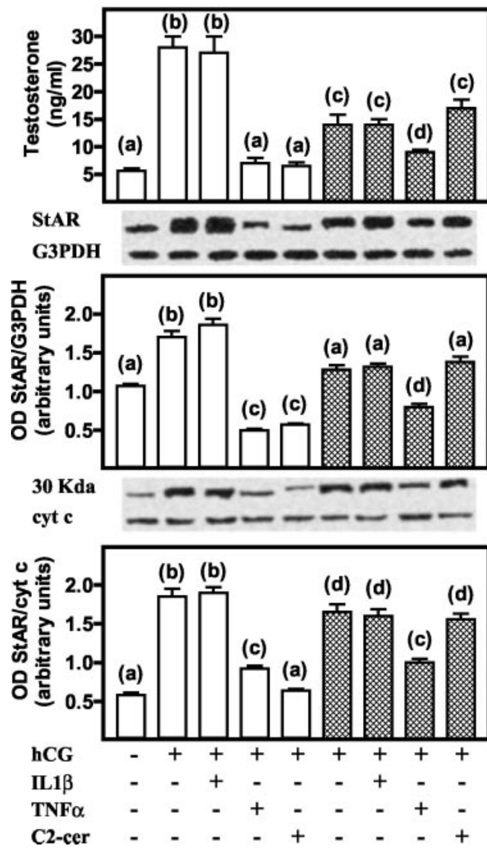

FIG. 4. Effect of TNFα and ceramide on gonadotropin-induced StAR expression and steroidogenesis. Equal number of rats (n = 8/group) were bilaterally injected with cytokines (200 ng/ kg BW per testis) or C2-cer (30 μg/kg BW per testis) and treated 30 min later with a single ip dose of hCG (100 IU) or 0.5 ml saline (controls). Blood samples were collected from the retroorbital plexus 4 h (*open bars*) and 24 h later (*dashed bars*) and serum obtained and stored frozen until assayed for testosterone concentrations. In additional experiments, animals were treated in a similar manner, blood samples obtained by heart puncture, and the testes rapidly removed and used for RT-PCR and Western analysis. Serum testosterone levels (mean ± SEM) represent pooled data of two experiments, using the same number of animals per group. Quantitation of five different RT-PCR (*middle panel*) and Western blot (*lower panel*) analysis, and a representative experiment showing mRNA and protein levels are depicted. Groups with *different letters* (a–d) are significantly different ($P < 0.05$).

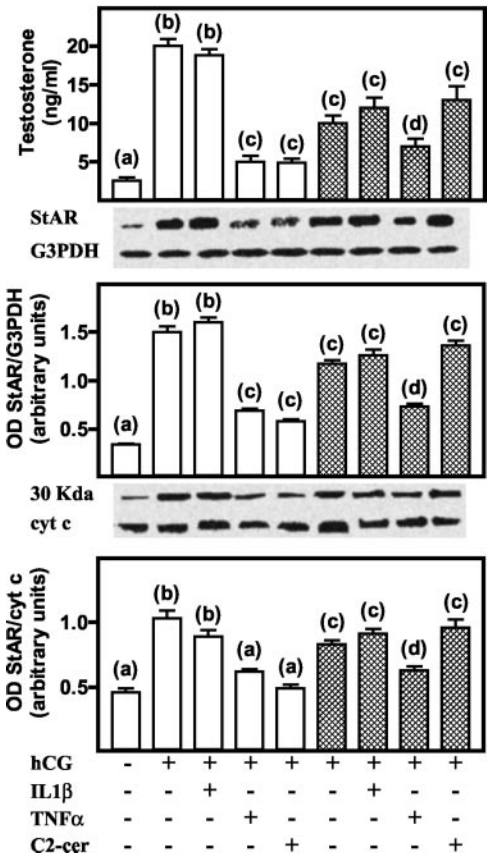

FIG. 5. Effect of TNFα and ceramides on hCG-induced StAR expression and testosterone biosynthesis in hypophysectomized rats. Hypophysectomized rats (n = 5 per group) were subjected to needle stress (C), injected in each testes with equivalent amounts of TNFα and IL-lβ1 (200 ng/kg BW per testis) or with C2-cer (30 μg/kg BW per testis) and treated 30 min later with a single ip dose of hCG (100 IU). Blood was collected from the retroorbital plexus 4 h (*open bars*) and 24 h later (*dashed bars*) and serum obtained and stored frozen until assayed for testosterone concentrations (*upper panel*). In parallel experiments groups of animals (n = 5 per group) were killed 4 h or 24 h after treatment, and blood samples were collected and the testes were removed and used to analyze mRNA levels (middle panel) and mitochondrial protein levels (*lower panel*) of StAR. Serum testosterone levels (mean ± SEM) represent pooled data of both experiments, which were replicated two times with the same number of animals per group. Representative experiments and quantitation of StAR transcription and protein levels are shown in the *middle* and *lower panels*. Groups with *different letters* (a–d) are significantly different ($P < 0.05$).

sepsis (47–49). Compared with intact controls, preliminary experiments revealed that needle stress induced a slight increase in adrenal StAR expression in some animals (results not shown). Even though this response was unrelated to a specific agent delivered via the scrotal wall, in the next experiments we used hypophysectomized animals to confirm that the inhibitory actions of TNFα and C2-cer on Leydig cell StAR expression and steroidogenesis are unrelated to alterations in the HPA axis (Fig. 5). The extremely low serum testosterone levels (*upper panel*), Leydig cell mRNA (*middle panel*), and protein levels of StAR (*lower panel*) were significantly augmented 4 h after gonadotropin treatment and remained elevated even 24 h after hCG administration. As expected, gonadotropin-stimulated steroidogenesis remained unchanged in rats pretreated with IL1β, but the direct delivery of TNFα and C2-cer dramatically blunted hCG-induced StAR expression 4 h later. By 24 h, this inhibitory effect was still apparent in animals bilaterally treated with TNFα, but similar to intact rats (Fig. 4), testosterone concentration, StAR message, and protein levels were significantly higher in these animals than in unstimulated controls.

Discussion

In contrast to the large amount of experimental evidence that demonstrates the ability of TNFα and IL1β to directly abrogate hCG-stimulated Leydig cell steroidogenesis *in vitro* (30–39), information concerning similar actions under *in vivo* conditions is less abundant. In this study we show that in-

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tratesticular delivery of TNFα and cell-permeant ceramides directly abrogate testosterone biosynthesis in intact and hypophysectomized and extend these findings to demonstrate for the first time that this response is causally related to an inhibitory action on basal and hCG-induced StAR expression in Leydig cells.

In contrast, and despite evidences demonstrating the capacity of IL1β to prevent hCG-and cAMP-stimulated steroidogenesis in cultured Leydig cells (30, 32, 33, 36, 39), local delivery of this cytokine did not significantly reduce serum testosterone levels and StAR expression in unstimulated or gonadotropin-treated animals. These divergent results were somewhat unexpected because binding of TNFα and ILβ to specific TNFR and IL1βR expressed in the testis (37, 58–60) trigger a common set of inhibitory actions on hCG-stimulated testosterone biosynthesis *in vitro* (30–39). Nevertheless, although testicular alterations in endotoxin-treated animals (20–23) correlate with the systemic release of cytokines (9–14), it must also be considered that the access of circulating IL1β to the interstitial space is limited by the blood-testis barrier (60), and simultaneously endotoxin treatment dramatically reduces IL1βR expression in the testis (61).

Otherwise intracerebroventricular delivery of IL1β induced a rapid decrease in pituitary FSH and LH release and a drastic a reduction of testicular StAR expression and steroidogenesis (17, 18). Although the ability of circulating cytokines to traverse the blood-brain barrier remains controversial (49), recent evidence suggests that LPS induces a rapid increase in IL1β content in the hypothalamic extracellular fluid that chronologically precedes the reduction in gonadotropin release and the elevated systemic concentrations of ACTH and corticosteroids (62). Nevertheless inhibition of StAR expression and steroidogenesis after intracerebroventricular delivery of IL1β was unrelated to pituitary gonadotropin secretion or activation of the HPA axis (17, 18, 63). On the other hand, these studies revealed that basal steroidogenesis remained unchanged after systemic administration of IL1β, but this treatment induced a small, albeit significant, reduction in testosterone concentrations in hCG-stimulated animals (18). Although the inhibitory action induced by iv treatment with the cytokine was accompanied by a dramatic increase in serum IL6 levels, administration of ILβ into the cerebral ventricles abrogated steroidogenesis before increases in serum IL6 levels were noted (18). In considering that IL6 activates the HPA axis and induces Leydig cell resistance to LH action in *vivo* (64), it seems reasonable to conclude that the release of this cytokine may account for the reduced inhibitory action on hCG-stimulated steroidogenesis observed after systemic treatment with IL1β.

These data, in conjunction with the limited ability of IL1β to cross the blood-testis barrier (60) and the lack of inhibitory effect after intratesticular delivery of the cytokine presented in this study, strongly support the contention that the contribution of IL1β to the general testicular failure associated with sepsis and inflammation may be exerted at the hypothalamic-pituitary level and/or mediated by the release of other factors that directly inhibit Leydig cell function.

Otherwise, the inhibitory effect induced after direct intratesticular administration of TNFα suggests that the local release of this cytokine by resident macrophages may significantly contribute to abrogate Leydig cell steroidogenesis during chronic inflammation (21, 22). In considering that administration of TNFα reduces testosterone biosynthesis in human volunteers and experimental animals (14, 15), the present data also support the notion that elevated systemic levels of this cytokine achieved during endotoxemia can transverse the blood-testis barrier and directly exert inhibitory actions on Leydig cell function.

Of interest to note, inhibition of testosterone biosynthesis and StAR expression after intratesticular delivery of TNFα occurred in a time frame (4–24 h) similar to that previously observed in LPS-challenged animals (20–23). Nevertheless, inhibition of testicular steroidogenesis in LPS-treated mice was causally related to a rapid (2 h) inhibition of StAR mRNA translation into the mature form of the protein, this early action being accompanied by a reduction of the hormone-regulatable components P450scc and *Cyp*17 at the longer time periods (24 h) studied (20). The data presented herein however clearly demonstrate that TNFα prevents basal and hCG-stimulated StAR transcription and translation under *in vivo* conditions in rats (Figs. 3–5). These divergent results may reflect species differences and/or the systemic treatment with LPS used in other reports (20–23), compared with intratesticular delivery of a single agent (TNFα) used in this study. It must be noted, however, that despite the fact that P450scc and *Cyp*17 enzymes are important components of the steroidogenic machinery in Leydig cells (8), StAR-mediated delivery of cholesterol to the inner mitochondrial membrane represents the first and true rate-limiting step for the biosynthesis of steroid hormones (5–7). Evidence for the paramount role of this initial step in steroid biosynthesis derived from experiments in which steroidogenic cells were cultured in the presence of the 22*R*-hydroxycholesterol or 25-hydroxycholesterol (25HC) analogs of the precursor (4–7). These hydroxylated compounds freely diffuse across the mitochondrial intermembrane space, accumulate at the P450scc complex and are converted to pregnenolone (4–7). Hence, under these experimental conditions steroidogenic cells produced high levels of steroids in the absence of hormone stimulation, indicating that full catalytic activity of P450scc is limited by the slow diffusion of cholesterol through the aqueous space between the outer and inner mitochondrial membrane (5).

But perhaps the most striking evidence of its paramount role in steroidogenesis concerns the demonstration that nonsense mutations of the human StAR gene leads to congenital adrenal hyperplasia (57), a fatal disease in which steroid biosynthesis is virtually eliminated. Hence, because inhibition of StAR expression is sufficient to explain the dramatic decrease in testosterone biosynthesis induced by TNFα and cell-permeant ceramides, no further attempts were made to elucidate whether these treatments intercept additional steps along the steroidogenic pathway. Because TNFα also abrogates P450scc and *Cyp17* in cultured Leydig cells (31, 34, 35), these results do not exclude that treatment *in vivo* with TNFα intercepts additional components of the steroidogenic pathway located downstream of StAR activity (8).

This assumption deserves further consideration in view of recent reports that demonstrate that testicular macrophages express the 25-hydroxylase enzyme required to convert cho-

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lesterol into the polar secretory product 25HC (65, 66). Although the release of low levels of 25HC may facilitate testosterone biosynthesis in unstimulated Leydig cells (4–7), these studies document that prolonged exposure to high concentrations of the oxysterol precursor abrogated LH responsiveness and induced nonspecific cytotoxic actions in testicular cells (66). Nevertheless, because concentrations of androgens similar to those found in the interstitial space abolished 25-hydroxylase transcription and oxysterol release by testicular macrophages (67), it has been proposed that 25HC and testosterone integrate a communication system between resident macrophages and Leydig cells, which could provide a physiologically relevant mechanism required to sustain basal and LH-stimulated steroidogenesis. If such a mechanism operates *in vivo*, the early inhibition of testosterone biosynthesis induced by TNFα and C2-cer may simultaneously up-regulate 25-hydroxylase expression and subsequently the release of 25HC (67), which is then converted to testosterone in a StAR-independent manner in surrounding Leydig cells that retain functional P450scc and Cyp17 enzymes (4–7). Hence, the recovery of serum testosterone levels after ceramide treatment suggests a functional steroidogenic machinery downstream of StAR protein. On the contrary, the prolonged inhibition of steroidogenesis observed in TNFα-challenged rats sustains the notion that this cytokine induces additional inhibitory actions at the P450scc and *Cyp*17 enzyme steps.

Ceramide generation in response to Fas/Apo1 ligation or p55TNFR activation has been associated with the cytotoxic and/or apoptotic actions mediated by the TNFα/Fas system (41, 43). In contrast to the well-established role of a ligand-regulated SM pathway in mediating cellular function and life-and-death decisions in the ovary, there is little information concerning a similar role for ceramide in the testis (reviewed in Ref. 68). This study shows that testosterone biosynthesis and StAR expression gradually recovered (6–8 h) and reached control levels 24 h after treatment with C2-cer (Figs. 1 and 3) a time point at which testicular steroidogenesis still remained low in animals treated with TNFα. Nevertheless in TNFα-treated and hCG-stimulated animals, testicular testosterone biosynthesis and StAR expression remained higher than in saline-injected controls (Figs. 3 and 4). Based on these data, it seems reasonable to conclude that the direct inhibition of testicular steroidogenesis reported in this study is not related to nonspecific toxic actions of TNFα or its putative second-messenger ceramide moiety.

The ability of C2-cer and C6-cer to reproduce the short-term inhibitory actions of TNFα is a ceramide-specific event because no such effect was observed in animals treated with DHC, the by-product of SMase action phosphorylcholine (43) or the two ceramide metabolites sphingosine and sphingosine-1P (reviewed in Ref. 69). Nevertheless, these data do not demonstrate that inhibition of testosterone biosynthesis and StAR expression *in vivo* is causally related to a ligand-induced activation of the SM pathway. Activation of the SM pathway is specifically coupled to the p55TNFR and has been demonstrated in a variety of cellular models, but such a relationship is difficult to assess in organs and tissues of TNFα-challenged animals (41, 43).

In addition to the cellular complexity of the testis, analysis of the SM pathway *in vivo* is further complicated because ligand-induced ceramide generation may accumulate in cells for variable periods of time, be recycled back to SM, or be degraded to sphingosine by ceramidase and further transformed by sphingosine kinase into the sphingosine-1P moiety (43, 69). Although the role of sphingolipid metabolites in the testis remains poorly defined (68), it has been recently shown that the total testicular concentration of sphingosine-1P is considerable higher (up to 100 nmol/g) than in other tissues tested (70). In agreement with these findings, metabolic labeling experiments performed with MA-10 Leydig tumor cells (38) and primary cultures of pig Sertoli cells (71) demonstrated that ceramide generated in response to TNFα was rapidly converted to sphingosine-1P in these cellular models. Furthermore, although cell-permeant ceramides reproduced the inhibitory action of TNFα on StAR expression in MA-10 Leydig cells (38), the conversion of ceramide to sphingosine was required for TNFα-induced glucose metabolism and LDH expression in Sertoli cells (71).

In this study we show that intratesticular delivery of cell-permeant ceramides induced a rapid and transient inhibition on testicular steroidogenesis (Fig. 1A), but no such effect was observed after bilateral treatment with sphingosine or sphingosine-1P (Fig. 1B). If it is assumed that the sequential action of testicular ceramidase and sphingosine kinase can convert exogenous short-chain ceramides to sphingosine-1P (43, 70), such a scenario provides a plausible explanation for the transient inhibition of steroidogenesis observed after intratesticular treatment with the C2-cer analog. Conversely, in considering that C2-cer reproduced only the early (up to 4 h) actions of TNFα on testicular steroidogenesis, it must be assumed that additional effector mechanisms unrelated to the SM pathway are ultimately responsible of the long-term abrogation of testosterone biosynthesis and StAR expression. Finally, because sphingosine-1P exerts stimulatory actions on cell growth and survival (43, 70), the release of sphingosine and its conversion to sphingosine-1P may constitute a physiological and ultimately protective mechanism to counteract the deleterious actions of ceramide and TNFα on germ cell survival and Leydig cell steroidogenesis.

In conclusion, the present study highlights that the intratesticular delivery of agents provides a useful model to evaluate the physiological relevance of putative intragonadal regulatory agents. This study also revealed several novel elements concerning the testicular response to inflammation. In the first place, we demonstrate that the direct intratesticular delivery of TNFα prevents basal or hCG-stimulated steroidogenesis and identified StAR as the target for the inhibitory action of this cytokine under *in vivo* conditions. In addition, we document the ability of cell-permeant ceramides to reproduce the early inhibitory actions of TNFα on StAR gene transcription and protein expression in unstimulated or hCG-treated animals. Although this study does not establish a causal relationship between TNFα-induced SM hydrolysis and testicular function *in vivo*, the present data demonstrate for the first time a direct and specific inhibitory action of ceramide on testicular StAR expression and steroidogenesis in intact animals.

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