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Tumor Necrosis Factor-α Activates Transcription of Inducible Repressor Form of 3′,5′-Cyclic Adenosine 5′-Monophosphate-Responsive Element Binding Modulator and Represses P450 Aromatase and Inhibin α-Subunit Expression in Rat Ovarian Granulosa Cells by a p44/42 Mitogen-Activated Protein Kinase-Dependent Mechanism

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The proinflammatory cytokine TNFα has important actions at the level of the ovary, including inhibition of P450 aromatase ($P450_{AROM}$) activity and the secretion of inhibin, two proteins that are markers of the granulosa cell's differentiated status. Because the transcription of both $P450_{AROM}$ and inhibin α-subunit can be suppressed in the ovary by the inducible repressor isoform of cAMP-responsive element binding modulator (ICER), we have investigated whether TNFα and its intracellular messenger ceramide can induce ICER expression and the mechanisms whereby the induction is accomplished. ICER mRNA levels were assessed by RT-PCR in granulosa cells treated with TNFα, the ceramide-mobilizing enzyme sphingomyelinase (SMase), or C6-cer, a cell-permeant ceramide analog. Rapid (3 h) yet transient increases in the four isoforms of ICER were observed in response to all treatments. Likewise, ICER protein measured by immunoprecipitation with a specific antibody increases after TNFα, SMase, or C6-cer treatment. The mandatory phosphorylation of cAMP-responsive element binding was also observed in response to TNFα, SMase, or C6-cer and shown to be prevented by the p44/42 MAPK-specific inhibitor PD098059 but no other kinase blockers. Activation of p44/42 MAPK by the cytokine and its messenger was subsequently demonstrated as well as the inhibition of ICER expression by PD098059. Finally, the blocking of p44/42 MAPK activation prevented TNFα inhibition of FSH-dependent increases in $P450_{AROM}$ and inhibin α-subunit mRNA levels, thus indicating that p44/42 MAPK-mediated ICER expression may be accountable for the effects of TNFα on the expression of both proteins. (*Endocrinology* 147: 5932–5939, 2006)

THE BEST CHARACTERIZED effect of TNFα is the ability to induce signals that trigger cell death (1–3), but this proinflammatory cytokine has pleiotropic effects in mammalian cells (4–6). TNFα is primarily secreted by monocytes and macrophages in response to bacterial lipopolysaccharide, and although resident or infiltrating macrophages secrete TNFα in the ovary (7), secretion by granulosa-luteal cells has also been reported (8, 9), and TNFα mRNA has been detected in ovarian tissue (10). Whatever is the source for the TNFα acting in the ovary, the cytokine has multiple and important effects in this organ that may be summarized as inhibition of FSH-dependent differentiation of granulosa cells (11, 12) and induction of apoptosis (13).

Two of the more prominent specific actions of TNFα in the ovary are the inhibition of FSH-stimulated P450 aromatase ($P450_{AROM}$) catalytic activity and inhibin secretion by granulosa cells (14, 15). The acquisition of the ability to synthesize estrogens and inhibin is a hallmark of the granulosa cell preovulatory phenotype, because follicle development, ovulation, and cyclic secretion of pituitary hormones all rely to some extent on the production of adequate amounts of estrogens and inhibin at the different phases of the ovarian cycle (reviewed in Refs. 16–19).

The transcription of $P450_{AROM}$ and inhibin α-subunit is tightly regulated by pituitary hormones. Upon binding to its G protein-coupled receptor in granulosa cells, FSH induces cAMP production, protein kinase A (PKA) catalytic subunit activation (20) and cAMP-responsive element binding protein (CREB) phosphorylation (21), resulting in the transcription of, among others, aromatase and inhibin α-subunit genes (22–25). Likewise, LH receptor is coupled to cAMP generation and CREB phosphorylation (21), although during the preovulatory surge of this hormone, both aromatase and inhibin α-subunit expression are down-regulated (26, 27). In response to LH, the cAMP-inducible repressor isoform of cAMP-responsive element

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Abbreviations: CREB, cAMP-responsive element binding protein; CREM, cAMP-responsive element binding modulator; ICER, inducible repressor isoform of CREM; $P450_{AROM}$, P450 aromatase; PI, propidium iodine; PMSG, pregnant mare serum gonadotropin; SMase, sphingomyelinase.

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binding modulator (CREM) (ICER) (28) is expressed in the rat ovarian granulosa cells, where it possibly participates in the mechanism underlying the LH-triggered down-regulation of aromatase and α-inhibin genes (29–31).

TNFα effects initiate after the interaction with two 55- and 75-kDa receptors that are coexpressed in virtually all cell types (32). The sequence of events that immediately follow TNFα binding to its receptor is not completely understood but includes, in some instances, the activation of MAPKs (33–35).

Although CREB is the best characterized substrate of PKA (36, 37), CREB Ser133 phosphorylation may also be performed by Ca^{2+}/calmodulin-dependent protein kinases and MAPKs (38, 39). That TNFα could induce CREB phosphorylation and ICER expression in granulosa cells seems reasonable in the light of the above mentioned observations and will possibly contribute to understanding the mechanism underlying TNFα effects on FSH-induced expression of $P450_{AROM}$ and inhibin α-subunit.

To test this hypothesis, granulosa cells were stimulated with TNFα or with its intracellular messenger ceramide, and CREB phosphorylation and ICER expression were assessed and shown to be induced by the cytokine. Both ICER expression and the inhibitory effects of TNFα on FSH-induced increase in aromatase and inhibin α-subunit mRNA levels were shown to depend on p44/42 MAPK activation, because TNFα effects were counteracted by PD098059 (40), the specific inhibitor of the dual kinase.

Materials and Methods

Reagents and hormones

All culture media and Trizol were from Invitrogen (San Diego, CA). Recombinant human FSH was obtained from Serono Laboratories (Rockland, MA). TNFα, bacterial sphingomyelinase (SMase; from *Staphylococcus aureus*), C6-cer, H89, GF109203X, SB203580, PD098059, and annexin V fluorescein isothiocyanate/propidium iodine (PI) apoptosis detection system were purchased from Calbiochem (Barcelona, Spain). [^{35}S]Methionine and [^{35}S]cysteine (Translabel) were from ICN (Costa Mesa, CA). Antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA) (anti-CREM and anti-CREB) or Cell Signaling Technology-New England Biolabs (Beverly, MA) (anti-pCREB, anti-p42 MAPK, and anti-p-p44/42 MAPK). Avian myeloblastosis virus reverse transcriptase (M519), RNAsin ribonuclease inhibitor (N211), and *Taq* DNA polymerase were from Promega (Madison WI). Diethylstylbestrol, pregnant mare serum gonadotropin (PMSG), protease, and phosphatase inhibitors and all other reagents were from Sigma Chemical Co. (Madrid, Spain).

Animals and cell culture

Immature 19- to 21-d-old female Sprague Dawley rats were purchased from Charles River Laboratories (Barcelona, Spain) and kept on 12-h light, 12-h dark cycles with free access to food and water. Both housing conditions and experimental procedures were approved by the University of Las Palmas Committee on Animal Care that enforces European Union rule 86/609. Granulosa cells were obtained by follicle puncture from the ovaries of rats implanted with diethylstilbestrol for 3 d or with a single injection of PMSG (10 IU) and cultured for the time periods indicated for each experiment in McCoy's 5a medium (modified, without serum).

Immunoprecipitation and Western blot analysis

To measure the cellular content of ICER protein, granulosa cells were incubated for 6 h in methionine- and cysteine-free McCoy's 5a modified medium, with 250 μCi/ml [^{35}S]methionine and [^{35}S]cysteine. The cells were thereafter washed in PBS and incubated with TNFα (10 ng/ml), SMase (1 U/ml), or C6-cer (1 μM) for the times indicated in McCoy's 5a medium supplemented with 4 mM methionine and 4 mM cysteine. To terminate the experiment, the cells were washed twice in ice-cold PBS and lysed for 30 min in 200 μl PBS containing 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 0.2% NaN_3, and a mix of protease and phosphatase inhibitors (1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 15 mM iodoacetamide, and 5 μg/ml aprotinin). After centrifugation at 14,000 × *g* for 15 min, aliquots from the supernatant containing the same amount of [^{35}S]methionine- and [^{35}S]cysteine-labeled proteins (1–2 mg) were immunoprecipitated overnight at 4 C with anti-CREM antibody preabsorbed to protein A-Sepharose and used at twice the manufacturer's recommendations to circumvent the low specificity for the ICER isoform. The labeled immunocomplexes were collected and subjected to SDS-PAGE. The dried gels were thereafter recorded as autoradiographs by photostimulatable-storage imaging followed by laser scanning in a Molecular Dynamics 400A PhosphoImager (Molecular Dynamics, Sunnyvale, CA).

Cell lysates were prepared with 1% Triton X-100, 10% glycerol in a buffer containing 150 mM NaCl, 1.5 mM $MgCl_2$, 5 mM EDTA, 50 mM HEPES, and 1 mM phenylmethylsulfonyl fluoride. Proteins were resolved using 12% SDS-PAGE, transferred onto nitrocellulose membranes, and immunoblotted with the appropriate specific antibody. The membranes were thereafter developed with a peroxidase-conjugated goat antirabbit second antibody and the ELC Western blot analysis kit (Amersham Biosciences Europe GmbH, Barcelona, Spain). To serve as controls of protein loading, membranes were routinely stripped out of the antibodies using Restore Western Blot (Pierce Biotechnology, Rockford, IL) and reprobed with a new antibody against the nonphosphorylated forms of CREB and p42MAPK, at twice the concentration recommended by the manufacturer. Recording and visualizing were performed in an imaging system with cooled CCD capture system for chemiluminescence (Diana III from Raytest, Straubenhardt, Germany) using the software provided by the manufacturer (AIDA, 1D, 2D).

PCR

Total RNA was extracted from triplicate granulosa cell cultures for each treatment using a modified guanidinium isothiocyanate method, and samples were stored frozen (−70 C) in diethylpyrocarbamate-treated water until use. After drying and OD determination, RT was performed by standard protocols. Briefly, equal amounts of RNA (1 μg) were incubated for 75 min at 42 C in 20 μl (final volume) of 1× PCR buffer (10 mM Tris-HCl, 50 mM KCl, 5 mM $MgCl_2$, and 0.1% Triton X-100, pH 9), 500 ng polydeoxythymidine primers, 1 mM dNTP, 5 U avian myeloblastosis virus reverse transcriptase, and 20 U RNAsin ribonuclease inhibitor. Each cDNA was amplified using specific oligonucleotides in 25 μl of 1× PCR buffer containing (final concentrations) 2.5 μM digoxigenin-dUTP, 0.625 U *Taq* DNA polymerase, and 125 ng (10–15 pmol each) of the appropriate specific primers for $P450_{AROM}$, AROM-5′-TGCACAGGCTCGAGTATTTCC and AROM-3′-ATTTCCACAATGGG GCTGTCC; α-inhibin, INHIB-5′-GAGGATGTCTCCCAGGCCAT and INHIB-3′-CAGGTCT ATTCTGTGGA; or ICER, ICER-5′-ACTTAGGATCCACTGTGTACGGCCAAC and ICER-3′-GTTAAATAGAATTCACTAATCTGTTTTGGG. PCR products were detected by chemiluminescence, and because of the method's high sensitivity, the thermal profile had to be adjusted as follows: 94 C for 1 min for denaturation and 1.5 min at 58 C and 72 C for annealing and elongation, respectively. Under these conditions, nonspecific background signals were reduced to undetectable levels and linearity ensured for up to 20 cycles for aromatase and α-inhibin, 29 cycles for ICER, and 16 cycles for the ribosomal protein L19 that was used as internal amplification control. The amplified products were resolved by 1.8% agarose gel electrophoresis and transferred to positively charged nylon membranes. Detection was performed with a commercially available digoxigenin luminescent detection kit (Roche Diagnostic SL, Barcelona, Spain) following the instructions of the manufacturer. The same system as in Western blot was used to visualize membranes.

Analysis of apoptosis by flow cytometry

Granulosa cells (1×10^6 per tube) were obtained from the ovaries of immature rats that were injected 2 d before the experiment with 10 IU

PMSG to induce aromatase expression and prevent apoptosis. The cells were incubated in McCoy's 5a medium (modified, without serum), supplemented with FSH (0.002 IU/ml) and testosterone (10 ng/ml) to ensure the continuity of aromatase activation and provide aromatase substrate, respectively. The experiments were carried out for 6 h, and thereafter the cells were washed twice at 1000 × *g* with cold PBS and resuspended in 0.5 ml cold binding buffer. Annexin V-fluorescein isothiocyanate and PI were then added following the instructions provided by the manufacturer. The tubes were kept in the dark, and cytometric analysis (Epics XL; Coulter, Hialeah, FL) was conducted right after the end of incubations.

Results

TNFα inhibits P450$_{AROM}$ and inhibin α-subunit mRNA levels

We and others have previously reported that TNFα decreases the FSH-induced activity of P450$_{AROM}$ and the secretion of the inhibin α-β dimer (15, 41). Although the changes in the catalytic activity of P450$_{AROM}$ and the rate of secretion of inhibin A should correlate with changes in the transcriptional rate of the enzyme and the inhibin α-subunit, we first wanted to test whether TNFα and its intracellular messenger ceramide did in fact inhibit FSH-induced increases in P450$_{AROM}$ and inhibin α-subunit mRNA. For that purpose, granulosa cells were cultured with FSH for 48 h, and TNFα (10 ng/ml), SMase (1 U/ml), or C6-cer (1 μM) was added 6 h before the completion of the experiment. As shown in Fig. 1, the cotreatment of granulosa cell cultures with TNFα, as well as with bacterial SMase that mobilizes ceramide or with the membrane-permeable analog of ceramide C6-cer, inhibits the FSH-induced increases in P450$_{AROM}$ and inhibin α-subunit mRNA levels. As can also be seen in Fig. 1, addition of cycloheximide counteracts TNFα and its intracellular messenger action, indicating that the effect is protein synthesis dependent.

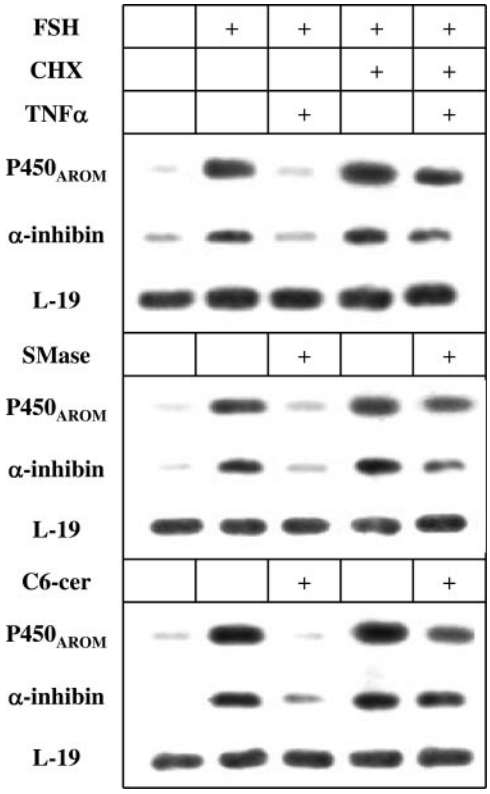

FIG. 1. TNFα inhibits P450$_{AROM}$ and α-inhibin subunit expression in granulosa cells. Granulosa cells (10^6 viable cells per well) were cultured with or without FSH (0.002 IU/ml) for 48 h in McCoy's 5a medium. TNFα (10 ng/ml) (A), SMase (1 U/ml) (B), or C6-cer (1 μM) (C) were added 6 h before the completion of the experiment. Cycloheximide (CHX) (1 μM) was also added 3 h before the end of the experiment where indicated. P450$_{AROM}$, α-inhibin subunit, and microsomal protein L-19 mRNA levels were assessed by RT-PCR as described in *Materials and Methods*. Triplicate dishes were used in each experiment, and the results of one of at least three different experiments are shown.

TNFα, SMase, and ceramide induce ICER expression

To test whether the protein synthesis-dependent effects of TNFα on P450$_{AROM}$ and inhibin α-subunit mRNA levels might be attributed to the repressor isoform of CREM (ICER), ICER mRNA and protein levels were assessed after 3, 6, and 12 h of treatment of granulosa cells with the cytokine or its intracellular messenger. Increases in ICER mRNA as determined by RT-PCR with the ICER gene-specific primers were found at 3 and 6 h after TNFα (Fig. 2A), SMase (Fig. 2B), or C6-cer (Fig. 2C) addition to cultured granulosa cells. After 12 h of treatment, ICER mRNA had already returned to basal, almost undetectable levels. ICER protein levels were also measured in extracts of granulosa cells labeled with [^{35}S]methionine after immunoprecipitation with an anti-CREM antibody. A band with an apparent molecular mass of 14 Kda, presumably corresponding to ICER-II, was observed to increase at all the times tested, suggesting that in fact ICER is being synthesized in response to TNFα, ceramide mobilization, and the ceramide analog (bottom of Fig. 2, A–C).

TNFα and ceramide induce CREB phosphorylation by a P44/42 MAPK-dependent mechanism

Because of the presence of four CRE-like sequences in a second CREM gene intronic promoter that directs ICER transcription, ICER is a unique, cAMP-inducible member of the CREM family (28). Therefore, we next investigated whether the treatment of granulosa cells with TNFα, SMase, or C6-cer resulted in the phosphorylation of CREB. Figure 3A shows that, as expected, CREB phosphorylation occurs in granulosa cells 15–30 min after the treatment with TNFα, SMase, or C6-cer, thus providing the grounds to explain the increasing levels of ICER mRNA in response to the cytokine. Because no coupling to cAMP production has been attributed to any TNF receptor, we next used a set of different kinase inhibitors to explore the nature of the kinase involved in the observed phosphorylation of CREB. Figure 3B shows that the inhibitors of PKA (H89) and PKC (GF109203X) did not affect TNFα-induced CREB phosphorylation. SB203580 (43), an antagonist of p38 MAPK slightly decreased TNFα effects, whereas PD098059 (40), a specific inhibitor of p44/42 MAPKs, almost completely blocked the phosphorylation of CREB in response to TNFα, suggesting that the effect of TNFα on CREB phosphorylation is mediated by this kinase.

To prove that in fact TNFα activates p44/42 MAPK in granulosa cells, triplicate cultures were stimulated with the

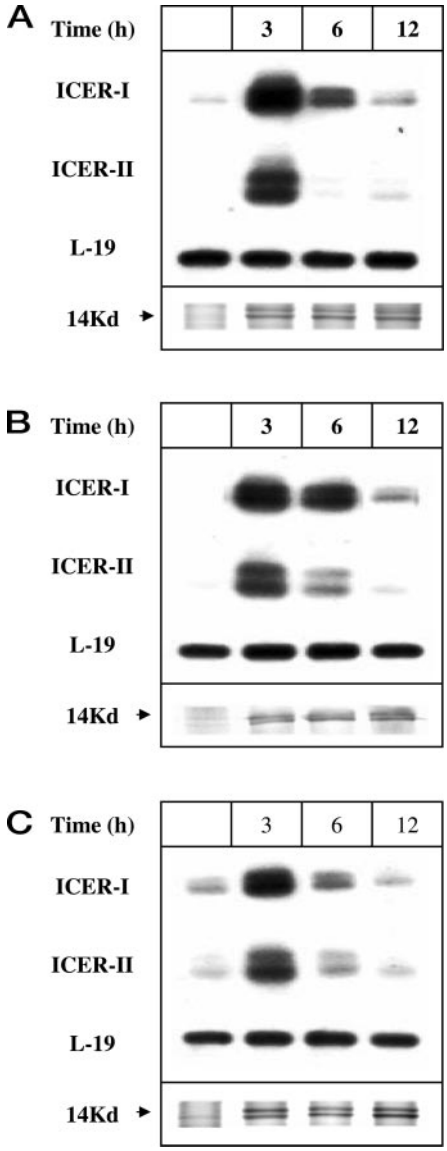

FIG. 2. TNFα increases ICER mRNA and protein levels in granulosa cells. Triplicate cultures of granulosa cells (10^6 viable cells per well) were treated with TNFα (10 ng/ml) (A), SMase (1 U/ml) (B), or C6-cer (1 μM) (C) for the times indicated in the figure. ICER mRNA levels were assessed by RT-PCR as described in *Materials and Methods*. ICER protein was determined after immunoprecipitation of protein extracts prepared from granulosa cells labeled with [^{35}S]methionine. Similar results were obtained in three other experiments.

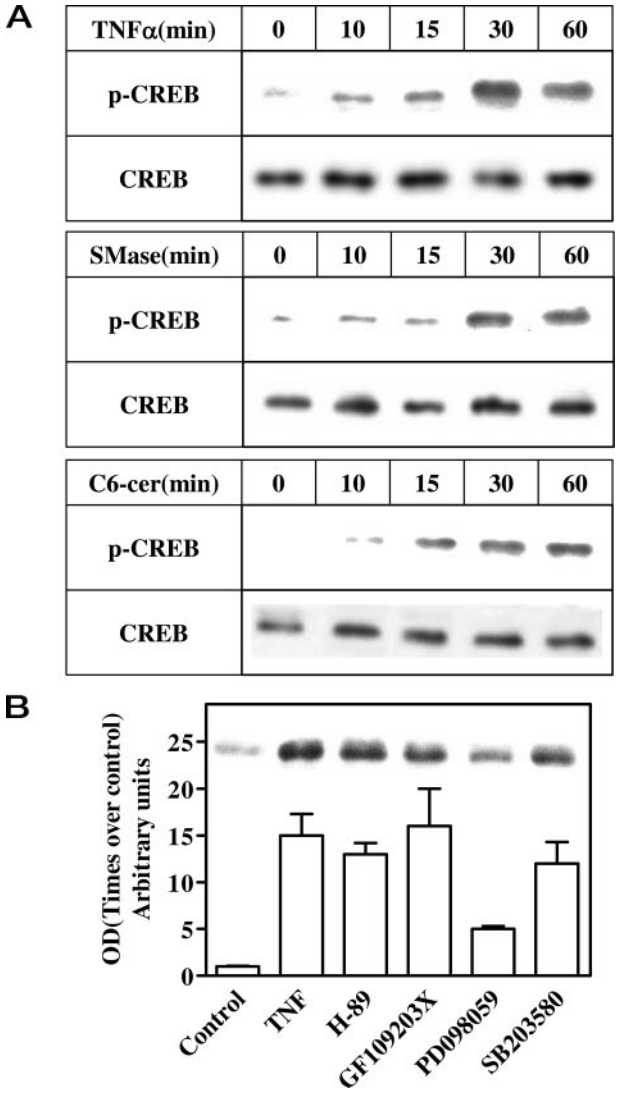

FIG. 3. TNFα promotes CREB phosphorylation in granulosa cells. Granulosa cells were treated with TNFα (10 ng/ml), SMase (1 U/ml), or C6-cer (1 μM) for the times indicated (A) or for 30 min (B) with TNFα (10 ng/ml) alone or in combination with a pretreatment (60 min) with H89 (10 μM), GF109203X (1 μM), SB20203580 (20 μM), or PD098059 (10 μM). Phosphorylated levels of CREB, as well as unphosphorylated CREB used as total protein control, were assessed by immunoblot as described in *Materials and Methods*. Densitometric analysis of four experiments (mean ± SEM) was performed, and a representative immunoblot is shown.

cytokine, bacterial SMase, or C6-cer, and p44/42 MAPK activation was assessed using immunoblot with an antibody that specifically recognizes dually phosphorylated ERK1 and ERK2. Figure 4 shows that TNFα (10 ng/ml) induces a rapid (5-min) phosphorylation of both kinases that reaches its maximum at 30 min after the treatment with the cytokine. Likewise, SMase (1 U/ml) and C6-cer (1 μM) activate both MAPKs during the first 30 min after they are added to the cells. Despite the discrete effect of SB203580 on TNFα-induced CREB phosphorylation, neither TNFα nor SMase or C6-cer were able to induce the activation of p38 MAPK (data not shown).

TNFα-induced ICER expression and P450$_{AROM}$ inhibition depends on p44/42 MAPK activation

To add support to the role of ERK1/ERK2 as mediators of TNFα effects, ICER, P450$_{AROM}$, and inhibin α-subunit mRNA levels were assessed in granulosa cells pretreated for 48 h with FSH (0.002 IU/ml) and cotreated for the last 6 h of the experiment with TNFα (10 ng/ml) alone or with the different kinase inhibitors (added 1 h before TNFα). Neither PKA nor PKC or p38 MAPK inhibitors prevented TNFα-induced ICER expression (Fig. 5A). However, PD098059, the well known specific inhibitor of p44/42 MAPK, completely abolished the increases in ICER mRNA levels induced by TNFα. Likewise, PKA, PKC, or p38 MAPK inhibitors did not affect TNFα

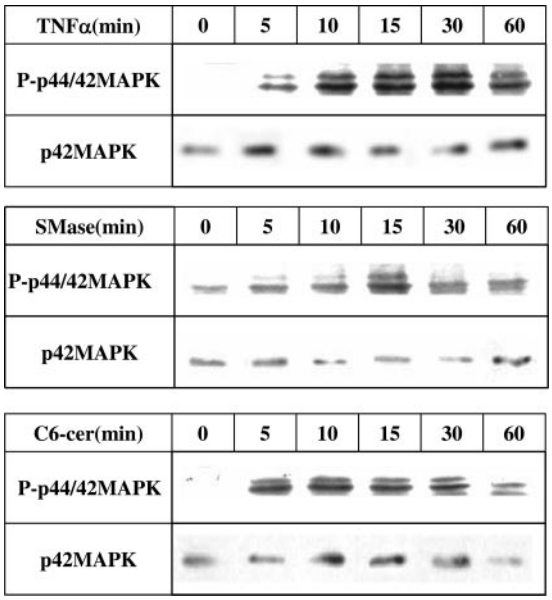

FIG. 4. TNFα, SMase, and C6-cer activate p44/42 MAPK. Granulosa cells were treated with TNFα (10 ng/ml), SMase (1 U/ml), or C6-cer (1 μM) for the times indicated. Activation (phosphorylation) of p44/42 MAPK as well as total p42 MAPK were assessed by immunoblot with an antibody against p-Thr and p-Tyr sites and anti p42 MAPK, respectively. One of three other experiments is shown.

effects on $P450_{AROM}$ and inhibin α-subunit mRNA levels, whereas the expression of both genes was restored to the levels of control, TNFα-untreated cells, when the MAP dual kinase was inhibited, indicating the involvement of ERK1 and ERK2 in the TNFα effect.

Effects of p44/42 MAPK inhibitors on TNFα-induced apoptosis

To prove a MAPK role on TNFα-induced apoptosis in granulose cells, flow cytometric analyses were performed with double-labeled (PI/annexin V) cells. Annexin V binds specifically to phosphatidylserine, which in live cells is located in the inner part of the cellular membrane. However, in the early phases of apoptosis, phosphatidylserine is exposed to the exterior, thus binding annexin V whose fluorescent signal (Fig. 6, *lower right quadrants*) provides a measure of the percentage of a cell population entering apoptosis. Live cells are also impermeable to PI, although in the late stages of apoptosis, cell membrane disruption allows for PI to enter the cell and bind DNA. The signal generated (Fig. 6, *upper right quadrants*) serves as an indicator of cells in the latter stages of apoptosis. As may be observed in Fig. 6, at time 0 of incubation, most (~99.7%) of the granulosa cells are live (*lower right quadrant*), whereas at the end of the experiment, a small number (~15%) of the cells undergoes spontaneous apoptosis, despite the presence of both FSH and testosterone.

After 6 h of incubation with TNFα, more than 60% of the cells are entering apoptosis and an additional 23% are at the terminal phase of the apoptotic process. The addition of PD098059 reduced to 34 and 12% the number of cells experiencing early and late apoptosis, respectively, thus demonstrating that p44/42 MAPK is part of the mechanism implicated in TNFα induction of apoptosis in granulosa cells.

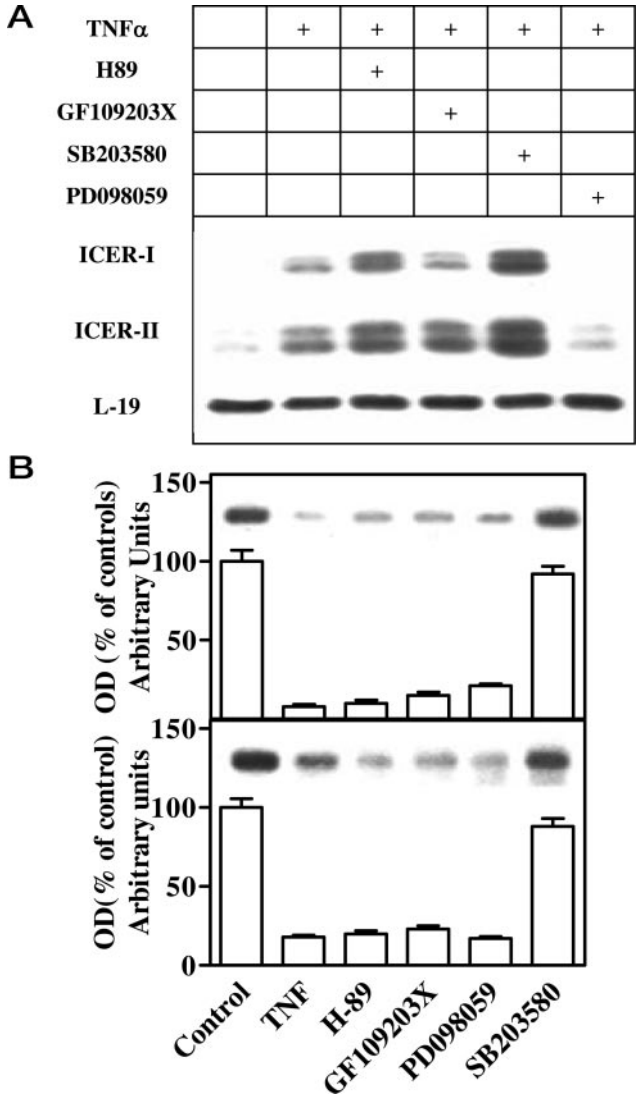

FIG. 5. TNFα induction of ICER expression and aromatase and inhibin α-subunit down-regulation depends on p44/42 MAPK activation. Granulosa cells were cultured 48 h with FSH (0.002 IU/ml) alone or with TNFα (10 ng/ml) that was added 6 h before the end of the experiment. H-89 (10 μM), GF109203X (1 μM), SB20203580 (20 μM), or PD098059 (10 μM) were added 60 min before TNFα. Levels of ICER (A), $P450_{AROM}$ (B, *bottom*) and inhibin α-subunit (B, *top*) mRNA were determined by RT-PCR. Densitometric analysis (mean ± SEM of OD arbitrary units is represented) was performed on the results of four different aromatase and inhibin α-subunit experiments. A representative RT-PCR is shown.

Discussion

We herein present experimental data that shows for the first time the induction of ICER expression in granulosa cells by TNFα and its intracellular messenger ceramide. To our knowledge, this is also the first time ICER induction in response to this cytokine has been reported in any cell or tissue.

We have also observed that in granulosa cells, phosphorylation of CREB in response to the cytokine and its messenger is achieved by a MAPK-dependent mechanism. Although

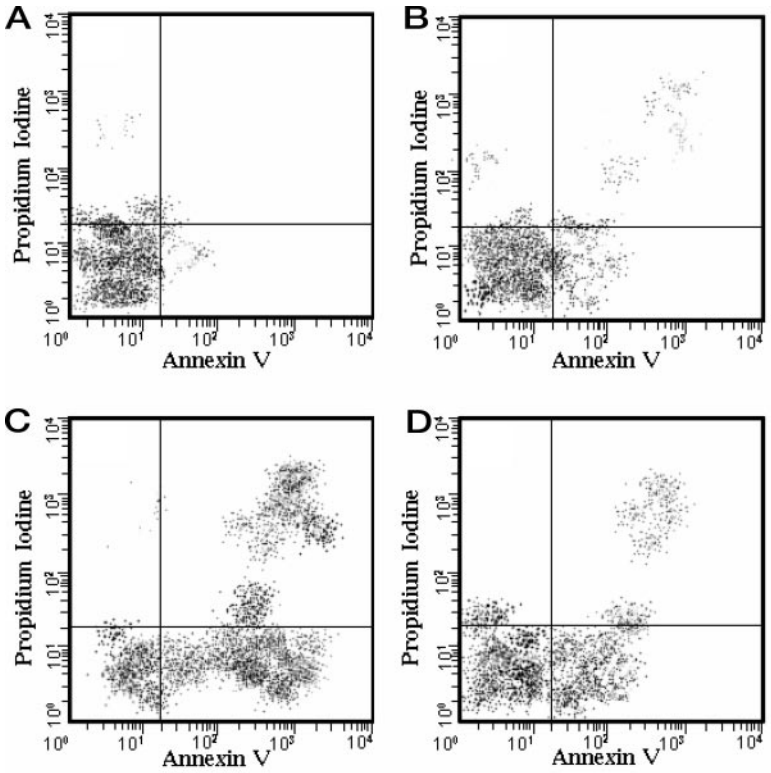

FIG. 6. Effects of p44/42 MAPK inhibitors on TNFα-induced apoptosis in granulosa cells. Granulosa cells were obtained by follicular puncture from the ovaries of immature rats that had been injected 2 d before with 10 IU PMSG. Cells were incubated in 6-ml Falcon culture tubes, washed, labeled with PI and annexin V as described in *Materials and Methods*, and counted immediately after the incubations. A, Control at time 0 of incubation; B, control at the end of the experiment; C, TNFα (10 ng/ml); D, cells pretreated for 60 min with PD098059 (10 μM) plus 6 h with TNF. The experiment was replicated twice, each using triplicate tubes.

CREB phosphorylation at the Ser133 residue (44) is preferentially performed by PKA (36, 37), the phosphorylation of CREB by Ca^{2+}/calmodulin-dependent protein kinases and MAPKs has been also extensively documented (38, 39). Thus, p44/42-MAPK- or p38-MAPK-dependent CREB phosphorylation has been shown *in vitro* and *in vivo* in several cell types (45–47) and is supposed to be achieved both through independent (46) or confluent pathways, these latter involving the phosphorylation of mitogen- and stress-activated kinases, which serve as substrates for p44/42 and p38 MAPK (47).

A p38-MAPK-dependent increase in the amount of phosphorylated CREB, after TNFα, SMase, or ceramide treatment has been reported in MC/9 and U937 myeloid cells (48). Our results in granulosa cells point to p44/42 as the kinase involved in TNFα effects because both CREB phosphorylation in response to TNFα and TNFα-induced transcription of ICER can be blocked by PD098059, a highly specific inhibitor of this kinase (40). Despite the fact that c-Jun N-terminal kinase, p38 MAPK, and other stress-activated kinases, were initially thought to be the only MAPK family members activated by cytokines (49), we have shown here, and others elsewhere, that TNFα can phosphorylate ERK1/ERK2 (33, 34, 50). Likewise, phosphorylation of the dual kinase has been reported in response to ceramide in cultured astrocytes (51).

To act as a key regulator of cAMP-dependent genes that are expressed in a rhythmic pattern was initially thought to be ICER's role (52), as a result of two distinctive features: 1) ICER is a repressor of cAMP-driven transcription because it competes with members of the bZIP superfamily of proteins that bind the CRE 8-bp palindromic sequence, and 2) four CRE-like elements in the CREM intronic promoter that directs ICER transcription enable ICER to down-regulate its own expression (28).

However, many other functions have been latter attributed to ICER, including regulation of insulin secretion (53), hormone receptor desensitization (54, 55), bone remodeling (56), and influencing T-cell and macrophage function (57, 58) and virus replication (59). ICER has also been proposed to be involved in the control of cellular proliferation (60, 61) and apoptosis in a limited number of cells and/or tissues (62–64).

Granulosa cells apoptosis is known to be determinant in ovarian follicular atresia (65), a process that is likely to be controlled by as many regulatory molecules as follicular growth (66). Because of the fact that decreases in $P450_{AROM}$ levels have been shown to be tightly associated with apoptosis in granulosa cells of atretic follicles and the death of these cells is largely prevented by treatment with estrogens (67, 68), estrogens have been proposed to be important promoters of granulosa cell survival. Thus, it is more than possible that ICER would be mediating TNFα effects on granulosa cell apoptosis by inhibiting FSH-induced $P450_{AROM}$ expression.

Other and more direct contributions to the activation of the apoptotic machinery by ICER could be subserving the TNFα proapoptotic effects in granulosa cells. Both in neurons and cardiomyocytes, the ICER proapoptotic role seems to be performed by suppressing cAMP-dependent expression of bcl-2 (63, 64). In the ovary, the ablation of bcl-2 results in a diminished and abnormal number of follicles, whereas the overexpression of this antiapoptotic gene leads to decreased ovarian somatic cell apoptosis and enhanced folliculogenesis (69, 70). These findings would suggest that granulosa cell

survival and the rescue of follicles from atresia will be favored by an augmented bcl-2 expression. However, no increases in bcl-2 expression have been encountered in granulosa cells in response to any of the antiapoptotic regulatory molecules, including the major survival factor for these cells, the gonadotropic hormone FSH, whose receptor is coupled to cAMP generation (66). Therefore, it seems unlikely that increases in bcl-2 expression would play a prominent role in salvaging granulosa cells from apoptosis.

Nevertheless, it has been reported that TNFα induction of granulosa cell apoptosis could by partly a result of the suppression of constitutive bcl-2 expression (42); thus, the possibility exists, and may be deserving of future studies, that in the ovary, as in neurons and cardiomyocytes, the ICER synthesized in response to TNFα could be repressing constitutive bcl-2 expression.

In conclusion, although it is more than likely that several other pathways would be contributing to the signaling of TNFα in granulosa cells, CREB phosphorylation by p44/42 MAPK followed by ICER induction and the repression of FSH-driven aromatase transcription plays a significant role in TNFα induction of apoptosis in these cells.

Acknowledgments

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