Endocrine, *vol. 27, no. 1, 17–24, June 2005* 0969–711X/05/27:17–24/$30.00 © 2005 by Humana Press Inc. All rights of any nature whatsoever reserved.

Tumor Necrosis Factor Alpha (TNF) Suppresses cAMP Response Element (CRE) Activity and Nuclear CRE Binding Protein in MA-10 Mouse Leydig Tumor Cells

Koji Y. Arai,[1] Katherine F. Roby,[2] and Paul F. Terranova[1,3]

Departments of [1]Molecular and Integrative Physiology, [2]Anatomy and Cell Biology, and [3]Obstetrics and Gynecology, Center for Reproductive Sciences, University of Kansas Medical Center, 3901 Rainbow Boulevard, Kansas City, Kansas 66160

TNF is known to suppress gonadotropin-induced steroid secretion by Leydig cells. However, the mechanisms by which this occurs are largely unknown. Because expression of many steroidogenic proteins is regulated by the PKA pathway, effects of TNF on CRE activity were examined using MA-10 mouse Leydig tumor cells. The cells were transfected with a CRE-luciferase construct, and stimulated with either LH or 8Br-cAMP in the presence or absence of TNF. TNF suppressed, LH-stimulated and 8Br-cAMP stimulated CRE activity. TNF also suppressed CRE activity stimulated with a PKA expression vector. Further experiments suggested that the effect of TNF on CRE activity was not mediated by the NF-κB pathway. TNF did not affect levels of either CREB or phospho-CREB in whole cell lysates; however, TNF decreased both CREB and phospho-CREB in nuclear extracts in a time-dependent manner. The decrease in nuclear CREB is likely to be a major mechanism of the suppressive effects of TNF on steroidogenesis in MA-10 Leydig cells.

Key Words: TNF; CREB; steroidogenesis; Leydig cell.

Introduction

Tumor necrosis factor alpha (TNF), a multifunctional cytokine, is principally secreted from monocytes and macrophages activated by agents such as lipopolysaccaride (LPS) *(1)*. Testicular interstitial macrophages and Leydig cells are two major cell types in the interstitium. Numerous studies have revealed that TNF suppresses gonadotropin-induced steroid synthesis by Leydig cells (*2–7*; for review, see ref. *2*). Thus, it is hypothesized that TNF derived from testicular macrophages plays a role in regulating Leydig cell steroidogenesis through a paracrine fashion. Although TNF contents in the testicular interstitial fluid are likely to be very low under the physiological condition *(2)*, LPS stimulates TNF secretion from testicular macrophages *(3,4)*, suggesting that the interstitial macrophages secrete TNF during bacterial infection. In patients with critical illness, burns, and sepsis, systemic levels of TNF elevated to the low nanograms per milliliter have been observed *(5–7)*, and those patients have shown depressed gonadal function *(8,9)*.

Many steroidogenic proteins are regulated by protein kinase A (PKA). Luteinizing hormone (LH) increases intracellular cAMP levels by activating adenylyl cyclase, then the catalytic subunit of PKA (PKA_C) is released from the regulatory subunit. PKA_C phosphorylates cAMP response element binding protein (CREB), which facilitates association of CREB with coactivators such as CREB-binding protein (CBP) and increases transcriptional activity of cAMP response element (CRE)–containing promoters.

Two major receptors for TNF are type I (TNFRI) and type II (TNFRII) *(10,11)*. Binding of TNF to TNFRI activates several pathways in the cell. Nuclear factor-κB (NF-κB), a TNF-induced transcription factor, is known to mediate TNFRI signaling *(12)*. NF-κB is composed of homo- or heterodimeric complexes of the Rel family [p50, p52, p65 (RelA), c-Rel, and RelB], which share a region of homology known as the Rel homology domain that mediates dimerization and DNA binding *(13)*. The best studied and most abundant of these complexes is the p50–p65 heterodimer. NF-κB activates transcription by binding to an NF-κB response element and interacting with the basal transcription machinery *(14,15)*. However, NF-κB represses transcription by competing for binding to coactivators, such as p300 and CBP *(16–20)*, which participate in steroidogenesis.

In the present study, we examined effects of TNF on CRE-reporter activity and protein levels of CREB and phospho-CREB in MA-10 mouse Leydig tumor cells. The data indicate that TNF suppresses CRE activity by reducing nuclear CREB.

Results

Effects of TNF on Steroidogenesis

The ability of TNF to inhibit Leydig cell steroidogenesis has been demonstrated by several investigators (*2–7*; for

Received February 17, 2005; Revised April 26, 2005; Accepted April 28, 2005.

Author to whom all correspondence and reprint requests should be addressed: Paul F. Terranova, Center for Reproductive Sciences, University of Kansas Medical Center, 3901 Rainbow Boulevard, Kansas City, KS 66160. E-mail: pterrano@kumc.edu

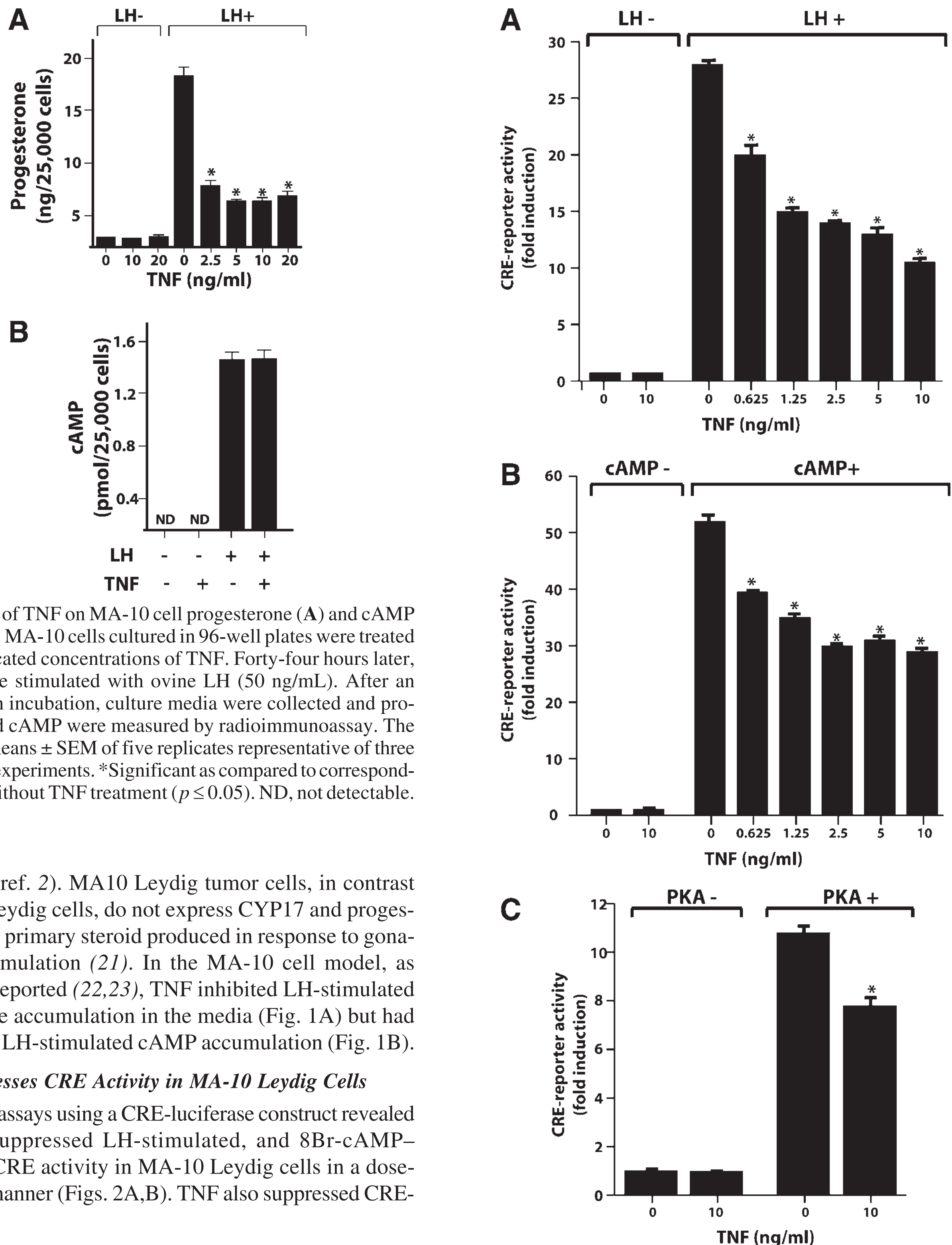

Fig. 1. Effect of TNF on MA-10 cell progesterone (**A**) and cAMP (**B**) secretion. MA-10 cells cultured in 96-well plates were treated with the indicated concentrations of TNF. Forty-four hours later, the cells were stimulated with ovine LH (50 ng/mL). After an additional 4 h incubation, culture media were collected and progesterone and cAMP were measured by radioimmunoassay. The data are the means ± SEM of five replicates representative of three independent experiments. *Significant as compared to corresponding control without TNF treatment ($p \leq 0.05$). ND, not detectable.

review, see ref. *2*). MA10 Leydig tumor cells, in contrast to normal Leydig cells, do not express CYP17 and progesterone is the primary steroid produced in response to gonadotropin stimulation *(21)*. In the MA-10 cell model, as previously reported *(22,23)*, TNF inhibited LH-stimulated progesterone accumulation in the media (Fig. 1A) but had no effect on LH-stimulated cAMP accumulation (Fig. 1B).

TNF Suppresses CRE Activity in MA-10 Leydig Cells

Reporter assays using a CRE-luciferase construct revealed that TNF suppressed LH-stimulated, and 8Br-cAMP–stimulated CRE activity in MA-10 Leydig cells in a dose-dependent manner (Figs. 2A,B). TNF also suppressed CRE-

Fig. 2. TNF suppresses CRE-reporter activity in MA-10 Leydig cells. **A** and **B**: MA-10 Leydig cells were transfected with CRE-reporter construct (pCRE-luc, 250 ng) and pRSV-β (100 ng) in 24-well culture plates. On the day after transfection, TNF was added to achieve the indicated concentrations of TNF. Two hours later, the cells were stimulated with either ovine LH (**A**, 10 ng/mL) or 8Br-cAMP (**B**, cAMP, 1 m*M*). The cells were incubated for an additional 4 h, and then harvested for luciferase assay. The data are the means ± SEM of triplicate transfections representative of three independent experiments. (**C**) The cells were transfected with pCRE-luc (250 ng), pRSV-β (100 ng), and 50 ng of either pRSV-CAT (control vector) or a hamster $PKA_C\alpha$ expression vector (RSV-CHO-PKA-Calpha V2). On the day after transfection, the cells were treated with TNF (10 ng/mL) for 6 h, and then harvested for luciferase assay. The data represents the mean ± SEM of three independent experiments. Luciferase levels were normalized with β-galactosidase levels. *Significant as compared to corresponding controls without TNF treatment ($p \leq 0.05$).

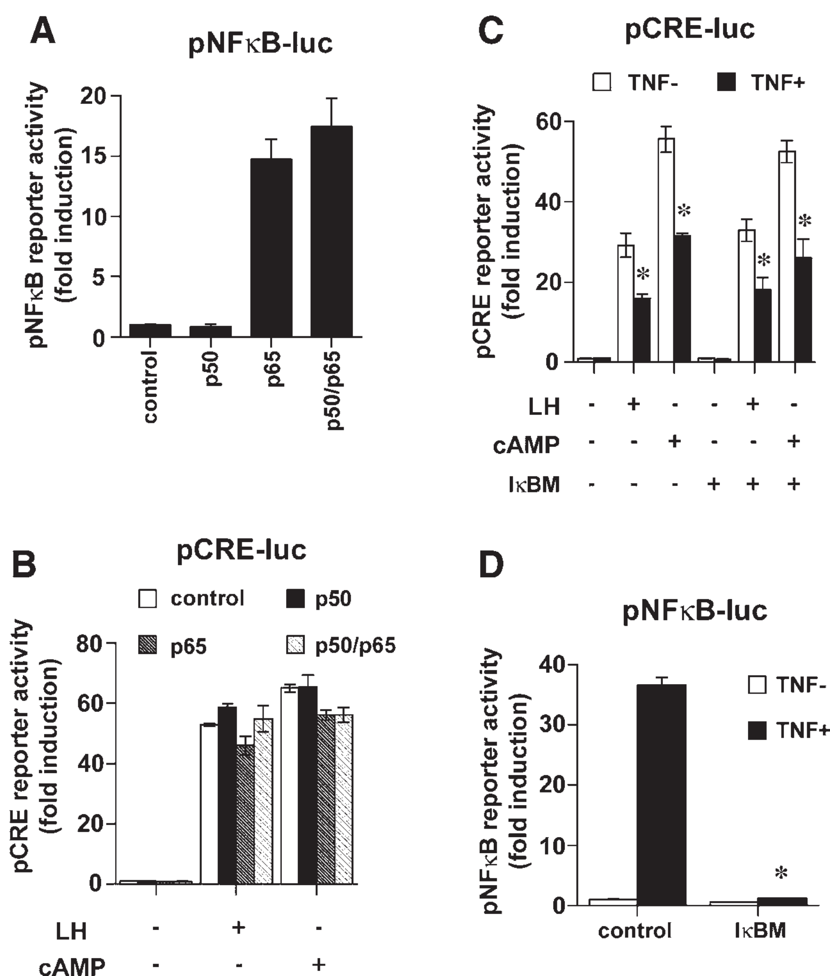

Fig. 3. NF-κB is not involved in the suppressive effect of TNF on CRE-reporter activity. (**A**) MA-10 cells cultured in 24-well plates were transfected with 250 ng of an NF-κB reporter construct (pNF-κB-luc), pRSV-β (100 ng), and 10 ng of p50 subunit expression vector (pcDNA1-p50) and/or the p65 subunit expression vector (pRc/RSV-p65). Twenty-four hours later, the cells were harvested for luciferase assay. (**B**) MA-10 cells were transfected with pCRE-luc (250 ng), pRSV-β (100 ng), and 10 ng of pcDNA1-p50 and/or pRc/RSV-p65. On the day after transfection, the cells were treated with either ovine LH (10 ng/mL) or 8Br-cAMP (cAMP, 1 m*M*) for 4 h, and then harvested for luciferase assay. In **A** and **B**, pcDNA3 and pRSV-CAT were used as control vectors for pcDNA1-p50 and pRc/RSV-p65, respectively. (**C**) MA-10 cells were transfected with pCRE-luc (250 ng), pRSV-β (100 ng), 50 ng of either an expression vector for IκBα with phosphorylation-resistant mutation (pCMV-IκBαM) or pRSV-CAT. On the day after transfection, the cells were incubated in the either presence or absence of TNF (10 ng/mL) for 2 h, and then stimulated with either ovine LH (10 ng/mL) or 8Br-cAMP (cAMP, 1 m*M*) for an additional 4 h, and then harvested for luciferase assay. (**D**) MA-10 cells were transfected with pNFκB-luc (250 ng) and pRSV-β (100 ng), and 50 ng of either pCMV-IκBαM or pRSV-CAT. On the day after transfection, the cells were treated with TNF for 6 h, and then harvested for luciferase assay. The data are the means ± SEM of triplicate transfections representative of three independent experiments. *Significant as compared to corresponding controls without TNF treatment ($p \leq 0.05$).

reporter activity stimulated with a $PKA_C\alpha$ expression vector (Fig. 2C). Thus, these results suggest that TNF modulates post-PKA site(s) in suppressing CRE activity.

Suppressive Effect of TNF on CRE Activity Is NF-κB Independent

Since activation of the NF-κB pathway represses many signaling pathways including the PKA pathway *(16–20)*, and because TNF signaling often utilizes NF-κB *(12)*, involvement of NF-κB on CRE activity was examined. Overexpression of the NF-κB p65 subunit with or without the p50 subunit strongly stimulated NF-κB responsive reporter activity (Fig. 3A). However, transfection of the MA-10 cells with p65 and/or p50 expression vectors did not suppress CRE reporter activity stimulated by either LH or 8Br-cAMP (Fig. 3B). In addition, overexpression of mutated IκBα (IκBM), which is not phosphorylated by IκB kinase and therefore inhibits activation of NF-κB, did not relieve TNF-induced suppression of CRE activity (Fig. 3C) although it suppressed stimulation of NF-κB responsive reporter activity by TNF (Fig. 3D). These results indicate that the suppressive effect of TNF on CRE activity is independent of NF-κB.

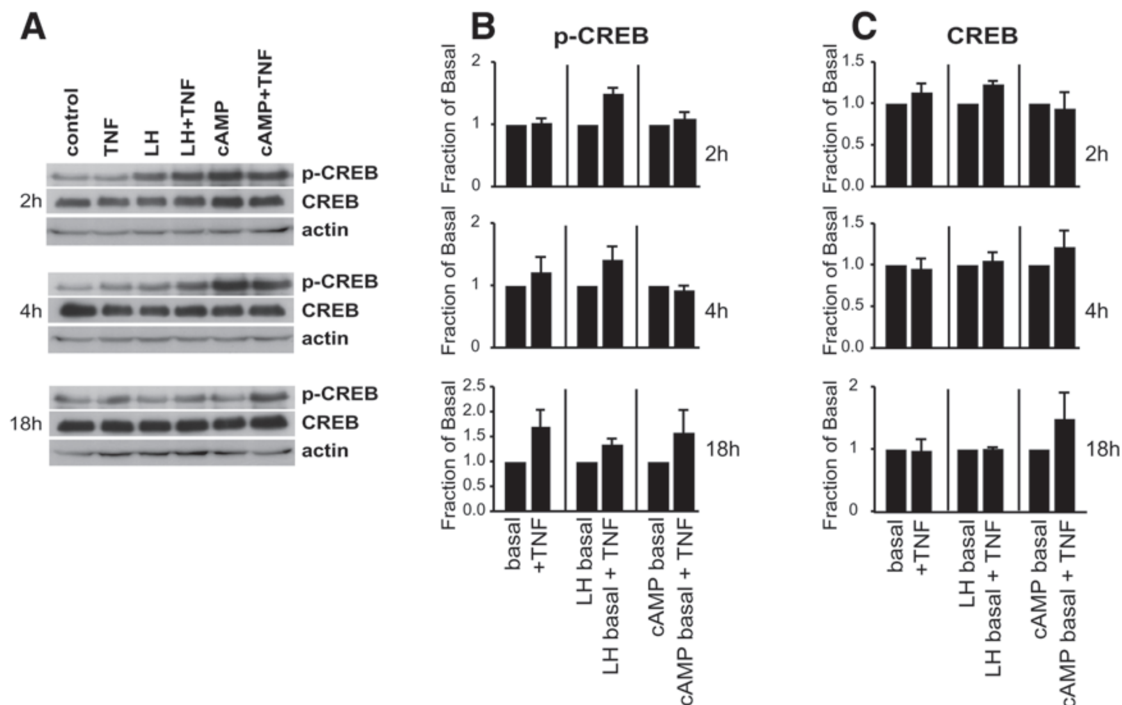

Fig. 4. TNF did not affect either CREB or phospho-CREB protein levels in whole cell lysates. MA-10 cells cultured in six-well plates were treated with TNF (10 ng/mL). Two hours later, the cells were stimulated with either ovine LH (50 ng/mL) or 8Br-cAMP (cAMP; 1 m*M*), and then harvested after 2, 4, and 18 h. Whole cell lysates containing 60 μg protein were subjected to immunoblot analysis for phospho-CREB (p-CREB), CREB, and actin proteins (**A**). Results for each time point were obtained from the same cell lysate. The data are representative of three independent experiments. (**B,C**) Immunoblots were analyzed by densitometry. Comparisons of basal vs TNF, LH vs LH+TNF, and 8Br-cAMP vs 8Br-cAMP+TNF are expressed as the fraction of basal. Data are results from comparisons of three independent experiments. *Significant as compared to corresponding basal treatment without TNF ($p \leq 0.05$).

Effects of TNF on CREB and Phospho-CREB Protein Expression in MA-10 Whole Cell Lysates

Treatment with LH or 8-bromo-cAMP (8Br-cAMP) increased phospho-CREB protein levels in whole cell lysates within 2 h (Fig. 4A). TNF did not change basal or stimulated phospho-CREB or CREB levels in whole cell lysates at 2, 4, and 18 h (Figs. 4A,B,C).

Effects of TNF on MA-10 Nuclear Levels of CREB and Phospho-CREB

TNF treatment alone suppressed levels of CREB and phospho-CREB in MA-10 nuclear extracts at 4 and 18 h but not at 2 h (Figs. 5A,B,C). LH or 8Br-cAMP treatment increased nuclear levels of CREB and phospho-CREB and in the presence of LH or 8Br-cAMP, TNF suppressed nuclear CREB and phospho-CREB (Figs. 5A,B,C). Because SF-1 is involved in regulation of many steroidogenic proteins, and because SF-1 is a nuclear transcription factor similar to CREB, effects of TNF on nuclear SF-1 levels were examined. TNF treatment suppressed basal and LH/8Br-cAMP–induced nuclear levels of SF-1 at 18 h; this effect was not detected at either 2 or 4 h (Figs. 5A,D).

Discussion

Testicular steroidogenesis is mainly regulated by pituitary gonadotropin. However, several studies suggest that inflammatory cytokines, such as TNF and interleukin-1, are also involved in the regulation of testicular steroidogenesis. TNF inhibits expression of StAR protein, P450c17, and P450scc in Leydig cells *(23–26)*. However, the mechanisms by which this occurs are largely unknown. In the present study, TNF clearly suppressed nuclear levels of CREB/phospho-CREB and CRE reporter activity stimulated with LH, 8Br-cAMP, or $PKA_C\alpha$ expression vector. The data imply that TNF-induced suppression of CRE activity via decreased nuclear phospho-CREB may be, at least in part, responsible for the suppression of Leydig cell steroidogenesis. The data also indicate that pre-cAMP sites are not likely to be involved in TNF-induced suppression of CRE activity since cAMP levels were unaffected by TNF.

TNF is known to exhibit cytotoxicity in some cell lines *(27)*. However, TNF did not show any effect on the viable cell number of MA-10 cells in either the absence or presence of PKA pathway stimulators (data not shown), suggesting that the effect of TNF on CRE-reporter activity was not due to cytotoxicity. In addition, NF-κB reporter activity was increased by TNF treatment using the same experimental protocol, again indicating TNF was not cytotoxic to the MA-10 cells and also imparting some specificity to the inhibitory effect of TNF treatment on the CRE. Leydig cells express TNF receptor I *(25,29–31)* and MA10 cells express both TNF receptor I and II *(22)*. Several studies

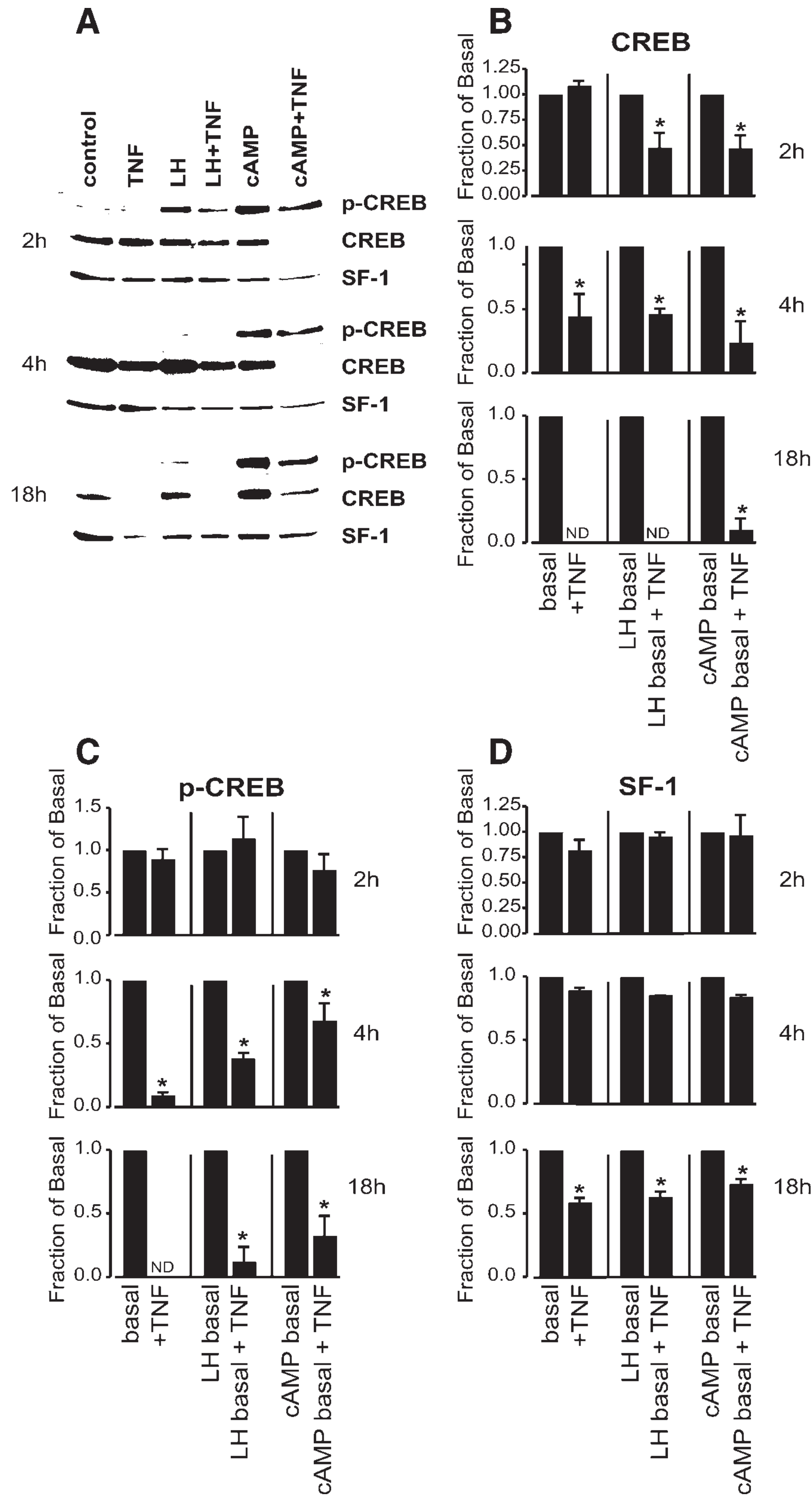

Fig. 5. TNF decreased CREB, phospho-CREB, and SF-1 levels in nuclear extracts. MA-10 cells cultured in six-well plates were treated with TNF (10 ng/mL). Two hours later, the cells were stimulated with either ovine LH (50 ng/mL) or 8Br-cAMP (cAMP, 1 m*M*), and then harvested after an additional 2, 4, and 18 h of incubation. (**A**) Nuclear extracts containing 20 μg protein were subjected to immunoblot analysis for phospho-CREB (p-CREB), then the same membranes were reprobed for CREB and SF-1. The data are representative of three independent experiments. (**B–D**) Relative changes in nuclear protein levels after TNF treatment as measured by densitometry. Comparisons of basal vs TNF, LH vs LH+TNF, and 8Br-cAMP vs 8Br-cAMP+TNF are expressed as the fraction of basal. Data are results from comparisons of three independent experiments. *Significant as compared to corresponding basal treatment without TNF ($p \leq 0.05$). ND, not detected.

have shown the effects of TNF on Leydig cell steroidogenesis is mediated through TNF receptor I *(22,25,30)*. Thus it is likely the effects of TNF on CRE are mediated through TNF receptor I.

CRE-reporter activity stimulated by either LH, 8Br-cAMP, or PKA expression vector was suppressed by TNF. Effects of TNF on CRE-regulated gene activity have been reported in other systems and effects have been shown to be both stimulatory and repressive *(32–34)*. In MA-10 cells TNF repressed CRE reporter activity. In a study using rat osteosarcoma cells TNF suppressed bone sialoprotein (BSP) gene expression in a manner that required an intact CRE *(33)*. In mouse juxtaglomerular cells, TNF inhibited renin gene expression through interaction with the CRE *(34)*. Interestingly, in that system, in contrast to the present findings in MA-10 cells, inhibitory effects of TNF mediated by interaction at the CRE also required NFκB *(34)*.

TNF is known to activate NF-κB, a transcription factor involved in numerous signaling pathways *(35)* and TNF suppresses several cell-signaling pathways including the PKA pathway, at least in part, through activation of NF-κB *(16, 17,19,20,35)*. In the present study, transfection of MA-10 cells with NF-κB subunit expression vectors did not repress CRE-reporter activity while the expression vectors noticeably stimulated NF-κB reporter activity. In addition, suppression of the NF-κB pathway by IκBαM overexpression did not affect TNF-induced repression of CRE-reporter activity. These data indicate that TNF-induced repression of CRE does not involve the NF-κB pathway. A recent report by Hong et al. *(36)* has investigated the role of TNF-mediated NF-κB signaling in testicular steroidogenic enzyme expression. In that study TNF treatment repressed StAR, P450c17, and 3β-HSD promoter–reporter constructs transfected into K28 mouse Leydig cells. Further experiments showed TNF-mediated suppression involved interactions between NF-κB, Nur77, and SF-1 *(36)*. The study by Hong et al. investigated entire promoters while our present studies assessed effects specifically at the CRE. Thus it appears that TNF-mediated inhibition of Leydig cell steroidogenesis occurs via multiple signaling pathways and interactions at several promoter elements.

Reduction of nuclear CREB and phospho-CREB by TNF was first observed at 4 h and then again at 18 h. In contrast nuclear SF-1 levels were reduced in response to TNF only at the 18 h time point. These data would indicate TNF-induced reduction in CREB may represent an early and rapid mechanism for reducing expression of CRE-containing genes. However, it is likely that the reduction of both nuclear CREB and SF-1 play a role in mediating the reduced steroid production in response to TNF.

Specific cellular mechanisms leading to decreased nuclear CREB and phospho-CREB in response to TNF are yet to be uncovered. Interestingly NF-κB, a major signaling mediator of TNF action, is not involved in the TNF-mediated change in nuclear CREB. A recent study reported TNF-repressed CRE-reporter activity correlated with decreased phosphorylation of CREB in pancreatic beta cells *(37)*. These studies further demonstrated the decreased phosphorylation of CREB resulted from decrease AKT activity *(37)*. Effects of TNF on Leydig cell AKT activity and the ability of AKT to phosphorylate CREB in Leydig cells has not been examined. The possibility that TNF-mediated reduction in nuclear CREB is mediated by altered AKT in Leydig cells will need to be addressed in future studies.

Alterations in nuclear CREB may also result from decreased import of CREB into the nucleus, rapid export of CREB out of the nucleus, or a rapid CREB/phospho-CREB degradation within the nucleus. Transport of proteins in and out of the nucleus is a highly regulated process mediated by a family of transporters known as importins. These proteins work in concert with the guanine nucleotide-binding protein, Ran, and regulatory proteins *(38,39)*. Nuclear export sequences for CREB have not been reported; however, the nuclear import of CREB mediated by importin-beta1 and Ran has been described *(40)*. Thus, future studies will need to address the hypothesis that transport of CREB into or out-of the nucleus in Leydig cells is altered in response to TNF.

In summary, the present study demonstrates that in MA-10 cells TNF suppresses CRE activity and this suppression is correlated with reduced nuclear levels of CREB and phospho-CREB. In addition, the suppressive effect of TNF on the CRE is not mediated by NF-κB.

Materials and Methods

Hormones and Chemicals

Recombinant murine TNF was purchased from R&D Systems Inc. (Minneapolis, MN). Ovine LH (NIH S-24) was provided by Dr. A. F. Parlow through the National Hormone and Peptide Program and NIDDK. 8Br-cAMP was purchased from Sigma (St. Louis, MO).

Plasmids

Luciferase reporter plasmids, pCRE-luc, pNFκB-luc, and an ubiquitin-dependent-degradation-resistant IκBα expression vector pCMV-IκBαM were purchased from BD Biosciences Clontech (Palo Alto, CA). Expression vectors for the p50 and p65 NF-κB subunits (pcDNA1-p50 and pRc/RSV-p65) *(41)* were provided by Dr. Tom Maniatis (Harvard University, Cambridge, MA). Hamster $PKA_C\alpha$ expression vector RSV-CHO-PKA-Calpha V2 *(42)* was provided by Dr. Keith L. Parker (University of Texas Southwestern Medical Center, Dallas, TX).

Cell Culture

MA-10 Leydig tumor cells were provided by Dr. Mario Ascoli, University of Iowa, College of Medicine (Iowa City, IA) and were handled as originally described *(21)* and as previously reported *(43)*. MA10 cells were routinely cultured in growth medium consisting of Dulbecco's modi-

fied Eagle's medium/F12, 1:1 containing 5% horse serum, 2.5% fetal bovine serum, and 15 m*M* Hepes. Cells were seeded onto tissue culture plates in growth medium and cultured until approx 70% confluence when the growth medium was removed and was replaced with serum-free medium (DMEM/F12, 15 m*M* Hepes). Following overnight incubation in serum-free medium treatments were added for times specified in each experiment.

Radioimmunoassay (RIA)

MA-10 cells were plated on 96-well plates at a density of 2.5×10^4 viable cells/well in the growing medium, and attached overnight. On the following day, medium was removed and was replaced with serum-free medium. After overnight incubation in serum-free media TNF was added to the final concentration desired and the cells were cultured for 48 h. The cells were treated with ovine LH for the last 4 h of culture. RIA for progesterone and cAMP was carried out as previously described *(44,45)*.

Transfection and Luciferase Assay

MA-10 cells were plated on 24-well culture plates and grown in growth medium. When the cells became approx 70% confluent, the cells were transfected with indicated amounts of plasmids in serum-free medium for 4 h using Lipofectamine Plus reagent (Invitrogen, Carlsbad, CA) according to the procedures recommended by the manufacturer. After transfection, medium was removed and replaced with fresh serum-free medium. On the day after transfection, cells were treated with indicated concentrations of TNF. Two hours after the addition of TNF, the cells were stimulated with either ovine LH (10 ng/mL) or 8Br-cAMP (1 m*M*), and incubated for an additional 4 h. The cells were then harvested with Reporter Lysis Buffer (Promega, Madison, WI). When the cells were stimulated with PKA expression vector, the cells were harvested 6 h after adding TNF. A CMV promoter-based expression vector pcDNA3 was used as control vector for pcDNA1-p50 and pIκBαM. pRSV-CAT vector was used as control vector for RSV-CHO-PKA-C alpha V2 and pRc/RSV-p65. In all transfection experiments, the cells were cotransfected with a β-galactosidase expression vector pRSV-β to monitor transfection efficiency. Luciferase and β-galactosidase levels were measured with the Luciferase Assay System (Promega) and Galacto-Light Plus (Applied Biosystems, Bedford, MA), respectively, using a plate luminometer. Luciferase levels were normalized with β-galactosidase levels.

Preparation of Whole Cell Lysates and Nuclear Extracts

MA-10 cells were plated on 6-well plates in growth medium. When the cells became approx 70% confluent, medium was removed and serum-free medium was added. On the next day, the cells were treated with either ovine LH (50 ng/mL) or 8Br-cAMP (1 m*M*) in the either presence or absence of TNF (10 ng/mL). TNF was added 2 h before adding LH or 8Br-cAMP. Cells were harvested 2, 4, or 18 h after the addition of PKA pathway stimulators. For preparation of whole cell lysates, cells were washed twice with PBS (1.4 m*M* KH_2PO_4, 4.3 m*M* Na_2HPO_4, 2.7 m*M* KCl, 137 m*M* NaCl, pH 7.4) and lysed with 200 µL cell lysis buffer (PBS containing 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 m*M* EDTA, 1 m*M* PMSF, 1 m*M* sodium orthovanadate, 1 m*M* NaFl, 1 µg/mL aprotinin, 1 µg/mL leupeptin, and 1 µg/mL pepstatin A). Samples were then sonicated for 10 s, and centrifuged for 10 min at 14,000*g*. Supernatant fluid was collected and stored at –70°C until Western blotting. To prepare nuclear extracts, cells were washed twice with PBS, and lysed with 200 µL sucrose buffer (0.32 *M* sucrose, 10 m*M* Tris-HCl, pH 8.0, 3 m*M* $CaCl_2$, 2 m*M* MgOAc, 0.1 m*M* EDTA, 0.5% NP-40, 1 m*M* DTT, 0.5 m*M* PMSF, 1 m*M* sodium orthovanadate, 1 m*M* NaFl) by gentle agitation with a pipet. Nuclei were collected by centrifugation at 500*g*, 4°C for 5 min. The nuclei in the pellet were washed with 1 mL sucrose buffer (without NP-40) by gentle agitation with a pipet. Nuclei were collected again by centrifugation at 500*g*, 4°C for 5 min. After removing the supernatant fluid, nuclei were resuspended in 20 µL low salt buffer [20 m*M* HEPES, pH 7.9, 1.5 m*M* $MgCl_2$, 20 m*M* KCl, 0.2 m*M* EDTA, 25% glycerol (v/v), 0.5 m*M* DTT, 0.5 m*M* PMSF]. Nuclear proteins were extracted by adding 20 µL high salt buffer [20 m*M* HEPES, pH 7.9, 1.5 m*M* $MgCl_2$, 800 m*M* KCl, 0.2 m*M* EDTA, 25% glycerol (v/v), 1% NP-40, 0.5 m*M* DTT, 0.5 m*M* PMSF, 4 µg/mL leupeptin, 4 µg/mL aprotinin, 4 µg/mL pepstatin, 1 m*M* sodium orthovanadate, 1 m*M* NaFl] and gentle agitation for 45 min at 4°C. Thereafter, samples were centrifuged at 4°C for 15 min at 14,000*g*. Supernatant fluid was collected and stored at –70°C until Western blotting or EMSA. Protein concentrations of these samples were measured using Bio-Rad Protein assay (Bio Rad) based on the method of Bradford *(46)*.

Western Blotting

Whole cell lysate and nuclear extract samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF membrane (Immobilon-P, Millipore, Bedford, MA). Subsequent immuno-detection analyses were performed as described previously *(43)*. Antibodies to CREB (Upstate Biotechnology, Lake Placid, NY) and phospho-CREB (Upstate Biotechnology) were used at a 1:1000 dilution. Antibodies to actin (I-19: Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used at a 1:1000 dilution. Equality of protein contents in the nuclear extracts was checked by SDS-PAGE and Coomassie Brilliant Blue (CBB) staining. Western analysis was repeated from at least three independent culture experiments. Data from at least three experiments were scanned and analyzed by densitometry (GelPro Analyzer 3.0, Media Cybermetics) and normalized to actin. Effects of TNF treatment were compared as basal vs TNF, LH vs LH+TNF, and 8Br-cAMP vs 8Br-cAMP+TNF, and are expressed as a fraction of the basal. Statistical differences were determined by Duncan's mul-

tiple range test. A value of $p \leq 0.05$ was considered to be significant.

Statistical Analysis

Values are represented as means ± SEM. To compare the mean values, results were subjected to analysis of variance, followed by Student's Neuman–Keuls test. A value of $p \leq 0.05$ was considered to be significant.

Acknowledgments

We are grateful to Dr. Tom Maniatis for pcDNA1-p50 and pRc/RSV-p65 (Harvard University, Cambridge, MA), and Dr. A. F. Parlow, the National Hormone and Peptide Program and NIDDK for supplying the ovine LH. We also thank to Dr. Keith L. Parker (University of Texas Southwestern Medical Center, Dallas, TX) for RSV-CHO-PKA-Calpha V2, and Dr. Mario Ascoli (University of Iowa, College of Medicine, Iowa City, IA) for providing the MA-10 cell line. This study was supported by grants from the NIH (CA50616), the Andrew W. Mellon Foundation, and by a Specialized Cooperative Centers Program in Reproduction Research from the NICHD (U54 HD 33994).

References

1. Akira, S., Hirano, T., Taga, T., and Kishimoto, T. (1990). *FASEB J.* **4,** 2860–2867.
2. Moore, C. and Hutson, J. C. (1994). *Endocrinology* **134,** 63–69.
3. Hutson, J. C. (1993). *J. Reprod. Immunol.* **23,** 63–72.
4. Xiong, Y. and Hales, D. (1993). *Endocrinology* **133,** 2568–2573.
5. Calandra, T., Baumgartner, J. D., Grau, G. E., et al. (1990). *J. Infect. Dis.* **161,** 982–987.
6. Cannon, J. G., Friedberg, J. S., Gelfand, J. A., Tompkins, R. G., Burke, J. F., and Dinarello, C. A. (1992). *Crit. Care Med.* **20,** 1414–1419.
7. Damas, P., Reuter, A., Gysen, P., Demonty, J., Lamy, M., and Franchimont, P. (1989). *Crit. Care Med.* **17,** 975–978.
8. Vogel, A. V., Peake, G. T., and Rada, R. T. (1985). *J. Clin. Endocrinol. Metab.* **60,** 658–665.
9. Woolf, P. D., Hamill, R. W., McDonald, J. V., Lee, L. A., and Kelly, M. (1985). *J. Clin. Endocrinol. Metab.* **60,** 444–450.
10. Rothe, J., Gehr, G., Loetscher, H., and Lesslauer, W. (1992). *Immunol. Res.* **11,** 81–90.
11. Tartaglia, L. A. and Goeddel, D. V. (1992). *Immunology Today* **13,** 151–153.
12. Chen, G. and Goeddel, D. V. (2002). *Science* **296,** 1634–1635.
13. Thanos, D. and Maniatis, T. (1995). *Cell. Biol. Int. Rep.* **80,** 529–532.
14. Grimm, S. and Baeuerle, P. A. (1993). *Biochem. J.* **290,** 297–308.
15. Kerr, L. D., Ransone, L. J., Wamsley, P., et al. (1993). *Nature* **365,** 412–419.
16. Ke, S., Rabson, A. B., Germino, J. F., Gallo, M. A., and Tian, Y. (2001). *J. Biol. Chem.* **276,** 39638–39644.
17. McKay, L. I. and Cidlowski, J. A. (1998). *Mol. Endocrinol.* **12,** 45–56.
18. Sheppard, K. A., Phelps, K. M., Williams, A. J., et al. (1998). *J. Biol. Chem.* **273,** 29291–29294.
19. Wadgaonkar, R., Phelps, K. M., Haque, Z., Williams, A. J., Silverman, E. S., and Collins, T. (1999). *J. Biol. Chem.* **274,** 1879–1882.
20. Waltner-Law, M., Daniels, M. C., Sutherland, C., and Granner, D. K. (2000). *J. Biol. Chem.* **275,** 31847–31856.
21. Ascoli, M. (1981). *J. Biol. Chem.* **256,** 179–183.
22. Budnik, L. T., Jahner, D., and Mukhopadhyay, A. K. (1999). *Mol. Cell Endocrinol.* **150,** 39–46.
23. Li, X., Youngblood, G. L., Payne, A. H., and Hales, D. B. (1995). *Endocrinology* **136,** 3519–3526.
24. Lin, T., Wang, D., Nagpal, M. L., and Chang, W. (1994). *Mol. Cell Endocrinol.* **101,** 111–119.
25. Mauduit, C., Gasnier, F., Rey, C., et al. (1998). *Endocrinology* **139,** 2863–2868.
26. Xiong, Y. and Hales, D. B. (1993). *Endocrinology* **132,** 2438–2444.
27. Heller, R. A. and Kronke, M. (1994). *J. Cell Biol.* **126,** 5–9.
28. De, S. K., Chen, H. L., Pace, J. L., Hunt, J. S., Terranova, P. F., and Enders, G. C. (1993). *Endocrinology* **133,** 389–396.
29. Lysiak, J. J. (2004). *Reprod. Biol. Endocrinol.* **2,** 9–19.
30. Mauduit, C., Hartmann, D. J., Chauvin, M. A., Revol, A., Morera, A. M., and Benahmed, M. (1991). *Endocrinology* **129,** 2933–2940.
31. Suescun, M. O., Rival, C., Theas, M. S., Calandra, R. S., and Lustig, L. (2003). *Biol. Reprod.* **68,** 2114–2121.
32. Hansen, W. R., Marvin, K. W., Potter, S., and Mitchell, M. D. (2000). *Placenta* **21,** 789–798.
33. Samoto, H., Shimizu, E., Matsuda-Honjo, Y., et al. (2002). *J. Cell Biochem.* **87,** 313–323.
34. Todorov, V. T., Volkl, S., Muller, M., et al. (2004). *J. Biol. Chem.* **279,** 1458–1467.
35. Sheppard, K. A., Rose, D. W., Haque, Z. K., et al. (1999). *Mol. Cell Biol.* **19,** 6367–6378.
36. Hong, C. Y., Park, J. H., Ahn, R. S., et al. (2004). *Mol. Cell Biol.* **24,** 2593–2604.
37. Jambal, P., Masterson, S., Nesterova, A., et al. (2003). *J. Biol. Chem.* **278,** 23055–23065.
38. Izaurralde, E. and Adam, S. (1998). *RNA* **4,** 351–364.
39. Kuersten, S., Ohno, M., and Mattaj, I. (2001). *Trends Cell Biol.* **11,** 497–503.
40. Forwood, J. K., Lam, M. H., and Jans, D. A. (2001). *Biochemistry* **40,** 5208–5217.
41. Thanos, D. and Maniatis, T. (1995). *Mol. Cell Biol.* **15,** 152–164.
42. Howard, P., Day, K. H., Kim, K. E., et al. (1991). *J. Biol. Chem.* **266,** 10189–10195.
43. Taylor, C., Limback, D., and Terranova, P. (1996). *Endocrinology* **137,** 5735–5738.
44. Chaturvedi, G., Arai, K., Limback, D., Roby, K. F., and Terranova, P. F. (2004). *Endocrine* **25,** 147–154.
45. Terranova, P. F. and Garza, F. (1983). *Biol. Reprod.* **29,** 630–636.
46. Bradford, M. (1976). *Anal. Biochem.* **72,** 248–254.