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The Effect of Tumor Necrosis Factor-α and cAMP on Induction of AP-1 Activity in MA-10 Tumor Leydig Cells

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The immunostimulant tumor necrosis factor-α (TNFα), produced by monocytes/macrophages in response to inflammatory disorders, regulates gene expression in part through induction of mitogen-activated protein kinases (MAPKs), including the stress-activated protein kinase (SAPK) (c-Jun *N*-terminal kinase [JNK]) and the extracellular signal-regulated kinases (ERKs). In testicular Leydig cells, the induction of steroidogenesis by cAMP is inhibited by TNFα. To examine the potential mechanisms governing the mutual inhibition between cAMP and TNFα in Leydig cells, the intracellular signaling pathways that contribute to AP-1-dependent gene expression were examined in the mouse MA-10 Leydig cell line. TNFα induced SAPK activity sixfold at 15 min, and the PKC inhibitor calphostin C reduced the induction of SAPK by 30%. cAMP induced SAPK activity twofold but reduced TNFα-induced SAPK activity. ERK activity was inhibited by both cAMP and TNFa. TNFa increased c-Jun protein, but only weakly induced FOS proteins (c-Fos, FosB, Fra-1, and Fra-2) whereas cAMP increased the abundance of several FOS proteins (c-Fos, FosB, Fra-1, and Fra-2), with little effect on c-Jun levels. AP-1 binding activity, assessed using electrophoretic mobility shift assays, was increased twofold by TNFα and fivefold by cAMP. Cyclic AMP alone induced AP-1-responsive reporter (p3TPLUX) activity threefold after 2 h with peak effect of 4-fold at 4 hr. AP-1 reporter was not induced by TNFα alone but in the presence of cAMP, TNFα induced AP-1 reporter activity 12-fold. In conclusion, TNFα and cAMP induce distinct components that separately contribute to the modulation of AP-1 activity in MA-10 cells.

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Introduction

Tumor necrosis factor-α (TNFα) is a multifunctional cytokine that is produced by a variety of cell types, including macrophages and lymphocytes, and is important in the initiation of the immune response *(1,2)*. In addition to antiviral and immunoregulatory effects, TNFα stimulates proliferation of normal cells, and has cytolytic or cytostatic effects against tumor cells *(1,2)*. TNFα produced during systemic infection and chronic inflammatory diseases reduces testicular steroid hormone production and fertility *(1–3)*. TNFα binding to its cell-surface receptor activates several signal transduction pathways, including protein kinase A (PKA) and protein kinase C (PKC), adenylate cyclase, sphingomyelin-ceramide, G-proteins, and NFκB *(1,4,5)*.

TNFα induces members of the mitogen-activated protein kinase (MAPK) family *(6)*. MAPKs include at least four related, but distinct serine/threonine kinases, including the extracellular signal-regulated kinases (p42 and p44 ERKs), the stress-activated protein kinases (SAPKs) or c-Jun-*N*-terminal kinases (JNKs), p38 and p38b, and ERK5 (BMK) *(7–12)*. Distinct MAPK or MEKs induce these MAPKs *(7–12)*. TNFα is capable of inducing several distinct MAPKs in a cell-type-dependent manner, including the ERKs *(13)*, SAPK and p38 kinases *(7–12,14)*. Downstream transcription factors phosphorylated by these MAPKs regulate gene expression at least in part through activity of the activator protein-1 complex (AP-1) composed of FOS/JUN proteins *(15,16)*.

Recent studies have examined the effect of cAMP on signaling by the Ras/ERK pathway. cAMP inhibits MAPK and AP-1 activity in several different cell types *(17–19)*. The interaction between the cAMP and TNFα signaling pathways is also complex, and may vary between cell types *(20–23)*. Although cAMP has previously been shown to inhibit MAPK and AP-1 activity, relatively

little is known about the effect of cAMP on SAPK activity. In T-lymphocytes, cAMP inhibition of SAPK activity was thought to contribute to cAMP-mediated inhibition of T-cell function *(24)*. The role of SAPK and the interaction with the cAMP pathway remains to be examined in other systems.

The interaction between the cAMP- and TNFα-mediated signal pathways is of particular importance in testicular Leydig cells *(3)*. Leydig cell steroidogenesis is primarily controlled by luteinizing hormone (LH), which binds a G-protein coupled receptor and induces a downstream signaling cascade through the second messenger cAMP. Leydig cell testosterone production depends on the activity of the 17α-hydroxylase/C17-20 lyase cytochrome P450 (P450c17), which is encoded by the *Cyp17* gene. cAMP is essential for P450c17 synthesis *(25)* and mRNA expression in mouse Leydig cells *(26)*. Moreover, the 5'-flanking region of the *Cyp17* promoter confers cAMP responsiveness to reporter constructs transiently transfected into MA-10 tumor Leydig cells *(27)*. cAMP-stimulated *Cyp17* gene expression is presumably mediated by activation of cAMP-dependent kinase A *(28)*. TNFα inhibits cAMP-stimulated *Cyp17* gene expression in Leydig cells *(29)* and in transfected MA-10 tumor Leydig cells *(30)*. In previous studies, we demonstrated that activation of PKC was required for the TNFα-mediated inhibition of cAMP-stimulated *Cyp17* gene expression *(30)*.

To gain further insight into the interaction between the cAMP and TNFα pathways in Leydig cells, we assessed the effect of cAMP and TNFα on several components of the AP-1 pathway, including SAPK and ERK activity, expression of the AP-1 proteins, AP-1 binding activity, and AP-1 reporter activity.

Results

TNFα and cAMP Induce an Increase of SAPK Activity

TNFα activates c-Jun protein phosphorylation in several different cell types *(31)*. Immunecomplex kinase assays were performed *(32)* to determine whether TNFα regulated SAPK activity in MA-10 cells. Extracts from cells treated with TNFα or cAMP were immunoprecipitated using a polyclonal antibody against SAPK, and GST-c-Jun (1-135) was used as substrate as previously described (33). TNFα induced SAPK activity 6-fold (5.8 ± 0.6 fold, $p < 0.01$) at 15 min (Fig. 1A). SAPK activity returned to control levels after 1 h (Fig. 1A,B). cAMP induced SAPK activity twofold (1.8 ± 0.4 fold, $p < 0.05$) (Fig. 1A,B).

To examine the effect of cAMP on TNFα-induced SAPK activity, cells were treated with both reagents for the time-points indicated in Fig. 1C. Although cAMP alone induced SAPK activity, when added to TNFa treated cells, cAMP reduced TNFα-induced SAPK activity by 40 to 60% at time-points between 15 min and 1 h (Fig. 1C).

In order to examine the possible involvement of the PKC pathway in TNFα-stimulated SAPK activity in MA-10 cells, the effect of the PKC inhibitor calphostin C was analyzed. MA-10 cells were treated with TNFα (1 and 10 ng/mL) for 15 min either alone or in the presence of calphostin C (1 μ*M*). SAPK activity was induced by the same fold by TNFα as in Fig 1A, and calphostin C inhibited TNFα-stimulated SAPK activity by 30% (Fig. 1D). This suggests that activation of the PKC pathway is not required for TNFα-stimulation of SAPK activity.

TNFα and cAMP Inhibit ERK Activity

To examine the effect of cAMP and TNFα on ERK activity in MA-10 cells, immune precipitation kinase assays were performed using cell extracts. cAMP inhibited ERK activity by 50–60% with maximal reduction at 2 h (Fig. 2). In contrast with several other cell types in which TNFα induced ERK activity *(13, 34–36)*, in MA-10 cells, ERK activity was reduced by TNFα treatment (Fig. 2). Furthermore, TNFα and cAMP together caused an even greater inhibition of ERK activity at 15 min (Fig. 2) than either agent did when added alone.

TNFα and cAMP Induce Distinct AP-1 Proteins

In order to examine further how cAMP and TNFα regulate AP-1 activity, the effect of these reagents on FOS and JUN protein abundance was determined. cAMP (500 μ*M*) preferentially increased FOS protein abundance (c-Fos, FosB, Fra-1, and Fra-2) at 2 h. c-Fos was induced 40-fold by cAMP (Fig. 3C). Fra-1 was induced 32-fold, and FosB was induced 14-fold (Fig. 3C). c-Jun abundance was induced sixfold (Fig. 3). TNFα (10 ng/mL) preferentially induced c-Jun protein abundance 23-fold (Fig. 3D), but induced FOS protein abundance only slightly (c-Fos, 4.5-fold; FosB, fourfold). Fra-1 was induced 12-fold and Fra-2 4-fold (Fig. 3).

Compared with cAMP alone, the addition of TNFα to cAMP-treated cells reduced Fra-1 abundance, but did not significantly affect the amount of the other FOS proteins (Fig. 3C). The addition of cAMP to TNFα induced c-Jun abundance further from 23- to 32-fold (Fig. 3D). Thus, although AP-1 proteins are induced by either cAMP or TNFα in MA-10 cells, specific members of the FOS /JUN family are preferentially induced.

TNFα and cAMP Induce AP-1 Binding Activity

Because DNA binding capacity may contribute to AP-1-dependent gene regulation *(15)*, the effect of cAMP and TNFα on AP-1 binding activity was assessed using electrophoretic mobility shift assays. γ^{32}P-labeled AP-1 sequences were incubated with MA-10 cell nuclear extracts. The AP-1 DNA–protein complex formed was specifically competed with unlabeled AP-1 oligomers (results not shown). The AP-1 DNA–protein complex was supershifted

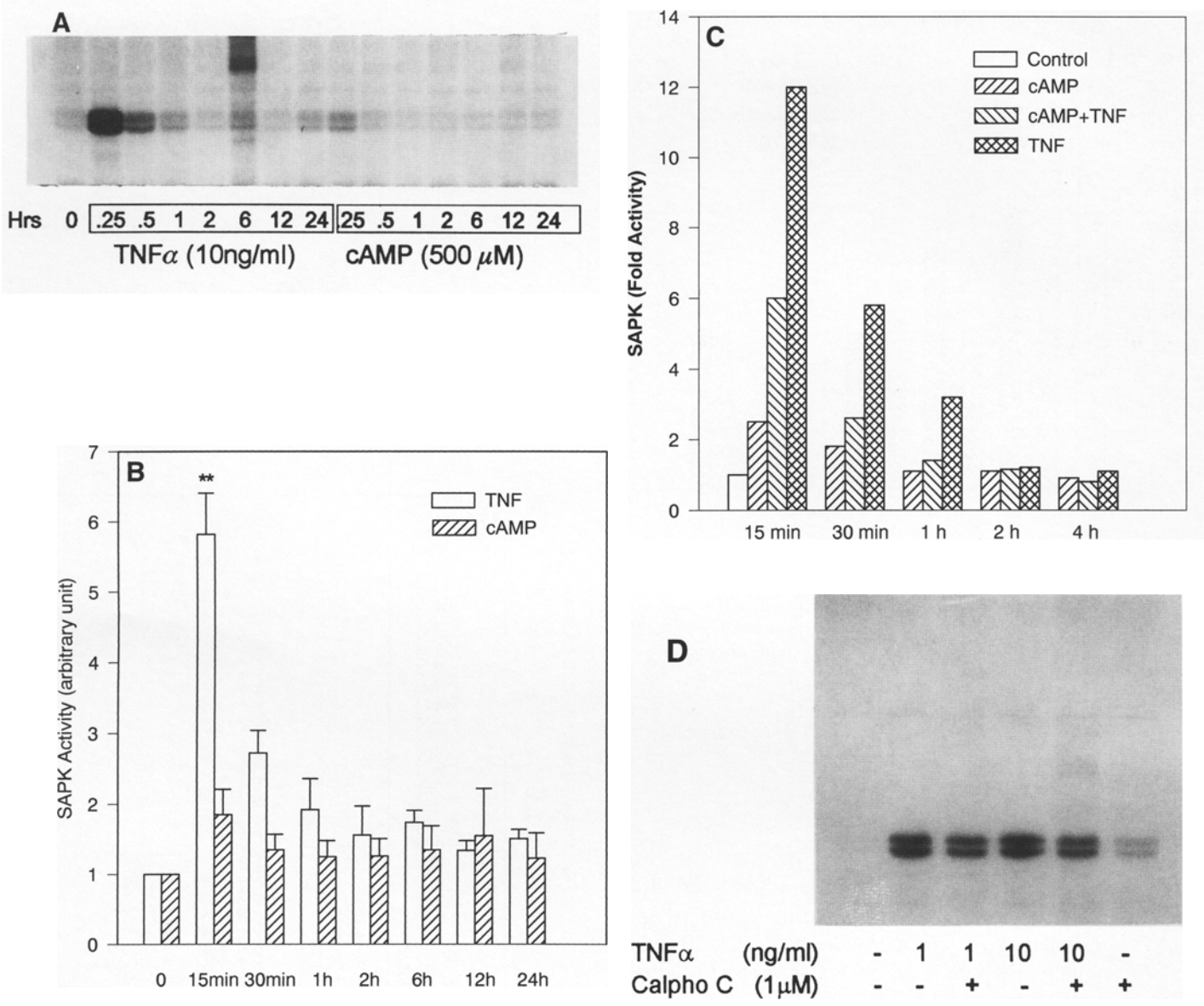


Fig. 1. TNFα and cAMP regulate of SAPK activity. MA-10 cells were treated for the time-points indicated, and immune complex kinase assays performed *(34)*. **(A)** Assays were performed using the polyclonal SAPK antibody and GST-c-Jun (1-135) as substrates in the presence of γ^{32}P-ATP. **(B)** The data represent the mean ± SEM for four separate experiments. **More than control ($p < 0.01$). **(C)** The effect of cAMP on TNFα-induced SAPK activity. The result is a representative of two separate experiments. **(D)** MA-10 cells were treated with 1 or 10 ng/mL TNFα, with or without 1 μ*M* calphostin C for 15 min. The result is a representative of two separate experiments.

by antibodies against the FOS family, c-Jun, JunB, and JunD (Fig. 4). The results of the supershift analysis indicate that the AP-1 DNA-protein complex contains FOS and multiple JUN family proteins.

cAMP treatment increased total AP-1 binding activity. c-Jun and JunB contributed to the AP-1 binding activity in cAMP treated cells, consistent with the finding that cAMP treatment induced c-Jun protein abundance (Fig. 4). TNFα induced a small increase in AP-1 binding activity (Fig. 4). Compared with the effect of cAMP alone, the addition of TNFα to cAMP-treated cells reduced the AP-1 binding activity (Fig. 4).

TNFα and cAMP Collaborate in Stimulating AP-1 Reporter Activity

A luciferase reporter gene construct with an upstream trimeric collagenase AP-1 site (p3TPLUX) was used to examine the effect of TNFα and cAMP on AP-1 transcriptional activity. TNFα alone did not cause a significant increase in the reporter activity during 12 h of treatment ($p > 0.05$); however, cAMP alone induced p3TPLUX activity by threefold (2.8- ± 0.7-fold) at 2 h, with a peak fourfold (3.9- ± 0.4-fold) induction at 4 h (Fig. 5). Strikingly, the addition of TNFα to cAMP-treated cells increased the reporter activity from three to ninefold at 2

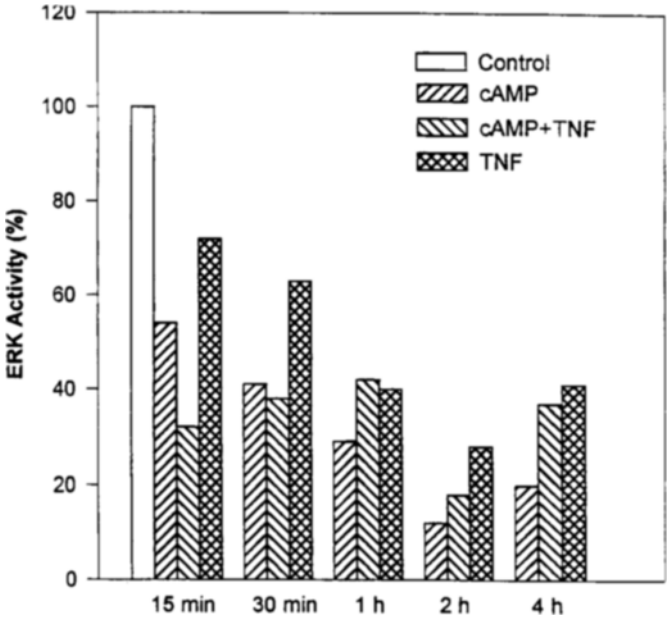


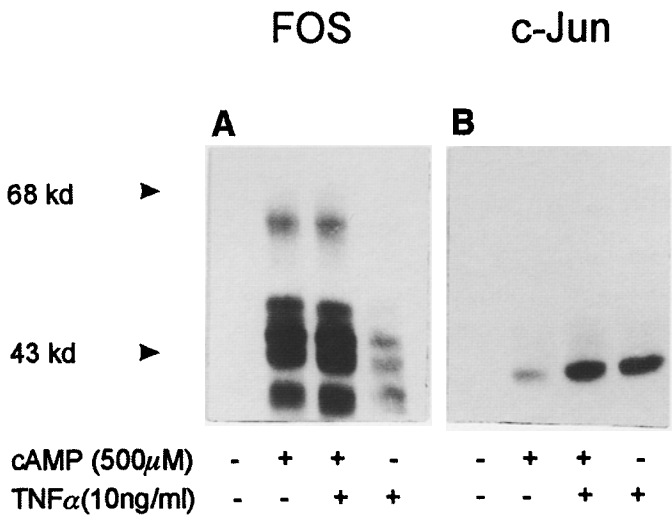
Fig. 2. TNFα and cAMP inhibited ERK activity. MA-10 cells were treated for the time-points indicated, and ERK activity assessed using MBP as substrates in the presence of γ^{32}P-ATP. The result is representative of two separate experiments.

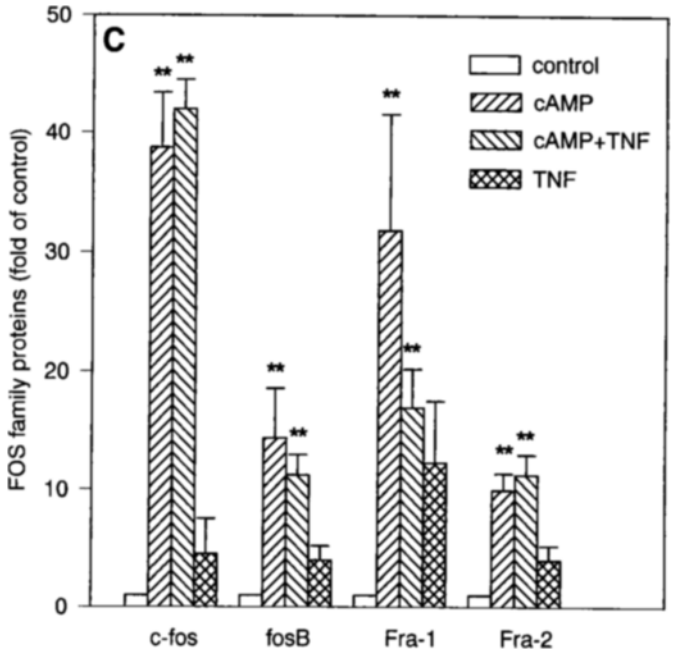
h ($p < 0.01$), with peak induction of 12.3- ± twofold (p ± 0.01) observed at 4 h.

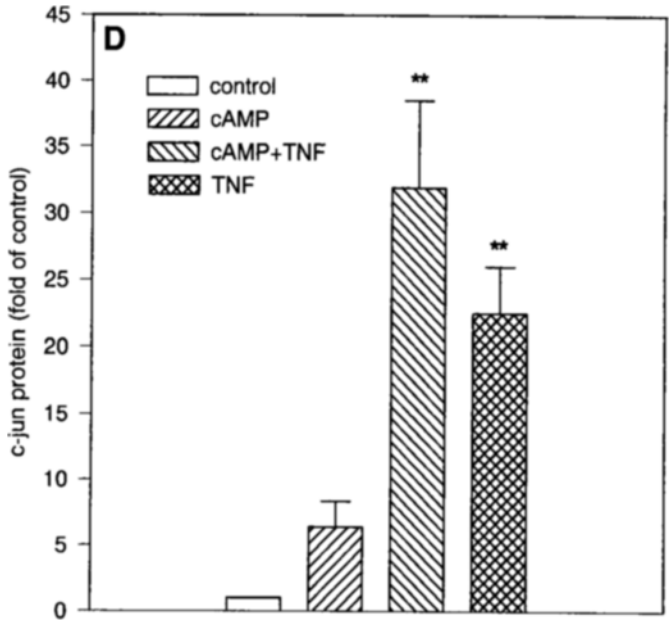
Discussion

The results of the present study demonstrate that cAMP inhibits ERK activity, but induces SAPK activity, FOS protein expression, AP-1 binding activity, and TNFα-dependent AP-1 reporter activity in MA-10 cells. TNFα inhibited ERK activity, but enhanced SAPK activity, primarily through a PKC-independent pathway, and increased c-Jun abundance and AP-1 binding activity. Because TNFα only minimally affected FOS protein abundance and cAMP induced c-Jun protein production, distinct components of the AP-1 pathway are induced by TNFα and cAMP in MA-10 cells. In combination, however, cAMP and TNFα caused a synergistic increase of AP-1 activity. Compared with cAMP alone, the addition of TNFα increased SAPK activity, reduced ERK activity, increased c-Jun abundance, and decreased AP-1 binding activity. The increase in SAPK activity likely contributed to the increase in c-Jun protein levels, and the reduction in ERK activity likely contributed to the reduction in AP-1 binding activity. Therefore, the synergistic effect of TNFα on cAMP-induced AP-1 activity is parallel to its effect on c-Jun protein production and SAPK activity.

cAMP reduced ERK activity as previously described in other cell types. Surprisingly, unlike a wide variety of other cell types in which TNFα induced ERK activity *(13, 34–36)*, TNFα inhibited ERK activity in MA-10 cells. The reduction in ERK activity to below basal levels was







delayed and sustained, continuing while the induction of SAPK activity was maintained. Although the mechanisms responsible for TNFα inhibition of ERK activity are not known, the induction of SAPK activity by TNFα may have contributed to the reduction in ERK activity, since SAPK can induce the ERK phosphatase MKP-1 *(37)*.

In previous studies performed in MA-10 cells, chorionic gonadotrophin (hCG), which activates the cAMP pathway, was reported to induce c-*fos* expression *(38)*. The present study demonstrates that cAMP induced FOS family proteins (c-Fos, FosB, Fra-1, and Fra-2) and also increased c-Jun abundance in MA-10 cells. The c-*fos* promoter contains a complex series of CREs, which bind CREB *(39)*, and c-*fos* expression is induced by cAMP in a wide variety of cell types. In contrast, the induction of c-Jun by cAMP in MA-10 cells is unusual, but consistent with the finding that SAPK activity was induced by cAMP in these cells. In the myelomonocytic cell line HL60, cAMP induced c-Jun abundance *(40)*, but c-Jun abundance is inhibited by cAMP in most cell types examined *(22, 41)*.

Cytokines have previously been shown to increase the abundance of c-Fos and c-Jun *(22)*. In our studies, TNFα increased both c-Jun and c-Fos levels. In previous studies, TNFα stimulated a prolonged activation of c-jun expression in fibroblasts (21). The induction of c-jun by EGF or anisomycin appears to involve a p55 kinase unrelated to ERK *(42)*, likely SAPK. The induction of c-jun transcription and subsequently c-Jun protein levels observed in these studies is likely mediated through induction of c-Jun and activating transcription factor 2 (ATF-2) proteins *(43)*, which bind to and activate the c-*jun* promoter *(15,44)*. Several different MAPK pathways contribute to the induction of c-Fos abundance. Recent studies demonstrated that the induction of c-Fos by growth factors involved the ERK pathway, but that the induction of c-Fos by UV irradiation or anisomycin involved the SAPK pathway through phosphorylation of TCF/Elk-1 *(45,46)*. Because ERK activity was inhibited by TNFα in our studies, the induction of c-Fos may have been mediated, at least in part, by TNFα induction of the SAPK pathway.

To determine if TNFα and cAMP induction of AP-1 protein synthesis results in an increase in AP-1 DNA-protein binding activity, electrophoretic mobility shift assays were assessed. The AP-1 DNA-binding complex in untreated MA-10 cells consisted primarily of JunD and FOS. c-Jun and JunB contributed proportionately more to the TNFα and cAMP-induced AP-1 DNA binding complex. These results are consistent with previous observations that JunD abundance is relatively unaffected by different stimuli and is constitutively present in most cells in relatively high amounts (47,48). TNF*a* induction of less AP-1 DNA binding is consistent with the protein abundance observed on Western blotting in which TNFα only causes an increase in c-Jun protein, but not FOS family proteins. The experiment suggests that a TNFα-stimulated increase in Jun protein synthesis and phosphorylation does not further increase cAMP-stimulated AP-1 DNA-protein binding activity (Fig. 4), but does result in an increase of cAMP-mediated AP-1 reporter activity (Fig. 5).

The intracellular signaling pathways linking the induction of SAPK activity with apoptosis, cellular proliferation, and the regulation of gene expression are complex. SAPKs are members of the MAPK superfamily and are induced by UV light *(49,50)* stress, osmotic shock, interleukin-1 *(51)*, and TNFα (21,44). The present study shows that treatment with TNFα stimulated SAPK activity in MA-10 cells at 15 min and that the PKC inhibitor calphostin C only partially reduced (30%) TNFα-stimulated SAPK activity. This finding suggests that TNFα activation of SAPK in MA-10 cells does not involve the activation of PKC. The induction of SAPK activity occurred independently of PKC activation in other systems. For instance, the PKC inhibitor GF109203X did not inhibit carbachol-induced SAPK activity or c-*jun* and *junD* expression in NIH-m1,

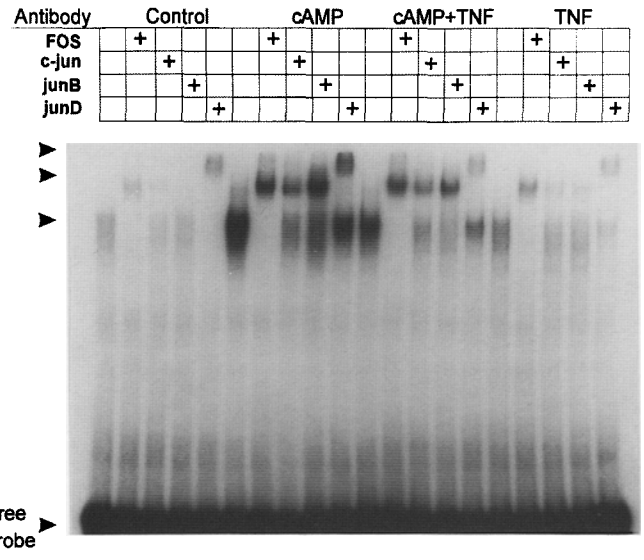


Fig. 4. TNFα and cAMP induce AP-1 DNA binding activity. The AP-1 site probe was incubated with 1 μg of protein extract in reaction buffer for 35 min (15 min on ice and 20 min at 25°C) in a total volume of 25 μL. For supershift assay, antibodies to FOS family, c-Jun, JunB, and JunD, were added.

Fig. 3. *(opposite page)* TNFα and cAMP induce distinct AP-1 proteins. MA-10 cells were incubated with 500 μ*M* cAMP, 10 ng/mL TNFα, or cAMP plus TNFα for 2 h. Nuclear proteins were extracted as described under Materials and Methods. Twenty-five micrograms of proteins of each sample were submitted to Western blot assay. Antibodies against FOS family **(A)** and c-Jun **(B)** were used to recognize specific proteins. The blots were visualized with ECL. Protein levels were quantitated by densitometry **(C, D)**. Data were expressed as fold of control and represent the mean + SE for four experiments. **More than control ($p < 0.01$).

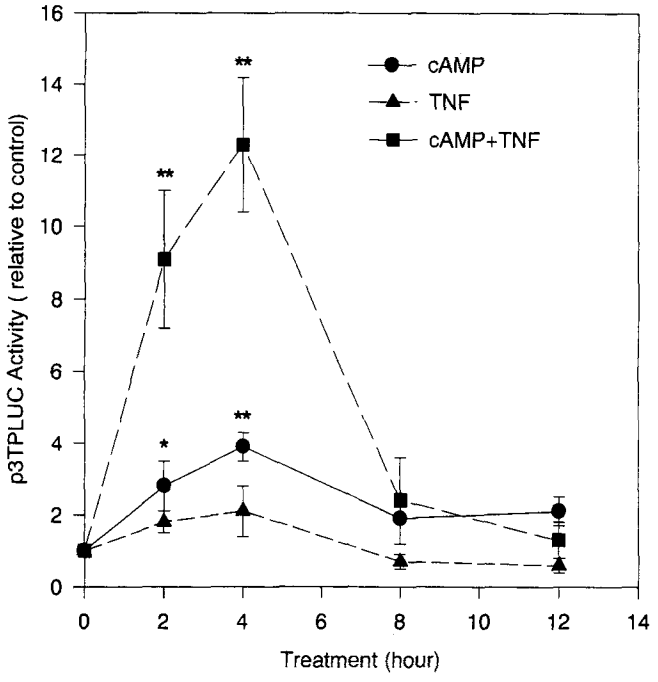


Fig. 5. TNFα and cAMP regulate AP-1 activity. MA-10 cells were transfected with p3TPLUX reporter and treated with cAMP (500 μ*M*), TNFα (10 ng/mL), or cAMP plus TNFα, for the time-points indicated. Data are shown as the mean ± SEM for four separate experiments. *more than control ($p < 0.05$), and **More than control ($p < 0.01$).

2 cells *(52)*. The mechanism through which TNFα induces SAPK activity in MA-10 cells is, as yet, uncertain.

The present study contrasted the effect of cAMP and TNFα. Compared with cAMP alone, the addition of TNFα increased SAPK activity, reduced ERK activity, and reduced AP-1 binding activity. These findings suggest that cAMP and TNFα may exert mutually antagonistic activities. In contrast, cAMP and TNFα exhibited functional synergy on the trimeric AP-1 site reporter (p3TPLUX) gene activity. Thus, the net effect on transcriptional activity may depend on the AP-1 site as assessed in the context of a given promoter. Also, the synergistic effect of TNFα is parallel to its effect on AP-1 protein production and SAPK activity. The functional antagonism between TNFα and cAMP may be owing to the different effects of phosphatases, such as protein phosphatase 2A (PP2A) and mitogen activated kinase phosphatase 1 (MKP-1) *(15,37,53,54)*.

TNFα, produced by testicular macrophages in response to bacterial lipopolysaccharide both in vivo and in vitro, inhibits basal and cAMP-induced Leydig cell steroidogenesis through reduction in *Cyp17* gene expression *(55,56)*. We demonstrated that the inhibitory effects of TNFα on cAMP-stimulated steroidogenesis in Leydig cells could be mimicked by the PKC activator phorbol 12-myristate 13-acetate, and abolished by the PKC inhibitor calphostin C (30). This suggests that activation of PKC is required for TNFα action. In the present study, we report that TNFα and cAMP utilize different intermediates, but interact to stimulate AP-1 activity in MA-10 cells. That activation of PKC is not required for TNFα stimulation of SAPK further supports the hypothesis that modulation of AP-1 activity by TNFα is not important to the TNFα-mediated inhibition of *Cyp17* gene regulation.

Materials And Methods

Western Blots

MA-10 tumor Leydig cells were grown in Waymouths MB752/1 medium (Gibco/BRL, Gaithersburg, MD) containing 20 m*M* HEPES, 1.1 g/L sodium bicarbonate, and 15% horse serum in 100-mm dishes at a density of 1×10^6 cells/dish *(57,58)*. Cells were grown for 40 h, then treated with medium, 500 mM cAMP, or cAMP plus 10 ng/mL TNFα for 2 hr. Cytosol and nuclear fractions were separated as described *(30,59)*. Briefly, cells were suspended in hypotonic buffer, incubated on ice for 10 min, vortexed, and centrifuged. The supernatant fraction was discarded, and nuclear proteins were extracted by resuspending pellets in high-salt buffer and incubating on ice for 20 min. The cellular debris was removed by centrifugation. The amount of protein in nuclear fractions was determined by BCA protein assay (Pierce, Rockford, IL). Equal amounts of protein (25 μg) from each sample were subjected to SDS-PAGE and transferred to nitrocellulose membrane. The membrane was incubated with anti-FOS family or anti-c-Jun antibody (1 μg/mL), and then horseradish peroxidase-conjugated goat anti-mouse (for c-Jun) or goat antirabbit antibody (for FOS family). The blot was developed with enhanced chemiluminescence (ECL, Amersham, UK) according to the manufacturer's protocol.

$p42^{ERK}$, $p44^{ERK}$, SAPK Immune Complex Assays

Assays were performed as previously described (32) on cell extracts treated with cAMP or TNFα. For assays, staphylococcal protein A-sepharose beads were incubated with either anti-MAPK antibody (C16) (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-SAPK antibody *(31)* for 1 h at 4°C. The antibody and beads were washed once with RIPA buffer and then incubated with cell lysates for 2 h at 4°C. The immunoprecipitates were washed with RIPA buffer once, with LiCl/0.1 *M* Tris base, pH 8.0, twice, and once in kinase buffer. The kinase reactions were performed at room temperature for 20 min in 30 mL of kinase buffer with 10 μCi of [γ^{32}P] ATP (3000 Ci/mmol; 1 Ci = 37 GBq) and 4 μg of myelin basic protein (MBP) or 4 μg of GST c-Jun fusion protein. The samples were analyzed by SDS/PAGE on termination of the reaction with Laemmli buffer and boiling. The phosphorylation of MBP or GST c-Jun was quantified by densitometry using a Fuji Bio Imaging Analyzer BAS 2000.

Electrophoretic Mobility Gel Shift Assays (EMSA)

EMSA were performed as described previously *(60)* except that the oligodeoxyribonucleotide probe used in these experiments contained two copies of the AP-1 sequences of the collagenase promoter (Gibco/BRL). The probe was labeled at the 5' end with γ^{32}P-ATP (NEN, Boston, MA). The labeled DNA was purified by G-50 Sephadex chromatography. The end-labeled AP-1 oligomers (3 x 10^4 cpm) were incubated with 1 μg of the protein extract in reaction buffer (10 m*M* Tris HCl, pH 7.5, 50 m*M* KCl, 5 m*M* $MgCl_2$, 1 m*M* DTT, 1 m*M* EDTA, 12.5% glycerol, and 0.1% Triton X-100) for 35 min (15 min on ice and 20 min at 25°C) in a total volume of 25 μl. For competitive assay, unlabeled AP-1 oligomers were added. For supershift assay, antibodies (anti-Fos family [K-25], c-Jun [KM-1], Jun-B [N-17], and Jun-D [329] [Santa Cruz Biotech, Inc. Santa Cruz, CA]) were added. Samples were analyzed on a nondenaturing 6% polyacrylamide gel in 0.5 x TBE (Tris-borate-EDTA buffer). The gels were dried and exposed to X-ray film at −70°C.

Transfection Assays

The equal amount of MA-10 cells (10^6/dish) were transfected as previously described *(27,30)* with the reporter plasmid p3TPLUX *(61)*, which contains three collagenase AP-1 sites (a gift from J. Massague, Sloan-Kettering Cancer Center, NY). Cells were treated with cAMP, TNFα, or cAMP plus TNFα. For luciferase assays, cells were harvested and extracted using a kit (Promega Co., Madison, WI). The luciferase activity was determined by a scintillation counter to detect the light emission during the initial 1 min of reaction between cell extracts and substrate. To generate a linear relationship between luciferase concentration and cpm from scintillation counter, the square root of the scintillation counts per minute was determined.

Statistics

The data from luciferase assay and densitometry analyses are presented as the mean ± SE of three or more independent experiments. For group comparison, one way analysis of variance was performed by a Newman-Keuls multiple-range test using the InStat2 Biostatistics software package (GraphPad Software, Inc., San Diego, CA). Differences were considered as significant at $p < 0.05$.

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