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Differential Effects of Tumor Necrosis Factor-α and Interleukin-1 on 3β-Hydroxysteroid Dehydrogenase/$\Delta^5 \rightarrow \Delta^4$ Isomerase Expression in Mouse Leydig Cells

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Immune–endocrine interactions are important to the regulation of Leydig cell steroidogenesis. We have shown previously that both tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1-β) inhibit 8-bromo-cAMP-(8-Br-cAMP)-stimulated steroidogenesis in mouse Leydig cells. TNF and IL-1 both inhibit cAMP-stimulated testosterone production as well as mRNA and protein levels of cholesterol side chain cleavage enzyme (P450scc) and 17α-hydroxylase/$C_{17,20}$ lyase (P450c17) in mouse Leydig cells. Neither TNF nor IL-1 affects basal levels of P450scc mRNA and protein. In the present study, we tested the effects of TNF and IL-1 on basal testosterone production and 8-Br-cAMP-stimulated 3β-hydroxysteroid dehydrogenase/$\Delta^5 \rightarrow \Delta^4$ isomerase (3βHSD) expression in Leydig cells. Purified and macrophage-depleted Leydig cells were cultured for 5 d with daily changes of media, and then treated with increasing concentrations of recombinant mouse TNF or IL-1 in the presence or absence of 8-Br-cAMP (50 μ*M*) for 24 h. The media were collected for testosterone RIA and RNA and protein were extracted from cells. Basal testosterone production was inhibited by TNF, but not IL-1. Treatment of Leydig cells with 8-Br-cAMP alone caused a marked increase in 3βHSD mRNA, and protein levels. Both TNF and IL-1 inhibited cAMP-stimulated 3βHSD mRNA and protein levels, but only TNF inhibited basal 3βHSD expression. These results demonstrate that TNF and IL-1 have different effects on basal steroidogenesis in Leydig cells and suggest that TNF-mediated inhibition of basal testosterone production may be owing to the inhibition of basal 3β-HSD expression in Leydig cells.

Key Words: Leydig cells; cytokines; steroidogenesis; immune–endocrine interactions.

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Introduction

Testosterone biosynthesis in the Leydig cells of the testis is a multistep process, involving the actions of several enzymes. The production of testosterone is primarily under the control of the pituitary gonadotrophin LH. In addition to LH, several endocrine, paracrine, and autocrine factors are now known to regulate Leydig cell steroidogenesis (for review, *see 1*). Cytokine-guided control of Leydig cell steroidogenesis has emerged as an important control mechanism. Proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1α and β (IL-1α,β), known to be secreted by activated testicular macrophages *(2–6)* are inhibitory to Leydig cell function (for review, *see 7*). Previously we have shown that both IL-1 and TNF inhibit LH or cAMP-stimulated steroidogenesis in Leydig cells *(2,8,9)*. The cAMP-stimulated expression of two key enzymes in testosterone production in Leydig cells, cholesterol side chain cleavage enzyme (P450scc) and 17α-hydroxylase/$C_{17,20}$ lyase (P450c17), is inhibited by IL-1 and TNF action. P450scc catalyzes the conversion of cholesterol to pregnenolone, and P450c17 catalyzes the conversion of progesterone to androstenedione, the immediate precursor of testosterone. There are no reports on the effects of IL-1 or TNF on the other two Leydig cell steroidogenic enzymes: 3β-hydroxysteroid dehydrogenase/$\Delta^5 \rightarrow \Delta^4$ isomerase (3βHSD) and 17β-hydroxysteroid dehydrogenase (17βHSD). 3βHSD catalyzes the conversion of pregnenolone to progesterone, and 17βHSD catalyzes the conversion of androstenedione to testosterone. The present study focuses on the cytokine-mediated regulation of basal and cAMP-stimulated 3βHSD. We have shown previously that cAMP-stimulated expression of both P450scc and P450c17 are sensitive to cytokine inhibition. P450c17 basal expression is below the level of detection, and thus, its expression is considered to be cAMP-dependent *(10)*. Interestingly, the basal expression of P450scc is not inhibited by either IL-1 or TNF, yet TNF, but not IL-1, appears to inhibit basal testosterone production *(2,9)*. The present study was designed to confirm the striking observation that IL-1 and TNF have different effects on basal

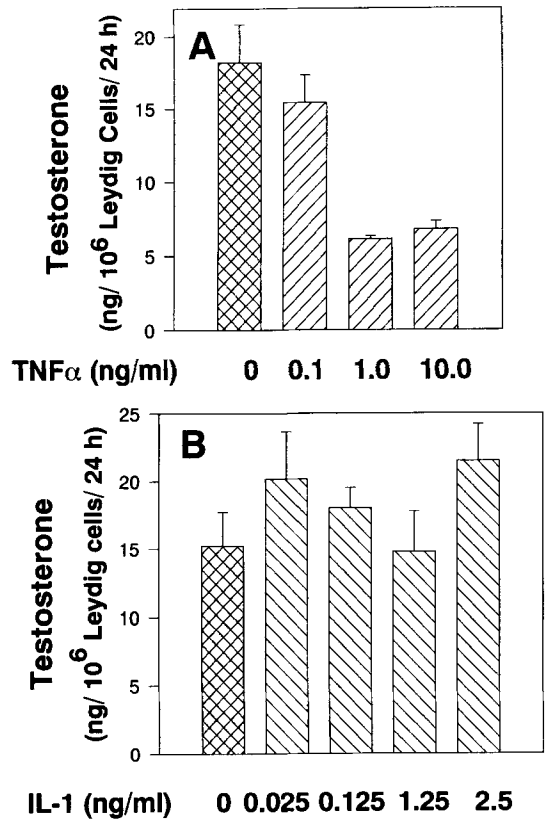

Fig. 1. Effects of TNF (**A**) and IL-1 (**B**) on basal testosterone secretion. Culture media were collected from dishes from which RNA and protein were extracted shown in Figs. 2 and 4. Basal testosterone levels were determined by RIA. Data represent the mean ± SE for 3–5 experiments, with replicate samples.

testosterone production and to determine the mechanisms through which TNF, but not IL-1, inhibits basal steroidogenesis.

Results

Effects of TNF and IL-1 on Basal Testosterone Production

We reported previously that TNF, but not IL-1 appears to inhibit basal testosterone production by Leydig cells (*2,9*). To confirm this observation, the effects of TNF and IL-1 on basal testosterone production was measured. Leydig cells were maintained in culture for 5–6 d prior to the initiation of treatment with increasing concentrations of either mrIL-1β (Fig. 1A) or mrTNF-α (Fig. 1B). Culture media were collected from the culture dishes from which RNA and protein were extracted, as described below, and testosterone measured by RIA. Treatment with TNF caused a significant and dose-dependent decrease in basal testosterone production. Testosterone was decreased from 18.2 ± 2.66 (ng/10^6 Leydig cells/24 h) to 15.3 ± 1.89, 6.11 ± 0.22 ($p < 0.05$), and 6.77 ± 0.56 ($p < 0.05$) with 0.1, 1.0, and 10.0 ng/mL TNF, respectively. This represents a decrease in basal testosterone by 15, 66, and 63%, compared to untreated controls cultures. In contrast, treatment with IL-1 caused no significant effect on basal testosterone production. Testosterone in control cultures was 15.2 ± 2.5 (ng/10^6 Leydig cells/24 h), compared to 20.15 ± 3.5, 18.0 ± 1.5, 14.75 ± 3.0, and 21.5 ± 2.7 in cultures treated with 0.025, 0.125, 1.25, and 2.5 ng/mL IL-1, respectively. These data confirm that TNF, but not IL-1 significantly inhibits basal testosterone production. In data not shown, hrIL-1α also had no effect on basal testosterone production. Concentrations as high as 10 ng/mL of IL-1 α or β were not inhibitory to basal steroidogenesis (not shown).

Effects of TNF on Basal and 8-Br-cAMP-Stimulated 3βHSD mRNA and Protein Levels

Leydig cells were treated with control medium, increasing concentrations of mrTNF-α, 50 m*M* 8-Br-cAMP, or 8-Br-cAMP plus TNF, as described in Materials and Methods. Total RNA and cellular protein were extracted, and 3βHSD mRNA and protein levels were quantitated. Representative Northern blots are shown in Fig. 2A and B. As shown in Fig. 2A, 3βHSD mRNA was constitutively expressed at a high level, and treatment with TNF resulted in a dose-dependent decrease in its basal expression. As shown in Fig. 2B, treatment with 50 m*M* 8-Br-cAMP for 24 h resulted in a marked increase in 3βHSD mRNA. TNF caused a dose-dependent decrease in cAMP-stimulated mRNA expression. The blot shown in Fig. 2A was exposed for 3 d, whereas the blot shown in Fig. 2B was exposed for <24 h, accounting for difference in signal intensities of basal 3βHSD mRNA in the respective autoradiographs. Results from five separate experiments were quantitated by densitometry and shown in Fig. 3A and B. Treatment with TNF resulted in a significant and dose-dependent decrease in basal 3βHSD mRNA levels: 69.5 ± 7.5, 88.3 ± 1.7, and 99.3 ± 0.2%, with 0.1, 1.0, and 10.0 ng/mL TNF, respectively (Fig. 3A). Cyclic-AMP increased 3βHSD mRNA by 2.2 ± 0.4-fold compared to control (Fig. 3B). Treatment with TNF resulted in a significant and dose-dependent inhibition of 8-Br-cAMP-stimulated 3βHSD mRNA expression: 39.9 ± 14.2, 91.0 ± 2.7, and 98.5 ± 0.6%, with 0.1, 1.0, and 10 ng/mL TNF, respectively (Fig. 3B).

The TNF-mediated decreases in basal and 8-Br-cAMP mRNA levels were reflected in decreases in 3βHSD protein levels. Representative Western blots are shown in Fig. 2C and D. Results from three separate experiments were quantitated by densitometry and shown in Fig. 3C and D. Basal 3βHSD protein levels (Fig. 2C and 3C) were decreased by 30.6 ± 6.9, 51.3 ± 9.9, 68.5 ± 7.4%, and 8-Br-cAMP-stimulated 3βHSD protein levels (Figs. 2D and 3D) were decreased by 20.5 ± 13.5, 40 ± 17.0, and 62.5 ± 13.6%, with 0.1, 1.0, and 10.0 ng/mL TNF, respectively.

Effects of IL-1 on Basal and 8-Br-cAMP-Stimulated 3β-HSD mRNA and Protein Levels

Analogous to the experiments described above, Leydig cells were treated with increasing concentrations of mrIL-1β in the absence and presence of 8-Br-cAMP, and both 3βHSD mRNA and protein levels were determined. Treat-

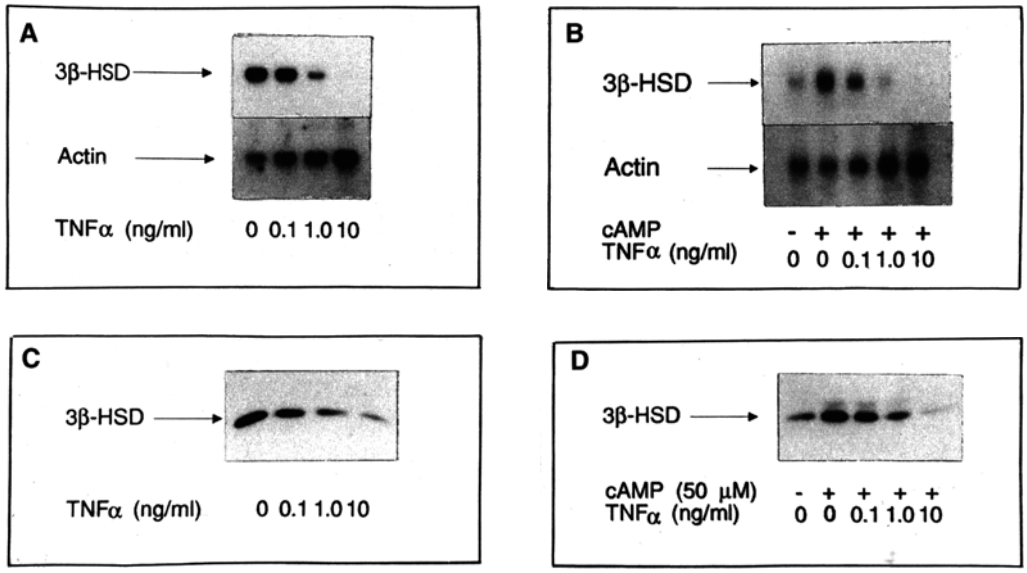

Fig. 2. Effects of TNF on basal and 8-Br-cAMP-stimulated 3β-HSD mRNA and protein levels. Leydig cells were maintained in culture for 5 d before the initiation of treatments. **(A)** Cells were treated for 24 h with control media or increasing concentrations of TNF (0.1, 1.0, and 10 ng/mL). Total RNA was extracted and subjected to Northern analysis. The blot was hybridized with ^{32}P-labeled 3βHSD cDNA (upper panel), and then stripped and rehybridized with ^{32}P-labeled β-actin cDNA (lower panel) **(B)**. Cells were treated for 24 h with control media, 50 μ*M* 8-Br-cAMP, or 8-Br-cAMP plus increasing concentrations of TNF (0.1, 1.0, and 10 ng/mL), and then subjected to Northern analysis as described in (A). **(C)** Cells were treated as described in (A), protein was extracted, subjected to Western blot and incubated with anti-3βHSD antibody (1:1000 dilution). Bound antibodies were visualized with ^{125}I-protein A and autoradiography. **(D)** Cells were treated as described in (B) and 3βHSD protein levels determined as described in (C). Autoradiographs are representative of at least three independent experiments.

ment with IL-1 inhibited 8-Br-cAMP-stimulated 3βHSD mRNA (Fig. 4B) by 21, 75, 93, and 94%, with 0.025, 0.25, 1.25, and 2.5 ng/mL IL-1, respectively (Fig. 5B). Previously, we demonstrated that 1.25 ng/ml IL-1 was completely inhibitory to cAMP-stimulated P450c17 mRNA expression *(8)*. The IL-1-mediated inhibition of 8-Br-cAMP-stimulated 3βHSD mRNA levels was reflected in a decrease in 3b-HSD protein levels (Fig. 4D), by 29, 31, 61, and 60% with treatments of 0.025, 0.25, 1.25, and 2.5 ng/mL IL-1, respectively (Fig. 5D). In marked contrast to the effect of TNF on basal 3βHSD expression, treatment with IL-1 did not inhibit basal 3βHSD mRNA (Figs. 4A and 5A) or protein (Figs. 4C and 5C) expression.

Discussion

The present study demonstrates that both TNF and IL-1 inhibit 8-Br-cAMP-stimulated 3βHSD mRNA and protein levels in a dose-dependent manner. In contrast, TNF, but not IL-1, represses basal testosterone production and 3βHSD mRNA and protein accumulation in Leydig cells. These data are consistent with the hypothesis that TNF, but not IL-1, inhibits basal testosterone production in Leydig cells by inhibiting the basal expression of 3βHSD.

We have reported previously that cAMP-stimulated testosterone production and cAMP-stimulated expression of P450scc and P450c17 are inhibited by TNF and IL-1 in Leydig cells *(2,8,9)*. In the current study, we show that cAMP-stimulated expression of 3βHSD is also inhibited by both TNF and IL-1. In marked contrast to the effects of TNF, however, IL-1 does not inhibit basal/constitutive expression of 3βHSD mRNA and protein or basal testosterone production. We reported previously that TNF inhibited cAMP-stimulated P450scc protein and mRNA expression, but that TNF did not inhibit basal P450scc expression *(9)*. The results of the present study therefore suggest that the TNF-mediated inhibition of basal 3βHSD expression may account, in part, for the TNF-mediated inhibition of basal testosterone production in primary cultures of mouse Leydig cells. Moreover, the effects of IL-1 and TNF on 3βHSD expression are reversible (data not shown), suggesting that these cytokines are not toxic Leydig cells, as we have shown previously *(8,9)*.

In contrast to TNF, IL-1 does not inhibit basal 3βHSD expression and steroidogenesis in Leydig cells. This striking observation reveals the difference between the action of these two proinflammatory cytokines in the regulation of basal Leydig cell function. TNF and IL-1 are cytokines principally synthesized and secreted from monocytes and macrophages. Both cytokines are encoded by different genes, have different amino acid sequences, and act via separate receptors, but exert similar biological activities (for review, *see 11–15*). Recent studies have provided considerable evidence to show overlapping and additive effects of TNF and IL-1 on the endocrine system. Though some controversy exists regarding the effects of these two cytokines, they appear to exert similar effects in the various

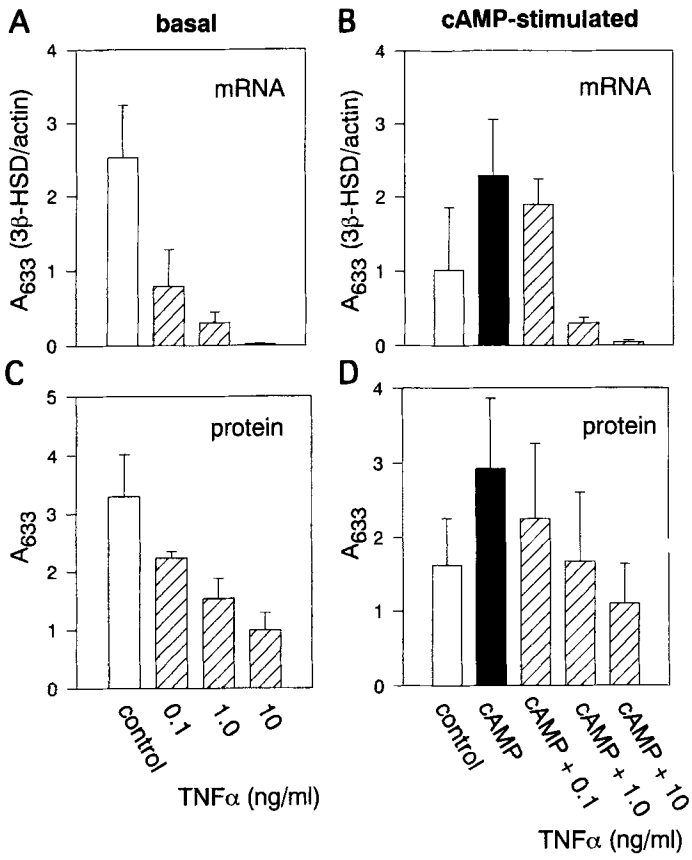

Fig. 3. Quantitation of the effects of TNF on 3βHSD mRNA and protein levels. Autoradiographs from at least three experiments were quantitated by densitometry. **(A)** Cells were treated as described in Fig. 2A and 3βHSD and β-actin RNA were quantitated by Northern blot. The ratio of A_{633} for 3βHSD/actin mRNA was determined. Data represent the mean ± SE for 5 experiments. **(B)** Cells were treated as described in Fig. 2B and 3βHSD and β-actin RNA were quantitated by Northern blot. The ratio of A_{633} for 3β-HSD/actin mRNA was determined. Data represent the mean ± SE for 5 experiments. **(C)** Cells were treated as described in Fig. 2C and 3βHSD protein was quantitated by Western blot. Data represent the mean ± SE for 3 experiments. **(D)** Cells were treated as described in Fig. 2D and 3βHSD protein was quantitated by Western blot. Data represent the mean ± SE for 3 experiments.

systems in which they have been analyzed. Both alter the steroidogenesis in testicular and ovarian cells (for review *see 7,16*), down-regulate parathyroid hormone receptors *(17)* and substance P receptors *(18)*, inhibit constitutive expression of cytochrome P-450-2C11 mRNA in rat hepatocytes *(19)*, stimulate prolactin secretion from rat anterior pituitary and dopamine release from the hypothalamus *(20)*, and stimulate the hypothalamo–pituitary–adrenocortical axis (for review, *see 21*). Differential effects of TNF and IL-1 on the endocrine system have also been reported. For example, TNF, but not IL-1, downregulates type II corticosteroid receptors in rat hippocampus *(22)*. In contrast, differential effects of TNF and IL-1 on one specific enzyme have never been reported. Both TNF and IL-1 inhibit cAMP-stimulated steroidogenic responses, but TNF alone inhibits basal steroidogenesis, indicating that these two proinflammatory cytokines act through different mechanisms.

Our finding that TNF, but not IL-1, inhibits basal 3βHSD expression is consistent with the view that these cytokines activate different signal transduction pathways in Leydig cells. Considerable progress has been made in the elucidation of the TNF signal transduction pathways in recent years. It is now well established that TNF signals through two receptors, type I (55 kDa) and type II (75 kDa). The TNF receptors belong to the low-affinity nerve growth factor receptor (NGF-R) superfamily, the defining characteristic of which is an extracellular domain containing cysteine repeats *(23)*. TNF homotrimers bind to type I or type II receptors, promote their aggregation, and activate intracellular domains. The activated intracellular domains bind other proteins and trigger signaling cascades*(23)*. Identification of the proteins that associate with the intracellular domains of TNF receptors, and steps following the association that lead to the activation of signaling cascades, are the subject of much current research *(24)*. Activation of three MAP kinase cascades (p42ERK, p38, and SAPK/JNK), activation of NF-κB, and stimulation of the sphingomyelin pathway (hydrolysis of sphingomyelin to ceramide) have been implicated in TNF signaling in various systems *(25)*.

We have demonstrated that TNF inhibition of P450c17 transcription involves activation of protein kinase C (PKC) in MA-10 tumor Leydig cells transiently transfected with *Cyp-17* reporter constructs, suggesting that TNF action in Leydig cells may also involve activation of PKC *(26)*. We have also shown that TNF activates AP-1 signaling pathways in MA-10 cells principally by stimulating the activity of stress-activated protein kinase/c-Jun nuclear kinase (SAPK/JNK). However, activation of AP-1 and SAPK/JNK is evidently not involved in blocking cAMP-stimulated steroidogenesis in Leydig cells *(27)*. Although activation of PKC may be involved in the TNF-mediated inhibition of cAMP-stimulated steroidogenesis, the mechanism(s) through which TNF inhibits basal steroidogenesis remain obscure.

Although much has been learned about TNF signaling, progress in understanding the mechanisms through which IL-1 signals has lagged behind. IL-1 comprises a family of three proteins, IL-1α, IL-1β, and IL-1 receptor antagonist (IL-1ra). Both IL-1α and β bind to the same cell-surface receptor and generally elicit similar cellular responses. IL-1α can be found on the surface of cells, whereas IL-1β is usually found in a soluble secreted form and not found associated with cells (for review, *see 15*). Two forms of IL-1 receptor have been identified. Type I IL-1 receptor is active and transduces IL-1 signals, but IL-1 type II receptor is a "decoy receptor" that binds IL-1 and prevents it from signaling. Within a few minutes of binding type I receptor, IL-1 induces several biochemical events. No sequential order or cascade for IL-1 signaling has been identified. Phospholipid, sphingomyelin, and guanine nucleotide hydrolysis following IL-1 receptor binding have been reported *(15)*. In general, the same intracellular signaling events attributed

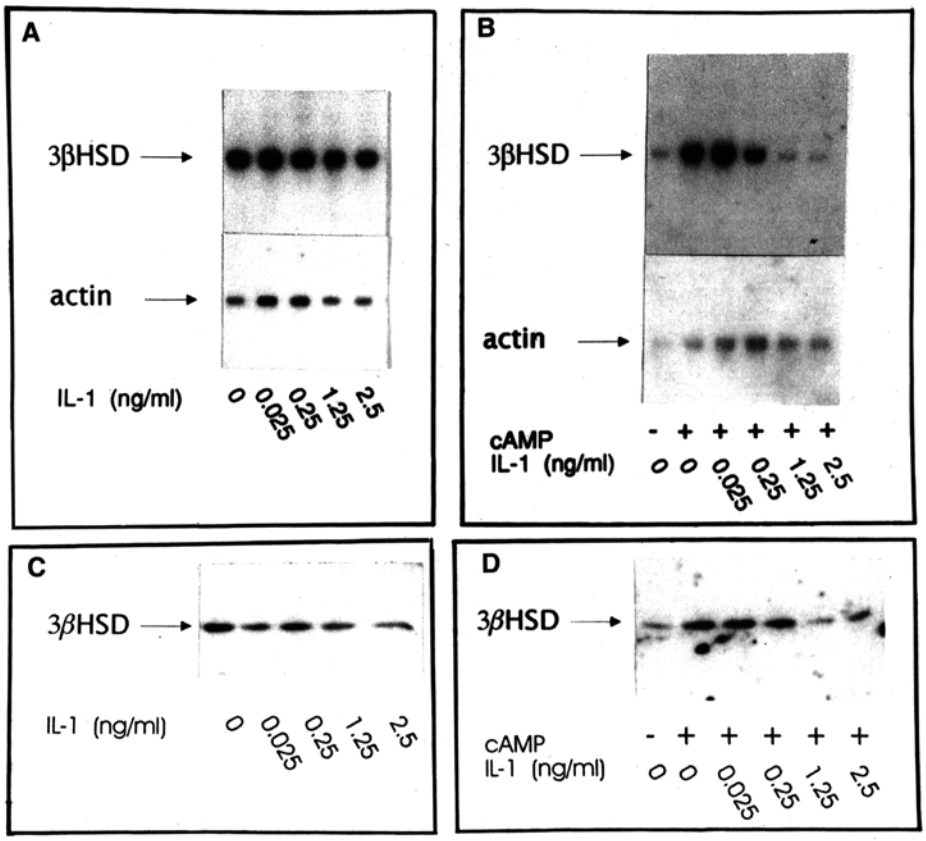

Fig. 4. Effects of IL-1 on basal and 8-Br-cAMP-stimulated 3βHSD mRNA and protein levels. Leydig cells were maintained in culture for 5 d before the initiation of treatments. **(A)** Cells were treated for 24 h with control media or increasing concentrations of IL-1 (0.025, 0.25, 1.25, and 2.5 ng/mL). Total RNA was extracted and subjected to Northern analysis. The blot was hybridized with ^{32}P-labeled 3βHSD cDNA (upper panel), and then stripped and rehybridized with ^{32}P-labeled b-actin cDNA (lower panel). **(B)** Cells were treated for 24 h with control media, 50 m*M* 8-Br-cAMP, or 8-Br-cAMP plus increasing concentrations of IL-1 (0.025, 0.25, 1.25, and 2.5 ng/mL), and then subjected to Northern analysis as described in (A). **(C)** Cells were treated as described in (A), protein was extracted, subjected to Western blot, and incubated with anti-3β-HSD antibody (1:1000 dilution). Bound antibodies were visualized with ^{125}I-protein A and autoradiography. **(D)** Cells were treated as described in (B) and 3βHSD protein levels determined as described in (C). Autoradiographs are representative of at least two independent experiments.

to TNF have been reported for IL-1 (for review, *see 28*). IL-1 signaling mechanisms in Leydig cells are unexplored, but the findings of the present study suggest that differences between IL-1 and TNF mechanism of action must exist to account for the differential effects that these two pro-inflammatory cytokines have on basal 3βHSD expression in mouse Leydig cells.

In summary, we have demonstrated that TNF, but not IL-1 inhibits basal 3βHSD mRNA and protein expression in Leydig cells, as well as inhibiting basal testosterone production. These data suggest that inhibition of basal 3βHSD expression may account for the TNF-mediated inhibition of basal steroidogenesis. Furthermore, the demonstration that IL-1 and TNF have differential effects on 3βHSD expression suggests these proinflammatory cytokines activate different signal transduction pathways in Leydig cells. Elucidation of the mechanisms through which IL-1 and TNF act in Leydig cells will offer greater insight into the mechanism of action of proinflammatory cytokines in immune and nonimmune cells.

Materials and Methods

Materials

Multiprimed labeling kit and [α-^{32}P]deoxycytidine triphosphate were purchased from Amersham Corp. (Arlington Heights, IL). Testosterone RIA kit was purchased from Diagnostic Products Corp. (Los Angeles, CA). [^{125}I]-protein A was purchased from ICN Biomedical, Inc. (Irvine, CA). Metrizamide, collagenase, 8-Br-cAMP, HEPES, BSA (fraction V), and bovine insulin were purchased from Sigma Chemical Inc. (St. Louis, MO). Medium 199, Dulbecco's Modified Eagle's Medium/Ham's nutrient mixture F-12 (DME/F12), penicillin-streptomycin, and guanidine isothiocyanate were obtained from Gibco-BRL (Gaithersburg, MD). Recombinant mouse TNFα was a gift from Genentech Corp. (South San Francisco, CA), and recombinant mouse IL-1β was purchased from R&D Systems (Minneapolis, MN). Mouse type I 3βHSD complementary DNA (cDNA) was a gift from A. H. Payne (Stanford University, Palo Alto, CA). Chicken β-actin cDNA was a gift from D. W. Cleveland (Johns Hopkins

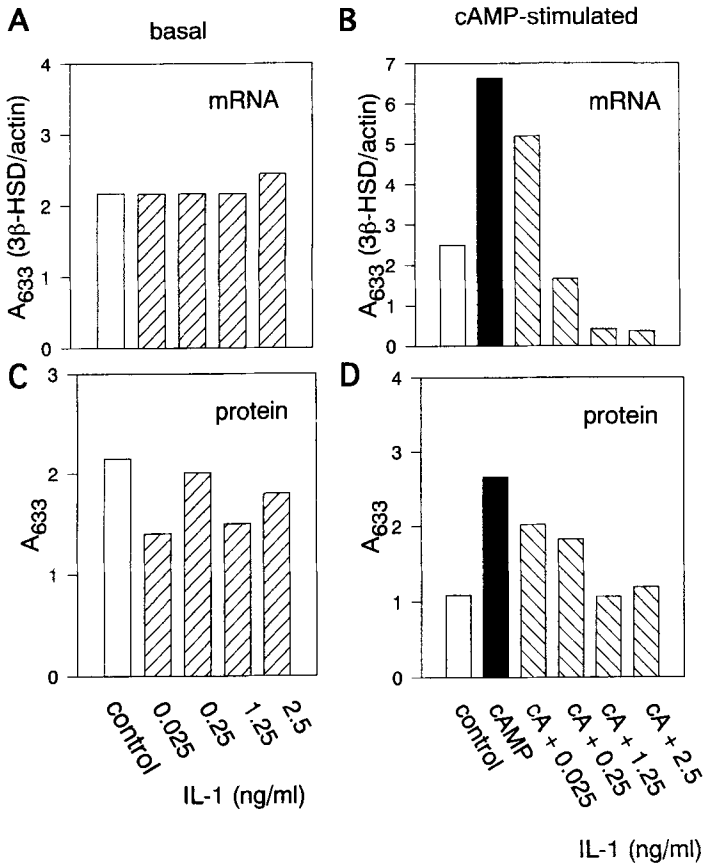

Fig. 5. Quantitation of the effects of IL-1 on 3βHSD mRNA and protein levels. Autoradiographs from at least two experiments were quantitated by densitometry. **(A)** Cells were treated as described in Fig. 4A, and 3βHSD and β-actin RNA were quantitated by Northern blot. The ratio of A_{633} for 3βHSD/actin mRNA was determined. **(B)** Cells were treated as described in Fig. 4B, and 3βHSD and β-actin RNA were quantitated by Northern blot. The ratio of A_{633} for 3βHSD/actin mRNA was determined. **(C)** Cells were treated as described in Fig. 4C, and 3b-HSD protein was quantitated by Western blot. **(D)** Cells were treated as described in Fig. 4D, and 3βHSD protein was quantitated by Western blot.

University, Baltimore, MD). Rabbit antihuman placental 3βHSD antisera were a gift from J. Ian Mason (Royal Infirmary, Edinburgh, Scotland, UK). All other reagents were from sources previously described *(9)*.

Animals

Adult, male outbred, pathogen-free CD-1 mice were purchased from Charles River Co. (Wilmington, MA). Mice were housed for at least 1 wk in groups of 5/cage. They were given food and water ad libitum and maintained on a 14-h light, 10-h dark schedule. The animals were procured, maintained, and used in accordance with the Animal Welfare Act and were killed by CO_2 asphyxiation. Experiments were performed when mice were 60–70 d old.

Isolation and Culture of Leydig Cells

Macrophage-depleted Leydig cells were isolated and cultured as described previously *(9)*. Leydig cells were cultured in serum-free DME/F12 culture medium (a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 supplemented with 2.2 g/L sodium bicarbonate, 10 m*M* HEPES, pH 7.4, 500 ng/mL insulin, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 1 mg/mL BSA) and incubated in a humidified atmosphere of 95% air, 5% CO_2 at 32°C. Leydig cell preparations were determined to be about 90% pure by histochemical staining for 3βHSD as described previously *(2)*. There was < 3% contamination by macrophages as determined by DiI-Ac-LDL staining *(29)*.

Treatment of Leydig Cells

Media were changed daily before the initiation of treatments on d 6 of culture. To determine effects of TNF and IL-1 on 8-Br-cAMP-stimulated 3βHSD mRNA expression, Leydig cells were treated for 24 h with control medium (supplemented DME/F12), 50 μ*M* 8-Br-cAMP, or 8-Br-cAMP plus increasing concentrations of TNF (0.1, 1.0, and 10.0 ng/mL) or IL-1 (0.025, 0.25, 1.25, and 2.5 ng/mL), and cells were lysed for RNA or protein extraction.

Total RNA Extraction and Northern Blotting Analysis

Total cellular RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform method as described previously *(8,9)*. The RNA was analyzed by Northern blotting as described *(8,9)*. Radioactivity was visualized by autoradiography and quantitated by densitometry. The results were normalized by comparison to β-actin levels.

Western Blotting Analysis

The total protein was extracted from Leydig cells by applying 100 μL of 0.1% SDS to each culture dish. Protein concentration was determined by the deoxycholate-trichloroacetic acid (TCA) precipitation modification of the Lowry method *(30)*. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli *(31)*, such that 20 μg of protein from cultures were applied to each lane of a given gel. Western blot was performed as described previously *(9)*. Blots were incubated with anti-3βHSD antisera (1:1000 dilution) at 4°C overnight. Bound antibodies were detected with ^{125}I-protein A, visualized by autoradiography, and quantitated by densitometry.

Testosterone RIA

Culture media were boiled for 5 min and centrifuged at 2000*g* for 20 min at 4°C. The supernatant was stored at –20°C until assayed for testosterone by using Coat-A-Count RIA kits. Results were normalized/10^6 Leydig cells.

Statistics

Data were presented as means ± SE of 3 or more independent experiments. For group comparison, one-way analysis of variance followed by a Student-Newmann-Keuls multiple-range test was performed using the GraphPad InStat, version 2.0, statistical software package (GraphPad

Software, San Diego, CA). Differences were considered as significant at $P < 0.05$.

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References

1. Saez, J. M. (1994). *Endocr. Rev.* **15,** 574–626.
2. Hales, D. B., Xiong, Y., and Tur-Kaspa, I. (1992). *J. Steroid Biochem. Mol. Biol.* **43,** 907–914.
3. Xiong, Y. and Hales, D. B. (1993). *Endocrinology* **133,** 2568–2573.
4. Hayes, R., Chalmers, S. A., Nikolics Paterson, D. J., Atkins, R. C., and Hedger, M. P. (1996). *J. Androl.* **17,** 41–49.
5. Kern, S., Robertson, S. A., Mau, V. J., and Maddocks, S. (1995). *Biol. Reprod.* **53,** 1407–1416.
6. Hutson, J. C. (1993). *J. Reprod. Immunol.* **23,** 63–72.
7. Hales, D. B. (1996). in: *The Leydig Cell.* Payne, A. H., Hardy, M. P., and Russell, L. D. (eds.) Cache River Press, Vienna, IL, pp. 451–466.
8. Hales, D. B. (1992). *Endocrinology* **131,** 2165–2172.
9. Xiong, Y. and Hales, D. B. (1993). *Endocrinology* **132,** 2438–2444.
10. Payne, A. H. and O'Shaughnessy, P. J. (1996). in The Leydig Cell, eds. Payne, A. H., Hardy, M. P., and Russell, L. D. (Cache River Press, Vienna, IL), pp. 259–285.
11. Akira, S., Hirano, T., Taga, T., and Kishimoto, T. (1990). *FASEB J.* **4,** 2860–2867.
12. Le, J. and Vilcek, J. (1987). *Lab. Invest.* **56,** 234–248.
13. Stewart, R. J. and Marsden, P. A. (1995). *Am. J. Kidney Dis.* **25,** 954–966.
14. Riches, D. W., Chan, E. D., and Winston, B. W. (1996). *Immunobiology* **195,** 477–490.
15. Dinarello, C. A. (1996). *Blood* **87,** 2095–2147.
16. Terranova, P. F., and Rice, V. M. (1997). *Am. J. Reprod. Immunol.* **37,** 50–63.
17. Katz, M. S., Gutierrez, G. E., Mundy, G. R., Hymer, T. K., Caulfield, M. P., and McKee, R. L. (1992). *J. Cell Physiol.* **153,** 206–213.
18. Johnson, C. L. and Johnson, C. G. (1991). *Brain Res.* **564,** 79–85.
19. Chen, J. Q., Strom, A., Gustafsson, J. A., and Morgan, E. T. (1995). *Mol. Pharmacol.* **47,** 940–947.
20. Yamaguchi, M., Koike, K., Yoshimoto, Y., Ikegami, H., Miyake, A., and Tanizawa, O. (1991). *Endocrinol. Jpn.* **38,** 357–361.
21. Besedovsky, H. O. and Del Rey, A. (1996). *Endocr. Rev.* **17,** 64–102.
22. Betancur, C., Borrell, J., and Guaza, C. (1995). *Neuroendocrinology* **62,** 47–54.
23. Naismith, J. H. and Sprang, S. R. (1996). *J. Inflam.* **47,** 1–7.
24. Fiers, W., Beyaert, R., Boone, E., Cornelis, S., Declercq, W., Decoster, E., et al. (1996). *J. Inflamm.* **47,** 67–75.
25. Introna, M. and Mantovani, A. (1997). *Arterioscler. Thromb. Vasc. Biol.* **17,** 423–428.
26. Li, X., Youngblood, G. L., Payne, A. H., and Hales, D. B. (1995). *Endocrinology* **136,** 3519–3526.
27. Li, X., Held Hales, K., Watanabe, G., Lee, R. J., Pestell, R. G., and Hales, D. B. (1997). *Endocrine*, in press.
28. Bankers–Fulbright, J. L., Kalli, K. R., and McKean, D. J. (1996). *Life Sci.* **59,** 61–83.
29. Pitas, R. E., Innerarity, T., Weinstein, J. N., and Mahley, R. W. (1981). *Arteriosclerosis* **1,** 177–185.
30. Bensadoun, A. and Weinstein, D. (1976). *Anal. Biochem.* **70,** 241–250.
31. Laemmli, U. K. (1970). *Nature* **227,** 680–685.