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THE ROLE OF CYTOKINES IN THE REGULATION OF LEYDIG CELL *P*450c17 GENE EXPRESSION

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Summary—Cytokines produced by immune-activated testicular interstitial macrophages (TIMs) may play a fundamental role in the local control mechanisms of testosterone biosynthesis in Leydig cells. We investigated whether *in vivo* immune-activation of TIMs can modulate Leydig cell steroidogenesis. To immune activate TIMs *in vivo*, mice were injected intraperitoneally (i.p.) with lipopolysaccharide (LPS, 6 mg/kg). TIMs and Leydig cells were purified for RNA analysis. LPS treatment resulted in a 47-fold increase in interleukin-1β (IL-1β) mRNA in TIMs. *P*450c17 mRNA levels in the Leydig cells from the same animals, decreased to less than 10% compared to control. The effect of LPS on IL-1β and *P*450c17 mRNA levels was reversible on both TIMs and Leydig cells, respectively. To determine if the effect of LPS on P450c17 was mediated by a possible decrease in pituitary LH secretion, mice were co-injected with LPS and hCG. Treatment with hCG did not change the effect observed with LPS alone, in TIMs or in Leydig cells. *In vitro*, LPS treatment of TIMs resulted in marked induction of IL-1β mRNA expression. In parallel, *in vitro* treatment of Leydig cells with recombinant IL-1 resulted in a dose-dependent inhibition of *P*450c17 mRNA expression and testosterone production. These data demonstrate that LPS treatment, *in vivo* and *in vitro*, induced IL-1 gene expression in TIMs, and that IL-1 inhibits *P*450c17 mRNA *in vitro*. Therefore, we suggest that immune-activation of TIMs might have caused the observed inhibition of *P*450c17 gene expression in Leydig cells *in vivo*.

INTRODUCTION

Leydig cells and testicular interstitial macrophages (TIMs) are the most prominent cells of the interstitial tissue in the mammalian testis. Leydig cells and TIMs are intimately associated and form specialized junctions [1]. This close association suggests that cytokines produced by TIMs may play a fundamental role in the local control mechanisms of testosterone biosynthesis in Leydig cells.

Pathological conditions, that are associated with immune-activation of macrophages, such as chronic inflammation and infection, often result in impaired male reproductive function. Testosterone is essential for the initiation of spermatogenesis and the maturation of sperm [2]. Macrophage-secreted cytokines, such as interleukin-1 (IL-1), have been shown to modulate steroid secretion by Leydig cells [3–13]. However, the expression of cytokines by testicular macrophages has not yet been investigated.

We have recently established a procedure to purify TIMs and Leydig cells from the same preparation of mouse testicular interstitial cells. This method allows an investigation of the possible role of macrophage-secreted cytokines in Leydig cell function. We examined whether *in vivo* immune-activation of TIMs by bacterial endotoxin (lipopolysaccharide, LPS) modulated Leydig cell steroidogenesis. The effect of immune-activation was examined by measuring mRNA levels of the cytokine, IL-1, in TIMs, and at the same time, Leydig cell function was determined by steroidogenic enzyme RNA analysis and by measuring testosterone production. Furthermore, we tested whether similar results could also be obtained in both TIMs and Leydig cells *in vitro*.

EXPERIMENTAL

Materials

Multiprimed labeling kit and [α-^{32}P]dCTP were purchased from Amersham (Arlington

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Hts, IL). LPS (rough strain from *E. coli*), 8-Br-cAMP (cAMP), hCG, *N*-2-hydroxymethyl-piperazine-*N'*-2-ethanesulfonic acid (HEPES), bovine insulin, Metrizamide, bovine serum albumin (BSA, fraction V), collagenase, paraformaldehyde, nitroblue tetrazolium, and 3β-hydroxy-androstan-17-one were obtained from Sigma (St Louis, MO). Sodium dodecyl sulfate (SDS) was purchased from Bio-Rad (Richmond, CA). Medium 199, Dulbecco's modified Eagle's medium/Ham's nutrient mixture F-12, penicillin–streptomycin, guanidine isothiocyanate, and agarose were obtained from GIBCO/BRL (Bethesda, MD). Acetylated low density lipoprotein labeled with 1,1'-dioctadecyl-1-3,3,3',3'-tetramethyl-indo-carbocyanine perchlorate (DiI-Ac-LDL) was purchased from Biomedical Technologies (Stoughton, MA). Testosterone radioimmunoassay (RIA) kits were obtained from Diagnostic Products Corp. (Los Angeles, CA.) Murine recombinant interleukin-1α (sp. act. = 8×10^6 U/mg) was purchased from Genzyme (Boston, MA). Rat *P*450c17 cDNA was a gift from Dr R. Fevold (University of Montana, Missoula, MT). Murine interleukin-1β cDNA was a gift from Genentech (South San Francisco, CA). Chicken β-actin cDNA was a gift from Dr D. W. Cleveland, (Johns Hopkins University, Baltimore, MD).

Animals

Adult, male, outbred, pathogen-free CD-1 mice were obtained from Charles River Co. (Wilmington, MA). Mice were housed for at least 1 week in groups of 5 in a cage. They were fed commercial feed and water *ad libitum* and maintained on a 12-h light–dark schedule. The animals were procured, maintained, and used in accordance with the Animal Welfare Act and were sacrificed by cervical dislocation or CO_2 asphyxiation. Experiments were performed when the mice were 60–70 days old.

Methods

In vivo experiments

LPS in vivo treatments. LPS was dissolved in sterile phosphate-buffered saline (PBS) and was injected intraperitoneally (i.p.) once (6 mg/kg). An equal volume of sterile PBS was injected i.p. into the control mice. Each treatment group consisted of 5 mice. Mice were sacrificed 24 or 120 h after the i.p. injection. Testes were removed and both TIMs and Leydig cells were isolated for RNA analysis as described below.

LPS and hCG in vivo treatments. Mice were divided into 4 groups of 5 animals each. The first two groups were injected with LPS or PBS alone as described above. The third and fourth groups were injected with LPS or PBS and, in addition, received 4 doses of 10 μg of hCG subcutaneously (s.c.) every 12 h, similar to the protocol described by Payne *et al.* [14]. Mice were sacrificed 48 h after the beginning of the treatment, the TIMs and Leydig cells were isolated for RNA analysis.

Isolation and culture of TIMs. Mice were sacrificed and the testes were removed aseptically. All other procedures were carried out under sterile conditions. Testes were decapsulated and dispersed in medium containing 0.75 mg/ml collagenase by vigorous shaking for 10 min at 37°C. Tubules were removed by sedimentation on ice for 2 min. The crude interstitial cells were collected by centrifugation at 600 *g* for 20 min then resuspended in medium 199 supplemented with 2.2 g/l sodium bicarbonate, 10 mM HEPES, pH 7.4, 500 ng/ml insulin, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 1 mg/ml BSA (M199). The method to isolate macrophages was adapted from Yee and Hutson [15]. The cell suspension was plated in culture dishes and incubated in a humidified atmosphere of 95% air, 5% CO_2 at 32°C, for 25 min to allow macrophages to attach to the plastic culture dish. The nonadherent cells were removed by collecting the supernatant and rinsing the dishes with M199 twice. TIMs were then either lysed for RNA analysis or cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine (RPMI) and incubated in a humidified atmosphere of 95% air, 5% CO_2 at 32°C. Using DiI-Ac-LDL staining [16], this cell preparation was determined to be 75–80% positive for TIMs. Histochemical staining for β-hydroxysteroid dehydrogenase (3β-HSD), as described below, demonstrated $<3\%$ contamination by Leydig cells. The other cells in this preparation were sperm, fibroblasts and peritubular cells.

Isolation and culture of Leydig cells. Leydig cells were purified from the nonadherent, TIMs-depleted, crude interstitial cell fraction by centrifugation through an 11–23% Metrizamide density gradient as described previously [17]. Purified Leydig cells were either lysed for RNA analysis or resuspended in serum-free DME/F12 culture medium (a 1:1 mixture of

Dulbecco's modified Eagles medium and Ham's nutrient mixture F-12 supplemented with 2.2 g/l sodium bicarbonate, 10 mM HEPES, pH 7.4, 500 ng/ml insulin, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 1 mg/ml BSA). Cells were plated at a density of 1×10^5 cells/cm^2 and incubated in a humidified atmosphere of 95% air, 5% CO_2 at 32°C. Media were changed every 24 h prior to the initiation of treatment on day 6 of culture.

Leydig cell preparations were determined to be 85–90% pure by histochemical staining for 3β-HSD, performed by a modification of the method described by Wiebe [18]. Twenty microliters of resuspended cell, appropriately diluted, were applied to glass slides and allowed to air dry for 15 min at 22°C. The cells were then covered with a solution of 1% paraformaldehyde and fixed for 30 min. The fixed cells were then incubated in a humidified chamber for 90 min at 32°C in PBS containing 0.1% BSA, 1.5 mM NAD+, 0.25 mM nitroblue tetrazolium, and 0.2 mM 3β-hydroxy-androstan-17-one. After staining, the cells were rinsed thoroughly with distilled H_2O and post-fixed with 3% formalin for 15 min. Cells were examined by light microscopy and cells containing dark blue formazan deposits, indicating the presence of 3β-HSD, were considered Leydig cells. A minimum of 500 cells were counted in 20 fields and the ratio of positive staining cells to total cells was calculated. There was <1% contamination by macrophages as determined by staining with DiI-Ac-LDL. Furthermore, there was no IL-1β transcript detected when Northern blots of Leydig cell RNA from control or LPS injected mice were probed with ^{32}P-labeled IL-1β cDNA (data not shown).

Analysis of RNA. Total cellular RNA was extracted and subjected to Northern analysis as described previously [19]. Briefly, cells were extracted with a solution of 4 M guanidine isothiocyanate, 25 mM Na citrate, pH 7.0, 0.5% Na lauroyl sarcosine, 0.1 M 2-mercaptoethanol. Prior to phenol–chloroform extraction 0.1 vol of 2 M Na acetate, pH 4.0 were added. RNA was precipitated twice with an equal volume of isopropanol and washed twice with ethanol. The precipitated RNA was dissolved in 10 mM Tris, pH 7.2, 1 mM EDTA and its concentration was determined by measuring its absorbance at 260 nm. Five micrograms of total RNA from Leydig cells or 10 μg of total RNA from TIMs were resuspended in 50% formamide, 2.2 M formaldehyde, 20 mM 3-[*N*-morpholino]propanesulfonic acid (MOPS), pH 7.0, and subjected to electrophoresis in 1.2% agarose containing 2.2 M formaldehyde and 20 mM MOPS, pH 7.0. RNA was transferred overnight by capillary blotting in 10 × SCC (1 × SSC = 0.15 M NaCl, 0.15 M Na citrate, pH 7.0) to nitrocellulose (Millipore Corp., Bedford, MA). The blots were then hybridized overnight at 48°C with 10^6 cpm/ml ^{32}P-labeled cDNA. DNA probes were radiolabeled with [α-^{32}P]dCTP using the multiprimed labeling kit. Following hybridization, the blots were washed in 2 × SSC/0.1% SDS twice in room temperature for 15 min, and then for 30 min at 60°C. Radioactivity was visualized by autoradiography and quantitated by densitometry.

In vitro experiments

LPS activation of TIMs in vitro. TIMs cultures were incubated for 24 h prior to initiation of treatment with control medium (RPMI) or 1 μg/ml LPS. Before and after 6 h of treatment, cells were lysed for RNA extraction.

IL-1 treatment of Leydig cells in vitro. Leydig cell cultures were maintained for 6 days prior to the initiation of treatment. Treatments consisted of control medium (DME/F12), 50 μM 8-Br-cAMP (cAMP), or cAMP plus increasing concentrations of IL-1 (0.2, 2, 5, 10 and 20 U/ml). Media were collected for testosterone RIA and replaced every 24 h for 48 h. After 48 h of treatment cells were lysed for RNA extraction.

Statistics. Comparisons between treatment groups were made using Student's *t*-test. A probability level of <0.05 was accepted as a significant difference between means. The results are presented as means ± SEM of three or more experiments.

RESULTS

In vivo experiments

LPS in vivo treatments: IL-1β expression in TIMs. LPS has been shown to immune-activate macrophages to secrete cytokines such as IL-1. To try to activate TIMs without direct manipulation of the testes, mice in groups of 5 were given a single i.p. injection of LPS. Twenty four and 120 h later, TIMs were isolated and IL-1β mRNA levels were quantitated. In TIMs from control animals, IL-1β mRNA was barely detected (Fig. 1). LPS treatment increased IL-1β mRNA by 47 ± 5.4-fold compared to control after 24 h, however, 120 h after LPS injection IL-1β mRNA levels returned to control levels

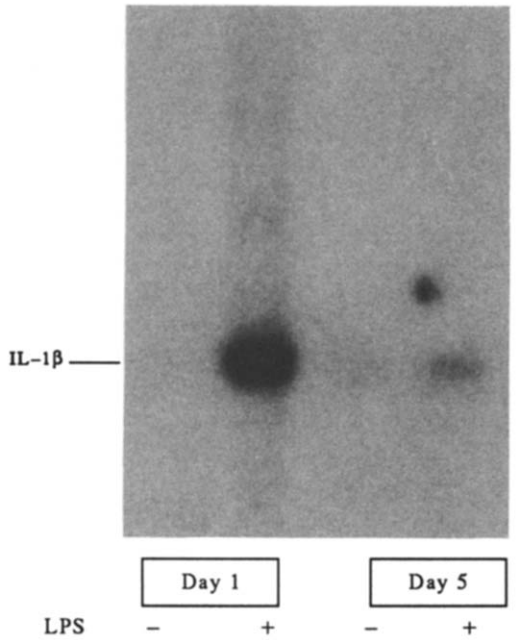

Fig. 1. IL-1β mRNA expression in TIMs following LPS treatment *in vivo*. Mice were injected i.p. with LPS and 24 h (day 1) or 120 h (day 5) later TIMs were isolated, RNA was extracted, subjected to Northern analysis, and hybridized with ^{32}P-labeled IL-1β cDNA.

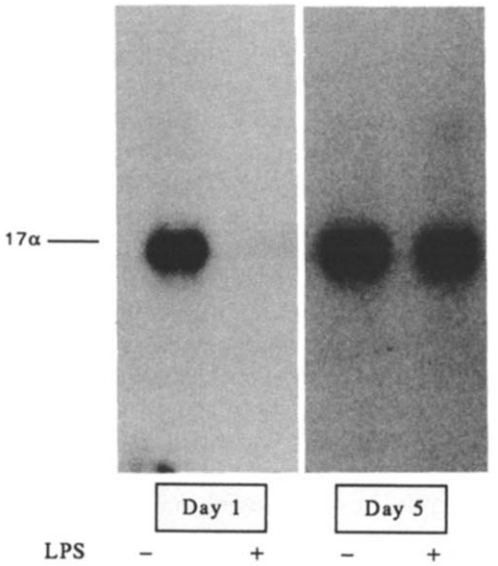

Fig. 2. *P*450c17 mRNA expression in Leydig cells following LPS treatment *in vivo*. RNA was extracted from Leydig cells isolated in parallel to the TIMs shown in Fig. 1, subjected to Northern analysis, and hybridized with ^{32}P-labeled *P*450c17 cDNA.

(Fig. 1). These data demonstrate that i.p. LPS treatment results in immune-activation of TIMs as measured by IL-1β mRNA induction. Furthermore, this effect was reversed 5 days after LPS injection.

LPS in vivo treatments: P450c17 expression in Leydig cells. Leydig cells were isolated in parallel from the same LPS-treated mice in which TIMs RNA was examined, as described above. *P*450c17 mRNA levels decreased to <10% of control 24 h after the LPS injection. *P*450c17 mRNA recovered to 80% of control at 120 h after the LPS injection (Fig. 2). These data demonstrate that i.p. LPS treatment results in a reversible inhibition of *P*450c17 gene expression in Leydig cells.

LPS and hCG in vivo treatments: IL-1β expression in TIMs and P450c17 expression in Leydig cells. In order to determine if the effect of LPS was due to a possible decrease in pituitary luteinizing hormone (LH) secretion, mice were co-injected with LPS and hCG. TIMs and Leydig cells were isolated 48 h after injection with LPS. Treatment with hCG had no effect on the LPS-induction of IL-1β mRNA in TIMs (data not shown). Treatment with hCG alone resulted in a 2-fold increase in Leydig cells *P*450c17 mRNA (Fig. 3). Treatment with LPS plus hCG decreased *P*450c17 to 32% compared to hCG alone, or 64% compared to PBS-injected control. Treatment with LPS alone decreased *P*450c17 to 43% compared to PBS-injected control (Fig. 3). Actin mRNA levels were unaffected by either LPS or hCG. Figure 3 demonstrates that the *in vivo* effect of LPS on Leydig cell *P*450c17 expression is not mediated through LH.

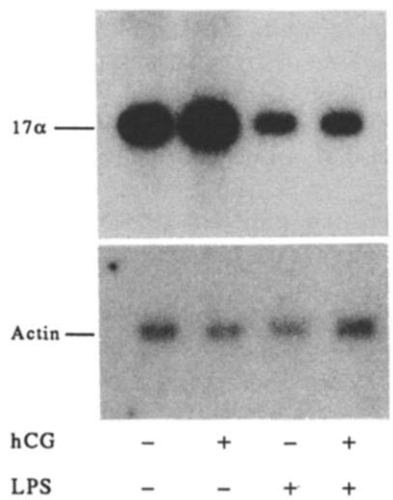

Fig. 3. The effect of LPS and hCG co-injection on *P*450c17 mRNA levels in Leydig cells. Mice were injected with PBS (−/−), hCG (10 μg, twice daily, +/−), LPS (−/+), or LPS plus hCG (+/+). Leydig cells were isolated 48 h post-LPS injection, subjected to Northern analysis, and hybridized with ^{32}P-labeled *P*450c17 cDNA (upper panel). Blot was stripped and re-hybridized with ^{32}P-labeled chicken β-actin cDNA (lower panel).

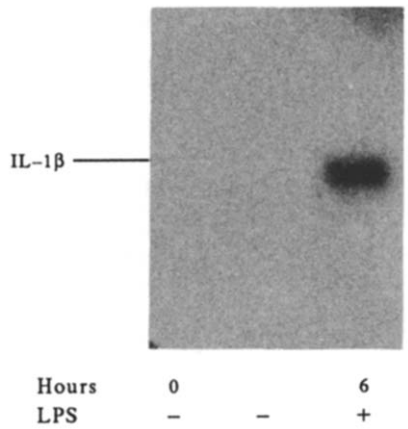

Fig. 4. Induction of IL-1β mRNA in TIMs *in vitro*. TIMs were isolated from untreated mice and cultured in RPMI medium containing 10% fetal bovine serum. Cultures were maintained for 24 h prior to the initiation of treatment with 1 μg/ml LPS. RNA was extracted before (0) or 6 h after the addition of LPS (+).

In vitro experiments

LPS activation TIMs in vitro. TIMs in primary culture were treated with LPS to determine if they express IL-1β mRNA, analogous to *in vivo* immune-activated TIMs. TIMs were cultured for 24 h prior to initiation of treatment with LPS to avoid complications due to adherence-stimulated induction of IL-1 mRNA [20]. RNA was extracted before and 6 h after the addition of LPS. Treatment with LPS caused a marked induction of IL-1β mRNA compared to untreated controls, where IL-1β mRNA was undetectable I(Fig. 4).

Table 1. Effect of IL-1 on cAMP-stimulated testosterone production in Leydig cells *in vitro*

Treatments	Testosterone[c]	% of cAMP alone
Control	17 ± 4.9	1.0 ± 28.0
cAMP[a]	1753 ± 181.8[d]	100.0
cAMP + 0.2 U/ml IL-1[b]	1097 ± 263.7[d,e]	62.6 ± 24.0
cAMP + 2 U/ml IL-1	707 ± 210.5[d,e]	40.3 ± 29.8
cAMP + 10 U/ml IL-1	45 ± 4.0[e]	2.6 ± 8.9
cAMP + 20 U/ml IL-1	50 ± 10.2[e]	2.9 ± 20.2

[a]cAMP: 50 μM 8-Br-cAMP.
[b]IL-1: murine recombinant IL-1α.
[c]Testosterone: ng · 10^{-6} Leydig cells · 24 h^{-1} ± SEM of 3 experiments, with duplicate incubations.
[d]Significantly different from control ($P < 0.05$).
[e]Significantly different from cAMP-alone ($P < 0.05$).

Effect of IL-1 in vitro on cAMP-stimulated P450c17 expression and testosterone production. To determine if cytokines affect Leydig cell function *in vitro*, similar to the observed inhibition in immune-activated mice *in vivo*, recombinant murine IL-1 was added to macrophage-depleted, purified Leydig cells in primary culture. Leydig cells were treated with 8-Br-cAMP (cAMP) alone and with cAMP plus increasing concentrations of IL-1 (Fig. 5). Treatment with cAMP alone caused a marked increase in *P*450c17 mRNA compared to untreated controls. IL-1 decreased cAMP-stimulated *P*450c17 mRNA levels in a dose-dependent fashion. Treatment with cAMP plus 0.2 U/ml IL-1 decreased *P*450c17 mRNA by 31 ± 9%, plus 2 U/ml by 82 ± 12%, and by 100% with 10 or 20 U/ml, compared to

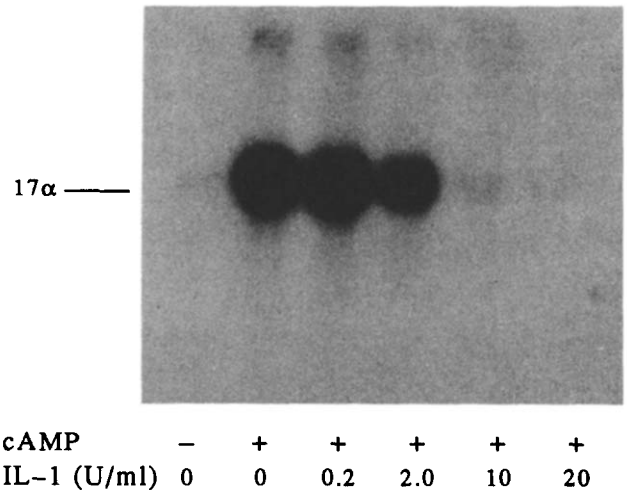

Fig. 5. The effect of IL-1 *in vitro* on cAMP-stimulated *P*450c17 mRNA levels in Leydig cells. Macrophage-depleted, purified Leydig cells were maintained in serum-free medium for 6 days prior to the initiation of treatment with control medium (–), 50 μM 8-Br-cAMP (cAMP), or cAMP plus increasing concentrations of murine recombinant IL-1. Cells were treated for 48 h, RNA was extracted, subjected to Northern analysis, and hybridized with ^{32}P-labeled *P*450c17 cDNA.

cells treated with cAMP alone. IL-1 caused a dose-dependent inhibition of cAMP-stimulated testosterone production (Table 1). Treatment with cAMP alone resulted in a 60-fold increase in testosterone accumulation in 24 h compared to untreated controls ($P < 0.05$). Treatment with cAMP plus 0.2 and 2 U/ml IL-1 caused a significant decrease in testosterone production to 63 ± 14, and $41 \pm 19\%$ of cAMP alone, respectively. Treatment with 10 and 20 U/ml IL-1 decreased testosterone production to $< 5\%$ of cells treated with cAMP alone.

DISCUSSION

The results of this study demonstrate that LPS treatment both *in vivo* and *in vitro* induced IL-1 gene expression in TIMs. This is the first report to demonstrate that immune-activation of resident testicular macrophages increases cytokine mRNA levels. In Leydig cells isolated in parallel from the *in vivo* immune-activated mice we demonstrated a significant and reversible decrease in *P*450c17 mRNA. Furthermore, treatment of Leydig cells *in vitro* with IL-1 resulted in a similar decrease in *P*450c17 mRNA and a significant decrease in testosterone production. Therefore, we suggest that immune-activation of TIMs, as measured by induction of IL-1 mRNA, might have caused the observed inhibition of *P*450c17 gene expression of Leydig cell *in vivo*. This study thus supports the hypothesis that cytokines produced by immune-activated TIMs may play a fundamental role in the local control mechanisms of testosterone biosynthesis in Leydig cells (Fig. 6).

Macrophage-secreted cytokines, such as IL-1, have been shown recently to be involved in the regulation of reproductive function [21]. Leydig cells are the site of testosterone production in the testis. Leydig cells and TIMs are intimately associated and form specialized junctions [1] which suggests that an important functional relationship exists between these cells. Recent studies [3–5, 7, 10, 11] demonstrated that IL-1 affects Leydig cell steroidogenesis by inhibiting gonadotropin and cAMP-stimulated testosterone production in rat Leydig cells *in vitro*. Tumor necrosis factor-α, another macrophage-secreted cytokine, has also been shown to inhibit Leydig cell steroidogenesis *in vitro* [6, 12, 13] and *in vivo* [8]. However, some investigators have demonstrated that IL-1 increased basal [3, 9, 11] or gonadotropin-stimulated [9] steroid production *in vitro*. The reason for the discrepancies in these results are not apparent, but may result from different experimental methodology.

In order to allow us to investigate the possible role of macrophage-secreted cytokines in Leydig cell function at the molecular level, we recently established a method to purify both TIMs and Leydig cells from the same preparation of

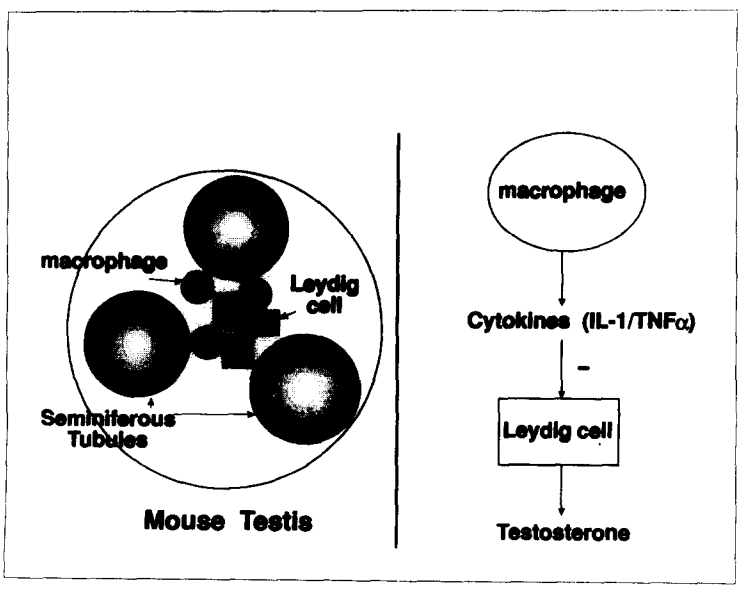

Fig. 6. Intra-testicular interaction of Leydig cells and macrophages. The left panel illustrates the close association of Leydig cells and macrophages (TIMs) in the interstitial tissue of the mouse testis. The right panel depicts immune-activated macrophages (TIMs) secreting cytokines [IL-1 and/or tumor necrosis factor-α (TNFα)] that exert a paracrine regulatory influence on Leydig cell function by inhibiting testosterone biosynthesis.

mouse testicular interstitial cells. This model allows an investigation of the effect of immune-activation of TIMs, as measured by induction of IL-1 mRNA, on Leydig cell steroidogenesis, both *in vivo* and *in vitro*. This model offers a procedure to immune-activate TIMs without direct manipulation of the testis. This is done by injecting LPS i.p. LPS is a constituent of the cell wall of most gram negative bacteria that has been shown to induce IL-1 expression in peritoneal macrophages, both *in vivo* [22] and *in vitro* [23]. We demonstrated here that i.p. LPS injection induces IL-1 mRNA in TIMs (Fig. 1). The dose of i.p. LPS (6 mg/kg) was empirically determined to achieve maximal immune activation of macrophages with no significant incidence of mouse-mortality compared to PBS-injected control. We have not yet shown secretion of cytokine protein from activated TIMs. However, supernatants from LPS-stimulated macrophages inhibited testosterone production in Leydig cells [24]. In addition, conditioned media from LPS-stimulated P388 cells, a murine monocyte-macrophage cell line, inhibited Leydig cell steroidogenesis (Hales D. B. and Okuno W. T. unpublished data). Therefore, we conclude that i.p. LPS immune activates TIMs to express, and probably, to secrete IL-1.

Since these Leydig cell preparations are TIMs-depleted, they provide a better system to investigate intratesticular immune-endocrine interactions. Moreover, there is no significant loss of Leydig cells during this modified isolation procedure. By 3β-HSD histochemical staining, and DiI-Ac-LDL uptake, this preparation was determined to contain 85–90% Leydig cells and $<1\%$ TIMs. Furthermore, there was no IL-1β transcript detected when Northern blots of Leydig cell RNA from control or LPS injected mice were probed with ^{32}P-labeled IL-1β cDNA (data not shown).

Leydig cells are the site of testosterone biosynthesis in the testis. *P*450scc and *P*450c17 are key enzymes in this important process. *P*450scc catalyzes the conversion of cholesterol to pregnenolone, and *P*450c17 catalyzes both progesterone 17α-hydroxylase/C17,20 lyase reactions, and is required for conversion of progesterone to testosterone. LPS treatment *in vivo* had no significant effect on the expression of *P*450scc (data not shown). However, *P*450c17 mRNA levels decreased to $<10\%$ of control levels 24 h after LPS injection and recovered to 80% of control at 120 h (Fig. 2). These data demonstrate that i.p. LPS treatment results in a reversible inhibition of *P*450c17 gene expression in Leydig cells.

In order to determine if the effects of LPS were due to a possible decrease in pituitary LH secretion, mice were co-injected with LPS and hCG. Treatment with hCG had no effect on either the LPS-induction of IL-1 mRNA in TIMs or on the LPS-mediated decrease in *P*450c17 mRNA levels in Leydig cells (Fig. 3). Thus the *in vivo* effect of LPS on Leydig cell *P*450c17 expression is probably not mediated through changes in LH, but rather, through an intratesticular regulatory mechanism.

LPS treatment of TIMs in primary culture resulted in a marked induction of IL-1β mRNA (Fig. 4). These data demonstrate that resident TIMs are LPS responsive and capable of expression IL-1β mRNA. Further studies will examine the synthesis and secretion of IL-1 protein from LPS-stimulated TIMs *in vitro*.

To determine if IL-1 affected Leydig cell function *in vitro*, recombinant murine IL-1 was added to primary cultures of Leydig cells. IL-1 had no effect on basal testosterone production (data not shown), however, IL-1 inhibited cAMP-stimulated *P*450c17 expression (Fig. 5) and testosterone production (Table 1) in a dose-dependent manner. LPS treatment of Leydig cell cultures, *in vitro*, had no effect on the level of cAMP-stimulated *P*450c17 mRNA (data not shown). This further supports the hypothesis that the decrease in *P*450c17 mRNA seen in immune-activated mice *in vivo* may be mediated by activation of TIMs, rather than by a direct effect of LPS on Leydig cells.

Recombinant murine IL-1α was used for the *in vitro* studies similar to other investigations on the effects of IL-1 on Leydig cell steroidogenesis [5, 11]. Molecular cloning studies have demonstrated the existence of two species of IL-1, the α and β forms. Both IL-1α and -1β are expressed following immune-activation of macrophages and are synthesized as 31 kDa precursors that are proteolytically cleaved to the 17 kDa secreted forms [25]. Both forms bind to the same receptor and have similar biological activities [25]. Calkins *et al.* [5] determined that recombinant murine IL-1α was more potent than human recombinant IL-1α and of equivalent potency to human recombinant IL-1β. While some difference in IL-1α and -1β effects have been observed [26], we used homologous IL-1α to avoid species differences.

We demonstrated that our model is suitable for the investigation of the role of TIMs-secreted cytokines in the regulation of Leydig cell function. These data demonstrate that LPS treatment, both *in vivo* and *in vitro*, induces IL-1 gene expression in TIMs. In addition, we have shown that IL-1 decreases *P*450c17 mRNA levels in Leydig cells *in vitro*. Therefore, we suggest that the immune-activation of TIMs, as measured by induction of IL-1β mRNA, might have caused the observed inhibition of *P*450c17 gene expression in Leydig cells *in vivo*. This study thus supports the hypothesis (Fig. 6) that cytokines produced by immune-activated TIMs may have a paracrine regulatory role in testosterone biosynthesis in Leydig cells.

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