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Bacterial Endotoxin Lipopolysaccharide and Reactive Oxygen Species Inhibit Leydig Cell Steroidogenesis via Perturbation of Mitochondria

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Chronic inflammatory disease and acute infection are well known to inhibit gonadal steroidogenesis. Previous studies have demonstrated that immune activation in response to lipopolysaccharide (LPS) results in reductions in serum testosterone, and this is a direct effect on the Leydig cell. We hypothesize that during the early onset of LPS endotoxemia in vivo, testicular macrophages produce reactive oxygen species (ROS) leading to perturbation of Leydig cell mitochondria and an inhibition in steroidogenesis. To investigate the mechanism of LPS inhibition of Leydig cell steroidogenesis, alterations in mitochondria and markers of oxidative stress were assessed in vivo and in Leydig cell primary culture. After a single injection of mice with LPS, serum testosterone was significantly decreased within 2 h. LPS injection of mice resulted in significant reductions in steroidogenic acute regulatory protein (StAR) and 3β-hydroxysteroid dehydogenase-Δ^4-Δ^5 isomerase (3β-HSD) proteins. LPS significantly increased lipid peroxidation of Leydig cell membranes, indicating that LPS results in oxidative damage in vivo. Mitochondria in Leydig cells isolated from LPS-injected mice were disrupted and showed a marked reduction in the mitochondrial membrane potential ($\Delta\Psi_m$). Similar to the effects of LPS, treatment of Leydig cells with hydrogen peroxide acutely inhibited steroidogenesis, reduced StAR and 3β-HSD protein levels, and disrupted $\Delta\Psi_m$. These results suggest that LPS acutely inhibits Leydig cell function by ROS-mediated disruption of Leydig cell mitochondria. Taken together, these results demonstrate the necessity of having respiring mitochondria with an intact $\Delta\Psi_m$ to facilitate StAR function and Leydig cell steroidogenesis. The acute effects of LPS demonstrate how sensitive Leydig cell mitochondrial steroidogenesis is to inflammation-induced oxidative stress.

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Introduction

Chronic inflammatory disease and acute infection are well known to inhibit gonadal steroidogenesis. Conditions that result in immune activation have been shown to inhibit GnRH and LH secretion, resulting in decreased testosterone levels *(1)*. Inflammatory mediators also act directly on the gonad to inhibit steroidogenesis. We have observed direct perturbation of Leydig cell steroidogenesis in three models of immune activation. Intracerebroventricular (ICV) injection of IL-1β in male rats, in addition to decreasing secretion of GnRH and LH, also causes a blunting of the Leydig cell response to hCG *(2)*. Experimental sepsis induced in male rats by cecal slurry results in a significant decrease in serum testosterone *(3,4)*. Following a single ip injection of the Gram negative endotoxin lipopolysaccharide (LPS) into male mice, serum testosterone levels are decreased by 80% within 2 h, and are still completely inhibited at 24 h *(5,6)*.

LH acting via its intracellular second messenger cAMP regulates testosterone synthesis in the Leydig cell, both acutely at the level of cholesterol transport into the mitochondria, as well as having more long-term influence on the levels of the steroidogenic enzymes [for reviews, see *(7,8)*]. Transfer of cholesterol across the inner-mitochondrial space is regulated by, and dependent on, the action of the steroidogenic acute regulatory protein (StAR). LH stimulation of the Leydig cell results in the activation of StAR transcription, subsequent translation of StAR protein, and transfer of cholesterol into the mitochondria to cholesterol side-chain cleavage cytochrome P450 (P450scc) *(9)*. P450scc converts cholesterol to pregnenolone, which diffuses out of the mitochondria to the smooth endoplasmic reticulum where it is further metabolized via the action of 3β-hydroxysteroid dehydrogenase Δ^4-Δ^5-isomerase (3β-HSD) to progesterone. It is noteworthy that 3β-HSD has also been shown to be associated with mitochondria, which would potentially enable the synthesis of progesterone at the organelle *(10)*.

Progesterone in turn is converted by a two-step process to androstenedione via the action of 17α-hydroxylase/$C_{17–20}$ lyase (P450c17). The conversion of androstenedione to testosterone is catalyzed by 17β-hydroxysteroid dehydrogenase (17β-HSD) [for reviews, see *(8,11)*].

There have been relatively few studies on the effects of reactive oxygen species (ROS) on steroidogenesis. ROS has been implicated in the ovary in the process of luteolysis *(12–14)*, and earlier studies indicated that ROS blocks the hormone-sensitive cholesterol transfer step in corpora lutea *(13)*. ROS has also been shown to inhibit steroidogenesis in MA-10 tumor Leydig cells, also at the level of cholesterol transfer *(15)*. We have recently shown that oxidative stress results in a perturbation of MA-10 cell mitochondria and an inhibition in steroidigenesis *(16)*. ROS have also been implicated in the senescence of steroidogenic function during aging *(17–19)*.

Leydig cells, which reside in the testicular interstitium, are particularly susceptible to extracellular sources of ROS owing to their close proximity to testicular interstitial macrophages *(20,21)*. The release of reactive oxygen species (oxidative or respiratory burst) by immune-competent cells such as macrophages is an essential part of the immediate immune reaction that takes place in the early onset of infectious events *(22)*. The release of ROS by activated macrophages not only affects invading microorganisms, but may also expose adjacent tissues and cells, such as Leydig cells, to oxidative stress. Testicular macrophages are known to produce ROS during inflammation or infection *(23)*. ROS cause tissue damage by a variety of mechanisms including DNA damage, lipid peroxidation, protein oxidation, depletion of cellular thiols, and activation of pro-inflammatory cytokine release.

Mitochondria are an important cellular source of ROS and are very susceptible to oxidative damage. Oxidative damage to mitochondria is considered to be central to cellular senescence associated with aging and a host of other pathologies *(24)*. Mitochondria are a key control point for the regulation of steroid hormone biosynthesis. The first and rate-limiting step in the biosynthesis of steroid hormones in the adrenals and gonads is the transfer of cholesterol across the mitochondrial membranes, a process which depends on StAR *(25,26)*. Site-specific mitochondrial-disrupting drugs such as carbonyl cyanide-*m*-chorophenyl hydrazone (CCCP) and nigericin have been shown to block cholesterol transfer and have revealed that an intact mitochondrial membrane potential ($\Delta\Psi_m$) is required for steroidogenesis *(27–30)*.

To investigate the mechanism of LPS inhibition of Leydig cell steroidogenesis, alterations in mitochondria and markers of oxidative stress were assessed in vivo and in Leydig cell primary culture. Results indicate that LPS endotoxemia inhibits testosterone production via elaboration of ROS, which perturbs Leydig cell mitochondria resulting in an inhibition in steroidogenesis.

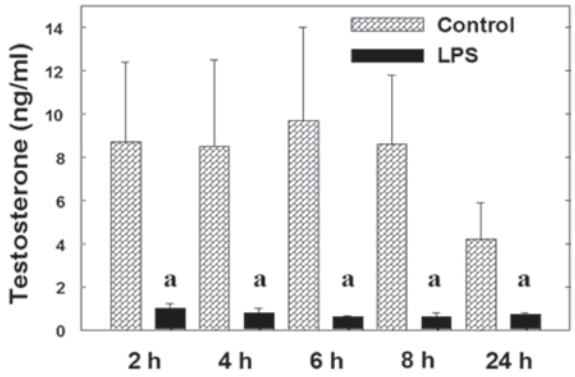

Fig. 1. Effects of LPS injection on serum testosterone levels in mice. Mice were injected ip with 200 μg/mouse of LPS, control mice were injected with PBS. Mice were sacrificed at the indicated times after injection and bled after treatment. Samples were subjected to testosterone RIA as described in Materials and Methods. Data are represented as mean ± SEM for fifteen animals ($n = 15$); [a], $p < 0.05$ vs control.

Results

Effect of LPS Injection on Serum Testosterone in Mice

A single injection of male mice with LPS results in a significant reduction in serum testosterone levels (Fig. 1). Mice were injected with LPS (200 μg), animals sacrificed, and serum testosterone was assessed. Serum testosterone was significantly reduced in LPS-injected mice within 2 h by nearly 70%, and this inhibition was sustained for up to 24 h. In addition, we have observed that LPS results in a prolonged reduction in serum testosterone for up to 21 d (data not shown).

To determine if LPS from different sources had similar effects on serum testosterone, mice were injected with different serotypes and lots of endotoxin as well as Gram positive preparations. Injection of the different preparations of *Escherichia coli* LPS and Gram positive *Salmonella typhimurium* LPS significantly reduced serum testosterone levels similarly (Table 1).

Effect of LPS on StAR and Steroidogenic Enzyme Proteins

To determine if components of the steroidogenic pathway in Leydig cells were inhibited during LPS endotoxemia, changes in steroidogenic proteins were analyzed by Western blot. Two hours post-injection of LPS, both StAR and 3β-HSD proteins levels were significantly decreased (Fig. 2). LPS treatment did not alter the levels of P450scc or P450c17. Two hours after LPS injection, 30 kDa StAR protein was significantly reduced by 25% (Fig. 2, $n = 6$; Con, 202 ± 40; LPS, 151 ± 34). In addition, the 37 kDa form of StAR protein increases relative to 30 kDa StAR in response to LPS, which indicates there is an effect at the level of StAR processing (Fig. 2A). This apparent increase in 37 kDa StAR, but decrease in 30 kDa StAR, in response to LPS is consistent with our previously published data investigating LPS effects on StAR protein *(31)*. Of the two forms

Table 1
Comparison of Different Sources and Preparations of LPS[a]

LPS type	Testosterone (ng/mL)	SEM	*n*	Percent naïve	Percent control
Naïve	4.022	0.64	70		
Control	4.90	0.96	31		
J5(RC mutant) *E. coli*	0.779	0.08	15	19%	16%
055:B5 *E. coli*	0.730	0.17	3	18%	15%
111:B4 *E. coli*	0.830	0.15	3	21%	17%
S. typhimurium	0.883	0.02	3	22%	18%

[a]Effectiveness of different preparations of LPS on inhibiting serum testosterone levels. Different batches, serotypes, and bacterial sources of LPS were examined. Mice were injected with 200 µg of each LPS and sacrificed after 2 h. Control mice were injected with PBS, naïve mice were not injected. Blood was collected and serum prepared for testosterone RIA. As shown in the table, each lot and type that was tested was equally effective and caused a greater than 80% decrease in serum testosterone.

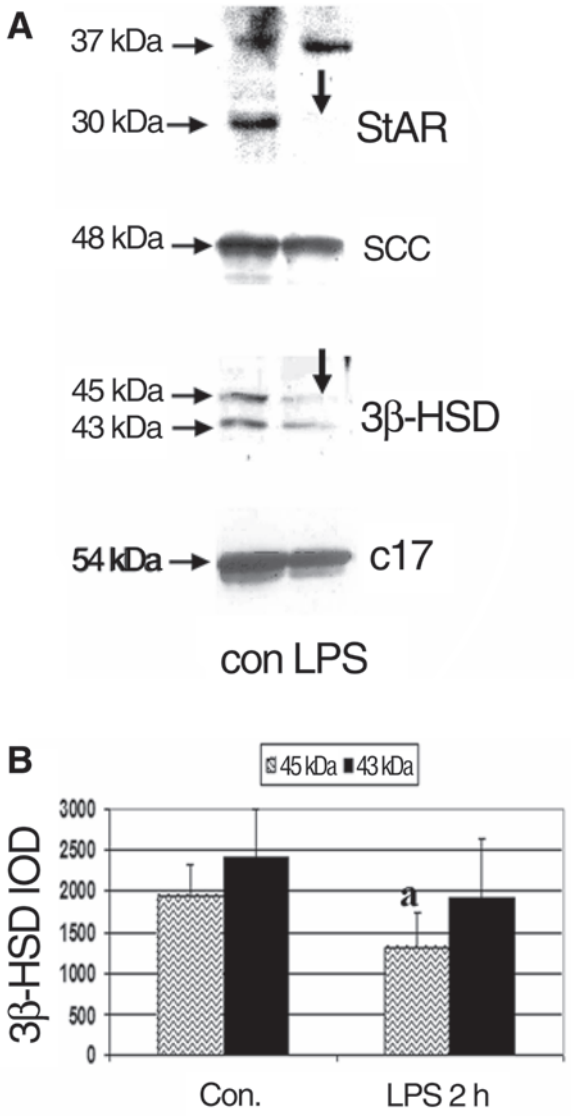

Fig. 2. Effect of LPS on StAR and steroidogenic enzyme protein levels. Mice were injected as described in Fig. 1 and sacrificed 2 h after LPS treatment. Leydig cells were isolated, lysed, and total protein was subjected to Western blot analysis as described in Materials and Methods. (**A**) Representative Western blots of StAR, P450scc, 3β-HSD, and P450c17 proteins. (**B**) Quantitation of 3β-HSD protein levels by scanning densitometry; 45 and 43 kDa forms of 3β-HSD were quantified separately. Data are represented as mean ± SEM for six independent experiments ($n = 6$); [a], $p < 0.05$ vs control.

of 3β-HSD analyzed, only the 45 kDa, type VI form of 3β-HSD was significantly reduced after LPS treatment by nearly 40% (Fig. 2C; $n = 6$; 45 kDa Con, 1950 ± 354; 45 kDa LPS, 1321 ± 406; 43 kDa Con, 2409 ± 562; 43 kDa LPS, 1906 ± 723). 3β-HSD has been suggested to associate directly with mitochondrial membranes *(10)*, and type VI 3β-HSD may represent a reduction of the mitochondrial localized pool of the protein. In addition, Leydig cells from LPS-injected mice were processed for histochemical staining for 3β-HSD. A marked decrease in 3β-HSD staining was evident in Leydig cells isolated from mice injected with LPS (Fig. 3). These results indicate that LPS injection of mice decreases StAR and 3β-HSD protein, which likely contributes to but may not completely account for the observed reductions in steroidogenesis (Fig. 1).

Effect of LPS on the Mitochondrial Membrane Potential ($\Delta\Psi_m$)

Mitochondria are essential organelles for steroidogenesis, and previous work has demonstrated that the mitochondrial membrane potential ($\Delta\Psi_m$) is important for StAR function *(27–30)*. To examine if LPS alters $\Delta\Psi_m$, Leydig cells isolated from mice injected with LPS were incubated with the potentiometric dye TMRE as described in Materials and Methods. Uptake of TMRE into mitochondria requires an intact $\Delta\Psi_m$, and visualizing TMRE fluorescence in cells can provide a semiquantitative measure of $\Delta\Psi_m$. To address the potential loss of LPS effects due to the duration of isolation of Leydig cells, two TMRE protocols were used to assess $\Delta\Psi_m$ ex vivo. Cells were either "pre-stained" with TMRE

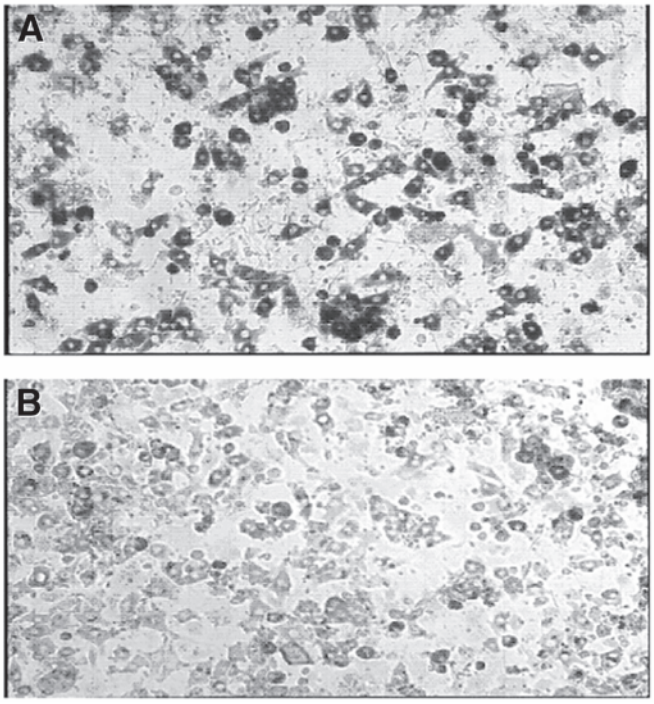

Fig. 3. Histochemical analysis for 3β-HSD enzyme activity in Leydig cells from control and LPS injected mice. Mice were injected as described in Fig. 1 and sacrificed 2 h after LPS treatment. Leydig cells were isolated, plated, and the 3β-HSD enzyme activty was assessed by histochemical analysis as described in Materials and Methods. (**A**) Representative 3β-HSD staining of Leydig cells isolated from vehicle injected mice. (**B**) Representative 3β-HSD staining of Leydig cells isolated from LPS injected mice.

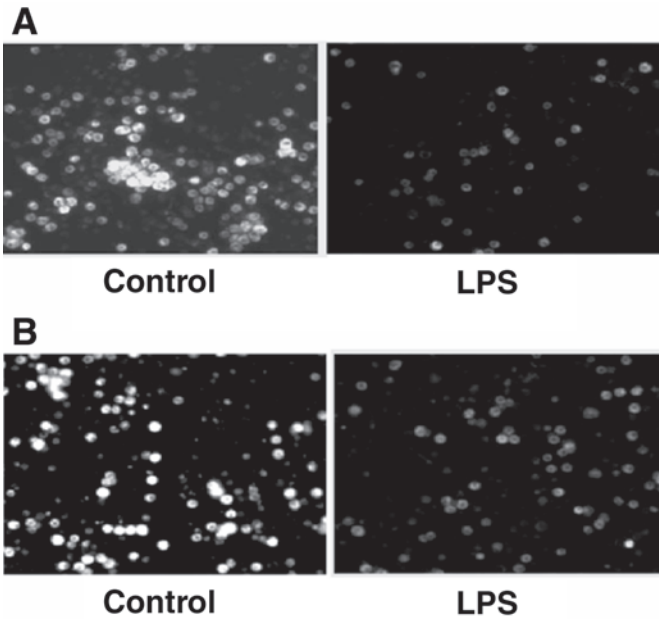

Fig. 4. Effect of LPS on the mitochondrial membrane potential ($\Delta\Psi_m$). Mice were injected as described in Fig. 1 and sacrificed 2 h after LPS treatment. Leydig cells were pre-incubated before isolation, or post-incubated after isolation with 40 n*M* tetramethylrhodamine ethyl ester dye (TMRE). The cells were then plated and examined for TMRE fluorescence using digital fluorescence microscopy as described in Materials and Methods. (**A**) TMRE fluorescence of Leydig cells pre-incubated with TMRE from LPS or vehicle-injected mice. (**B**) TMRE fluorescence of Leydig cells post-incubated with TMRE from LPS or vehicle injected mice.

prior to the isolation procedure, or "post-stained" with the dye after isolation as described in Materials and Methods. Leydig cells isolated from control mice showed a strong TMRE fluorescence indicating that mitochondria possess an intact $\Delta\Psi_m$ (Fig. 4). In contrast, Leydig cells isolated from LPS-injected mice showed a substantial decrease in TMRE fluorescence, indicating a decrease in $\Delta\Psi_m$. Leydig cells from both pre- and post-stained protocols showed similar results. The reduced TMRE fluorescence in post-stained Leydig cells suggests that LPS injection results in long-term disruption of $\Delta\Psi_m$ (4 h ex vivo). These data demonstrate that LPS injection of mice results in disturbances of Leydig cell mitochondria in vivo, and loss of $\Delta\Psi_m$ is consistent with oxidative stress induced perturbation of Leydig cell mitochondria.

Assessment of Membrane Lipid Peroxidation after LPS Injection

To assess if LPS exposure results in oxidative stress in Leydig cells, lipid peroxidation was measured. A useful indicator of oxidative damage of cells and tissues is the generation of lipid peroxidation products malondialdehyde (MDA) and 4-hydroxyalkenals such as 4-hydroxynonenal (4-HNE). To assess if LPS exposure results in oxidative damage, Leydig cells were isolated from LPS-injected mice and cell

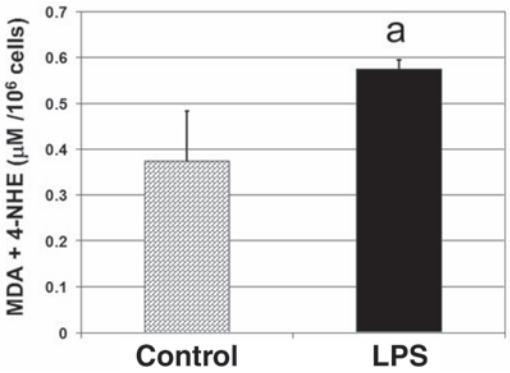

Fig. 5. Assessment of lipid peroxidation products in Leydig cells from mice injected with LPS. Mice were injected as described in Fig. 1 and sacrificed 2 h after LPS treatment. Leydig cells were isolated, and the levels of lipid peroxidation products malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE) were determined as describe in Materials and Methods. Data are represented as mean ± SEM for three experiments done in duplicate ($n = 3$); [a], $p < 0.05$ vs vehicle.

homogenates were assayed for lipid peroxidation products as described in Materials and Methods. LPS significantly increased lipid peroxidation products (MDA and 4-HNE) in Leydig cells (Fig. 5; Con, 0.37 ± 0.11; LPS, 0.58 ± 0.03, $n = 3$). Increased concentrations of MDA and 4-HNE indicates that LPS results in lipid peroxidation of Leydig cell membranes in vivo. This increased lipid peroxidation sug-

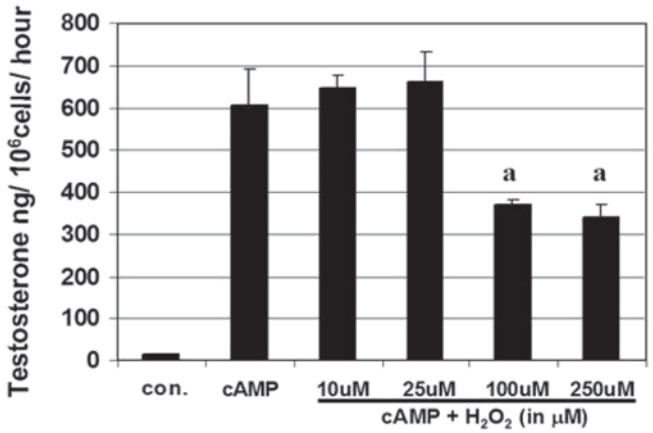

Fig. 6. Effect of H_2O_2 on cAMP-stimulated testosterone production in primary Leydig cells. Primary Leydig cells were isolated from mice, and cells were treated for 3 h in serum-free media that contained 1 m*M* 8-Br-cAMP (cAMP) or cAMP plus increasing concentrations of H_2O_2. Media were collected and subjected to testosterone RIA as described in Materials and Methods. Testosterone concentrations were normalized to the number of cells present in the culture and are represented as mean ± SEM for four independent experiments ($n = 4$); [a], $p < 0.05$ vs cAMP.

gests that LPS inhibits Leydig cell function and steroidogenesis through a reactive oxygen mechanism.

Effect of H_2O_2 on Testosterone Biosynthesis in Primary Leydig Cells

To further investigate the potential involvement of reactive oxygen in inhibiting Leydig cell steroidogenesis, primary Leydig cells were isolated and exposed to hydrogen peroxide in vitro. Treatment of primary Leydig cells with 100 and 250 μ*M* H_2O_2 significantly decreased cAMP-stimulated testosterone synthesis by approx 40% (Fig. 6, $n = 3$; Con, 14 ± 0.53; cAMP, 604 ± 87; 10 μ*M* H_2O_2, 644 ± 32; 25 μ*M* H_2O_2, 663 ± 70; 100 μ*M* H_2O_2, 368 ± 12; 250 μ*M* H_2O_2, 339 ± 30). Decreased testosterone synthesis after H_2O_2 treatment of Leydig cells in vitro demonstrates that ROS inhibits steroidogenesis, and supports the concept that reactive oxygen species may mediate the inhibitory effects of LPS on steroidogenesis in vivo.

Effect of H_2O_2 on StAR and 3β-HSD Proteins in Primary Leydig Cells

As LPS endotoxemia resulted in significant decreases in both StAR and 3β-HSD protein (Fig. 3), changes in these proteins were also examined in Leydig cells treated with H_2O_2 in vitro. Exposure of Leydig cells to H_2O_2 resulted in decreases in StAR protein (Fig. 7A). 250 μ*M* H_2O_2 signifi-

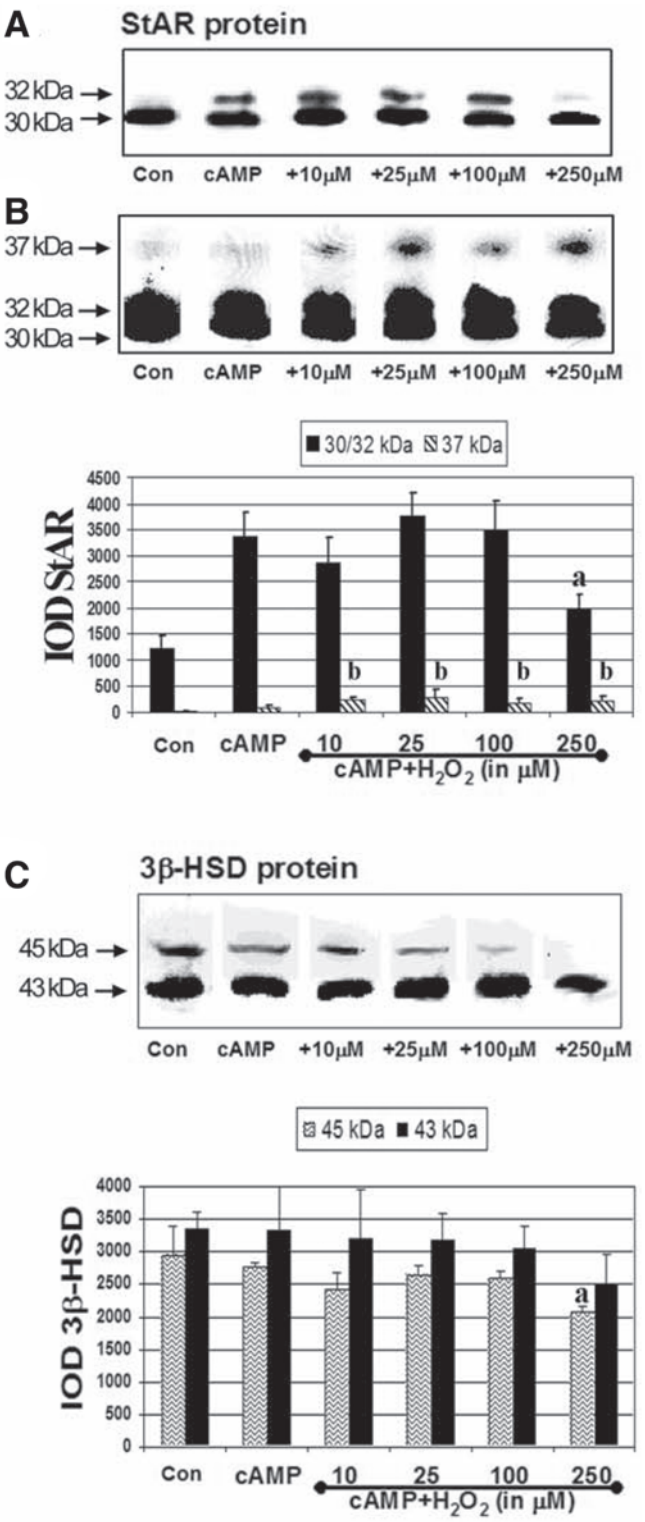

Fig. 7. Effect of H_2O_2 on StAR and 3β-HSD protein levels. Primary Leydig cells were grown in culture and treated for 3 h with 1 m*M* 8-Br-cAMP (cAMP) or cAMP plus increasing concentrations of H_2O_2 as described in Fig. 6. After treatment, cell preparations from Fig. 6 were lysed and subjected to Western blot analysis as described in Materials and Methods. (**A**) Representative Western blot of StAR protein showing the 30 and 32 kDa forms. (**B**) Overexposure of the same blot shown in Fig. 6A, reveals the 37 kDa form of StAR. The lower panel is a quantitation of the different forms of StAR protein determined by scanning densitometry. Data are represented as mean ± SEM for six independent experiments ($n = 6$); [a], $p < 0.05$ vs cAMP for 30 + 32 kDa StAR; [b], $p < 0.05$ vs cAMP for 37 kDa StAR. (**C**) Representative Western blot of 3β-HSD protein and quantitation of 3β-HSD protein levels by scanning densitometry. The 45 and 43 kDa forms of 3β-HSD were quantified separately. Data are represented as mean ± SEM for four independent experiments ($n = 4$); [a], $p < 0.05$ vs cAMP.

cantly decreased 30 + 32 kDa StAR protein by 40% versus cAMP alone (Fig.7B, $n = 6$; Con, 1228 ± 232; cAMP, 3367 ± 447; cAMP + 250 μ*M* H_2O_2, 1998 ± 248). In addition, H_2O_2 treatments resulted in an increase in the 37 kDa form of StAR protein relative to 30 kDa StAR (Fig. 7B). The 37 kDa form of StAR increased significantly after H_2O_2 treatments versus cAMP-stimulated cells (Fig. 7B, $n = 6$; Con, 18 ± 8; cAMP, 86 ± 32; cAMP + 10 μ*M* H_2O_2, 243 ± 23; cAMP + 25 μ*M* H_2O_2, 276 ± 151; cAMP + 100 μ*M* H_2O_2, 180 ± 71; cAMP + 250 μ*M* H_2O_2, 216 ± 79). This increase and apparent accumulation of 37 kDa StAR in response to H_2O_2 in vitro is similar the previosuly published results which demonstrate an increase in 37 kDa StAR after LPS injection in vivo *(31)*.

Reactive oxygen treatment of Leydig cells also decreased 3β-HSD protein, in particular the 45 kDa isoform (Fig. 7B); 250 μ*M* H_2O_2 treatment of Leydig cells significantly reduced 45 kDa 3β-HSD vs cAMP alone (Fig. 7B, $n = 4$; 45 kDa 3β-HSD, Con = 2936 ± 434; cAMP = 2752 ± 88; cAMP + 250 μ*M* H_2O_2 = 2054 ± 109). This significant decrease of 45 kDa 3β-HSD after H_2O_2 treatment is similar to the effects of LPS on selectively inhibiting the higher-molecular-weight isoform of 3β-HSD (Fig. 2C). These results demonstrate that ROS treatment of Leydig cells decreases StAR and 3β-HSD protein levels similar to the changes observed after LPS endotoxemia. As in the case of the in vivo effects of LPS, the in vitro effects of H_2O_2 on steroidogenesis are of a greater magnitude than that observed for the individual steroidogenic proteins.

Effect of H_2O_2 on the Mitochondrial Membrane Potential ($\Delta\Psi_m$) in Primary Leydig Cells

As shown in Fig. 4, LPS injection of mice causes a depolarization of the mitochondria and a marked loss of the mitochondrial membrane potential ($\Delta\Psi_m$). To examine of ROS can induce similar changes in $\Delta\Psi_m$, primary Leydig cells were pretreated with 100 and 250 μ*M* H_2O_2 for 3 h and then stained with TMRE as described in Materials and Methods. A strong TMRE fluorescence was evident in control Leydig cells as observed by epi-fluorescence microscopy, indicating mitochondria were polarized with an intact $\Delta\Psi_m$ (Fig. 8). In contrast, Leydig cells treated with 100 μ*M* H_2O_2 showed a marked decrease in TMRE fluorescence, and profound reduction in TMRE fluorescence was observed in cells exposed to 250 μ*M* H_2O_2. Reduced TMRE fluorescence in Leydig cells exposed to reactive oxygen indicates that H_2O_2 disrupts $\Delta\Psi_m$ in vitro, and this is similar to observed effects of LPS on $\Delta\Psi_m$ ex vivo. This disruption of mitochondria by reactive oxygen further supports the hypothesis that LPS may inhibit Leydig cell function by perturbing mitochondria through a reactive oxygen mechanism.

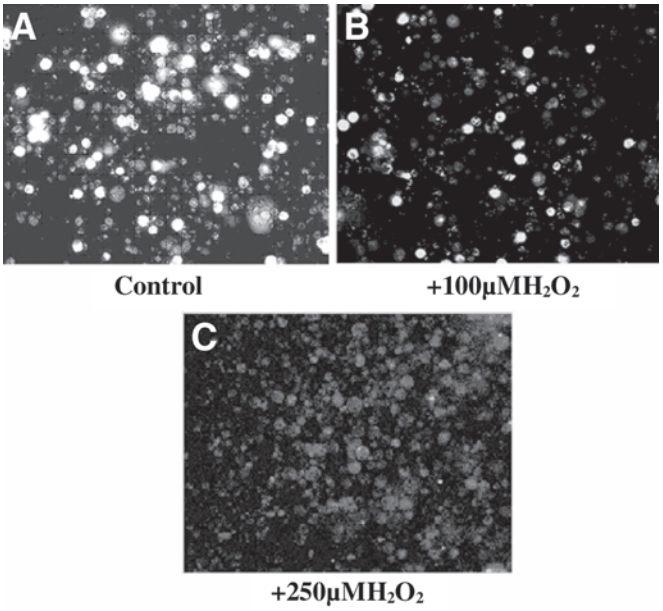

Fig. 8. Effect of H_2O_2 on the mitochondrial membrane potential ($\Delta\Psi_m$). Primary Leydig cells were incubated in serum-free media (SFM) for 1 h. Cells were then treated with 100 μ*M* or 250 μ*M* H_2O_2 for 3 h. After treatment, cells were incubated with 40 n*M* tetramethylrhodamine ethyl ester dye (TMRE) for 10 min and TMRE fluorescence was assessed using digital fluorescent microscopy as described in Materials and Methods.

Discussion

Using the LPS model of endotoxemia, we have examined the effects of LPS on Leydig cell function in vivo, with the aim of determining the mechanism by which an inflammatory stimulus inhibits Leydig cell steroidogenesis. A single injection of mice with LPS resulted in acute and long-term decreases in serum testosterone (Fig. 1). Testosterone levels were significantly decreased in mice as rapidly as 2 h after LPS injection. LPS exposure decreases the steroidogenic proteins StAR and 3β-HSD (Fig. 2), suggesting that the initial observed decrease in serum testosterone *to some extent* correlated to a reduction in these proteins. Notably, only the 45 kDa form of 3β-HSD was significantly decreased after LPS injection (Fig. 2C). Leydig cells isolated from LPS-injected mice showed a marked decrease in 3β-HSD histochemical staining (Fig. 3), consistent with a reduction in 3β-HSD. These results indicate that LPS-induced decrease in serum testosterone is in part due to the inhibition of StAR and 3β-HSD proteins. However, LPS inhibits steroidogenesis to a far greater degree than the decrease observed in StAR and 3β-HSD, indicating an additional site of inhibition.

Mitochondria are essential organelles in testosterone biosynthesis and are the site of the first enzymatic step in steroidogenesis. Previous investigation has demonstrated that an intact mitochondrial membrane potential ($\Delta\Psi_m$) is necessary for cholesterol transfer; therefore, we investigated the effects of LPS on $\Delta\Psi_m$. Leydig cells isolated from LPS injected mice showed a substantial decrease in TMRE fluorescence, indicating a decrease in $\Delta\Psi_m$ (Fig. 4). This disruption of Leydig cell mitochondria is likely a major factor

contributing to LPS-induced inhibition of steroidogenesis. Moreover, Leydig cells isolated from LPS-injected mice showed a significant increase in the lipid peroxidation products MDA and 4-HNE (Fig. 5), indicating that LPS causes oxidative stress in Leydig cells.

To test the hypothesis that ROS are involved in this LPS effect, primary cultures of Leydig cells in vitro were treated with hydrogen peroxide in an attempt to mimic the inhibitory effects of LPS. One experimental complexity is determining a physiologically relevant concentration of ROS to utilize in vitro that faithfully represents the ROS produced in vivo in response to LPS. Previous investigations of LPS-injected mice have reported peritoneal macrophages produce superoxide anion at concentrations from 70 to 150 μ*M* after 2 h of endotoxemia *(32)*. This suggests that LPS-exposed macrophages in vivo produce ROS within the micromolar ranges we have utilized when treating primary Leydig cells in vitro. ROS treatment of Leydig cells significantly inhibited cAMP-stimulated testosterone production (Fig. 6). ROS treatments also resulted in a reduction in TMRE fluorescence, indicating a decrease in $\Delta\Psi_m$ and disruption of mitochondria (Fig. 8). Similar to the effects of LPS, ROS treatment also significantly decreased levels of StAR and 3β-HSD protein (Fig. 7). ROS treatments also resulted in an increase in the 37 kDa form of StAR protein relative to 30 kDa StAR, indicating ROS exerts its effects at the level of StAR processing (Fig. 7B). This increase in 37 kDa StAR after ROS treatment is similar to the effects of LPS in vivo which also result in increased 37 kDa StAR (Fig. 2A). Notably, ROS treatment inhibited progesterone synthesis at a concentration of 100 μ*M* H_2O_2 (Fig. 6), but this concentration did not reduce StAR protein levels (Fig. 7B), indicating ROS can inhibit steroidogenesis independently of decreasing StAR protein. However, 100 μ*M* H_2O_2 does substantially alter mitochondria and disrupt $\Delta\Psi_m$ (Fig. 8), suggesting that mitochondria themselves may be the initial target of ROS that results in an inhibition of steroidogenesis. These data demonstrate mitochondria are more sensitive to perturbation by ROS, and that inhibition of StAR requires higher concentrations. This further supports the hypothesis that the state of the mitochondria is the primary target for ROS-mediated inhibition of steroidogenesis. These experimental results demonstrate that ROS exposure of Leydig cells in vitro results in similar inhibitory effects as LPS in vivo, and support the interpretation that LPS may inhibit Leydig cell function in vivo via disruption of mitochondria by reactive oxygen.

Leydig cells are highly sensitive to conditions resulting in immune activation, and inflammatory responses inhibit Leydig cell function *(1–6)*. We and others have shown StAR protein is very sensitive to inflammatory stimuli. In these studies, systemic LPS exposure decreased StAR protein within 2 h. It is notable that both LPS and ROS have similar inhibitory effects on StAR (Figs. 2 and 7); 37 kDa StAR increases relative to the 30 kDa form after LPS injection in vivo (Fig. 2) and after ROS treatments in vitro (Fig. 7B). This relative increase in 37 kDa StAR suggests that StAR is not imported and subsequently processed due to LPS- or ROS-mediated disruption of $\Delta\Psi_m$. This is consistent with the accepted paradigm that matrix-targeted proteins require an intact $\Delta\Psi_m$ for their import and processing *(33–35)*. 3β-HSD protein is also decreased after LPS injection of mice. Mouse Leydig cells express two isoforms of 3β-HSD, with corresponding molecular weights of 45 and 43 kDa *(36)*. In this study, the 45 kDa, Type VI 3β-HSD isoform, is particularly sensitive to LPS-induced endotoxemia (Fig. 3). It has been demonstrated that 3β-HSD is associated with mitochondria *(10)*. It is intriguing to speculate that type VI 3β-HSD is the mitochondrially associated form in the Leydig cell, and that the observed disturbances in the mitochondria after LPS (Fig. 4) could thus be responsible for this selective inhibition of Type VI 3β-HSD.

LPS induces the production of pro-inflammatory cytokines, principally IL-1, TNFα, and IL-6 by activated immune cells *(37)*. The LPS-induced elaboration of pro-inflammatory cytokines occurs several hours after LPS exposure in vivo, owing to the requirement for transcription/translation *(38)*. Thus, cytokines themselves cannot be responsible for the observed acute and rapid reductions in serum testosterone after LPS exposure. However, cytokine mediated decreases in steroidogenic enzyme mRNA likely account for the pronounced loss of steroidogenesis observed 12–24 h post-LPS *(38,39)*. In an attempt to elucidate the underlying mechanism for decreased testosterone at 2 h, many agents in addition to pro-inflammatory cytokines, known to be released during immune activation were tested in vitro. These included catecholamines (norepinephrine and β-adrenergic agonist isoproterenol), prostaglandins (PGF2 alpha and PGE2), and arginine vasopressin, but none of these inhibited Leydig cell steroidogenesis quickly enough to accurately mimic the LPS inhibition observed in vivo (unpublished data).

ROS are the other class of inflammatory mediators that are rapidly released from the immune system during immune activation *(40)*. The release of ROS by immune-competent cells such as macrophages is an essential part of the immediate immune reaction that takes place in the early onset of infectious events *(22)*. Testicular macrophages are known to produce ROS during infection and in response to LPS *(23)*, and LPS is known to induce oxidative stress *(32,40, 41)*. The release of ROS by activated macrophages not only affects invading microorganisms, but would also expose adjacent tissues and cells, such as Leydig cells, to oxidative stress. Leydig cells may be particularly susceptible to extracellular sources of ROS during immune reactions due to their close proximity to testicular interstitial macrophages *(20,21)*. We show increased lipid peroxidation and mitochondrial depolarization of Leydig cells exposed to LPS in vivo (Figs. 4 and 5), and ROS treatment of Leydig cells in vitro results in inhibitory effects similar to LPS. These data

support the hypothesis that LPS inhibits Leydig cell function by a ROS-mediated mechanism. It is worth noting that LPS endotoxemia may also result in the production reactive nitrogen species (RNS), which also are known to target and disrupt mitochondria. To address this as a mechanism, we also examined the expression of both inducible and endothelial nitric oxide synthases (NOS) in the testis isolated from LPS-injected animals. Interestingly, NOS expression was not altered during the first several hours after LPS injection, but was markedly upregulated after prolonged periods (manuscript in preparation). This suggests that nitric oxide and potentially RNS may be generated during LPS endotoxemia, but RNS are unlikely to contribute to inhibiting Leydig cell steroidogenesis during the time points examined in this study (2 hs post-injection LPS). However, RNS may be involved in the long-term inhibition of Leydig cell steroidogenesis that is observed post LPS.

In addition to being essential for steroidogenesis, mitochondria are also linked to the process of apoptosis. There is no evidence for apoptosis in the time frames we have examined (up to 24 h after LPS, Fig. 1). However, over a 7-d period after LPS injection, significant Leydig cell apoptosis is evident by TUNEL assay (manuscript in preparation). This is consistent with LPS mediating its chronic inhibitory effects on Leydig cells by mitochondrial-induced apoptosis and consistent with known effects of oxidative stress causing apoptosis. Subsequent to the oxidative burst of macrophages, there is a prolonged elevation in pro-inflammatory cytokine levels *(37)*, which also contributes to chronic inhibition of Leydig cell function. These elevated levels of cytokines and Leydig cell apoptosis may both contribute to the long-term reductions in testosterone which lasts up to 21 d post-injection [manuscript in preparation, *(5)*]. While cytokines and apoptosis are involved in the long-term inhibition of Leydig cell steroidogenesis, they do not appear to be responsible for the rapid effects of LPS.

Several studies have demonstrated the importance of $\Delta\Psi_m$ for steroidogenesis and StAR function *(16,27–30)*. We have previously observed depolarization of mitochondria which leads to post-transcriptional inhibition of StAR and an inhibition of steroidogenesis *(16)*. In this article we show that $\Delta\Psi_m$ is decreased in Leydig cells 2 h after LPS injection (Fig. 4), and that ROS similarly disrupts $\Delta\Psi_m$ in Leydig cells in vitro (Fig. 8). Taken together, these results and those of others demonstrate the necessity of having respiring mitochondria with an intact $\Delta\Psi_m$ to maintain StAR function and Leydig cell steroidogenesis. Even in the presence of adequate amounts of StAR and 3β-HSD, depolarization of the mitochondria results in the inhibition of steroidogenesis.

Our results as a whole suggest that LPS inhibits Leydig cell function by ROS-mediated disruption of mitochondria, which prevents StAR from facilitating cholesterol import into the mitochondria. We hypothesize that during the early onset of LPS exposure in vivo, testicular macrophages produce ROS, leading to oxidative stress within neighboring Leydig cells which disrupts mitochondria and collapses $\Delta\Psi_m$. Without an intact $\Delta\Psi_m$, StAR cannot facilitate cholesterol transfer into mitochondria, which would effectively inhibit steroidogenesis. It appears LPS inhibits steroidogenesis at multiple sites. LPS via ROS disrupts mitochondria and dissipates $\Delta\Psi_m$, inhibits StAR and 3β-HSD post-transcriptionally, and ultimately causes the repression of steroidogenic enzymes via cytokines.

Oxidative stress is central to a host of pathologies including inflammation, infection, alcohol toxicity, neurodegenerative disease, ischemia-reperfusion injury, cryptorchidism, endocrine disruption by environmental compounds, and damage from ultraviolet radiation *(24,42–51)*. Studies by Zirkin and co-workers have demonstrated that ROS is increased in the testis of aging rats, and this increased ROS is associated with decreasing Leydig cell steroidogenesis and StAR during aging *(17,52)*. We suggest the underlying mechanism for disorders involving hypogonadism may involve oxidative mediated disruption of Leydig cell mitochondria and inhibition of steroidogenesis.

Materials and Methods

Materials

Metrizamide was purchased from Accurate Chemical and Scientific Corp. (Westbury, NY); collagenase was purchased from Worthington Biochemical Corp, (Lakewood, NJ); ECL Western Blot Detection Kit™ (Amersham Pharmacia Biotech Inc., Piscataway, NJ); TMRE (tetramethylrhodamine ethyl ester) (T-669, Molecular Probes, Eugene, OR); HEPES, BSA (fraction V), bovine insulin, EDTA, sodium bicarbonate, hydrogen peroxide, and LPS (Lipopolysaccharide Gram negative serotypes 055:B4, 111:B4, J5(RC mutant) from *E. coli* and Gram-positive *S. typhimurium*) were purchased from Sigma Chemical Inc. (St. Louis, MO); Medium 199, DME/F12, penicillin, streptomyocin, were purchased from GIBCO BRL Life Technologies (Gaithersburg, MD).

Animals

Mice were housed for at least 1 wk in groups of five per cage. They were given food and water *ad libitum* and maintained on a 14 h light–10 h dark schedule. Adult (60–70 d old) male outbred pathogen-free CD-1 mice (Charles River, Portage, MI), averaging 33 g, were injected ip with LPS (as described in figure legends) or vehicle (PBS) alone, and blood and testis were collected at the times indicated. The mice were procured, maintained, and used in accordance with the Animal Welfare Act and approved by the University of Illinois at Chicago animal care committee. Animals were sacfriced by CO_2 asphyxiation prior to exsanguination.

Isolation and Purification of Leydig Cells

After CO_2 asphyxiation of mice, testes were surgically removed and collected via scrotal incision and were imme-

diately placed into ice-cold M-199 medium (Gibco BRL Life Technologies). Testes were removed, were subsequently decapsulated, and the testicular tissue was subjected to collagenase dispersion (Collagenase Type 4, CLS-4, Worthington Biochemical Corporation). Leydig cells were purified via metrizamide gradient centrifugation as described previously *(53)*. Content of Leydig cells in the cell preparation was estimated via histochemical staining for 3β-HSD as reported previously *(39)*. Purified Leydig cells were counted in an hemocytometer and plated into sterile cell culture dishes (Falcon™, Becton Dickinson Labware, Franklin Lakes, NJ) for primary culture. Treatment of cultured Leydig cells was performed at 32°C in a (5%/95% air) CO_2 incubator (Fisher Scientific) after 1 h of culture that allowed attachment of cells.

Histochemical Staining for 3β-Hydroxysteroid Dehydrogenase

Leydig cells preparations were determined to be 85–90% pure by histochemical staining for 3β-hydroxysteroid dehydrogenase/Δ^5,Δ^4-isomerase (3β-HSD), performed by a modification of the method described by Wiebe *(54)*. Twenty microliters of resuspened cells, 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 incubated in a humidified chamber for 90 min at 32°C in PBS containing 0.1% BSA, 1.5 m*M* nicotinamide adenine dinucleotide (NAD^+), 0.25 m*M* nitrobluetetrazolium, and 0.2 m*M* 5β-Androstan-3β-ol-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, indicated the presence of active 3β-HSD protein.

Measurement of Lipid Peroxidation

Lipid peroxidation was estimated by measuring malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), using the lipid peroxidation kit from Calbiochem (La Jolla, CA) according to the manufacturer's instructions. This assessment provided a convenient index of lipid peroxidation. Leydig cell homogenates were incubated with the chromogenic reagent *N*-methyl-2-phenylindole in acetonitrile followed by incubation with methanesulfonic acid, which yields a stable chromophore with a maximum absorbance at 586 nm allowing simultaneous quantitation of MDA and 4-HNE by spectrophotometry (Spectrophotometer Beckman Coulter DU 500, Fullerton, CA). Freshly isolated Leydig cells were pelleted at 400*g*, then resuspended in ice cold PBS, homogenized, centrifuged at 300*g*, and the supernatant was used for the assay. MDA and 4-HNE were quantified simultaneously and the concentration determined by comparison to a standard curve constructed with authentic MDA and 4-HNE. The concentration of MDA and 4-HNE were normalized to the number of cells homogenized for each assay.

Western Blotting and Immunodetection of Proteins

Total cellular protein was obtained by placing cells in lysis buffer (PBS/0.1% SDS) followed by brief sonication (Ultrasonic processor 50 T, 50% power for approx 2 s). Protein concentrations were determined by microBCA protein assay (Pierce Chemical Co., Rockford, IL). Thirty micrograms of total protein was separated by SDS-PAGE using 10% acrylamide/SDS separating gels and transferred to nitrocellulose membranes as described previously *(53,55,56)*. The preparation of the polyclonal antiserum to StAR, P450scc, and P450c17 has been previously described *(6,57,58)*. 3β-HSD antibody was a generous gift from Dr. Ian Mason (Edinburgh, UK). Detection of bound antibody on the blot was assessed with a horseradish peroxidase (HRP)-conjugated, goat anti-rabbit IgG antibody (Sigma, St. Louis, MO) visualized by enhanced chemiluminescence and quantitated, after densitometry (Personal Densitometer, Molecular Dynamics, Sunnyvale, CA) using Imagequant software (Molecular Dynamics, Sunnyvale, CA). Expression data for protein are represented as integrated optical density (IOD).

Assessment of the Mitochondrial Electrochemical Gradient ($\Delta\Psi_m$)

Mitochondrial electrical chemical gradient ($\Delta\Psi_m$) was assessed by measuring uptake and accumulation of the potentiometric dye, tetramethylrhodamine ethyl ester (TMRE). Analysis of TMRE fluorescence by microscopy allowed the semiquantitative assessment of the $\Delta\Psi_m$, as described *(58)*. Fluorescent images were obtained using an inverted microscope equipped for fluorescent microscopy (Nikon Eclipse, TE 300, 547, nm excitation, 579 nm emission); and digital images were processed using IPLab software (Scanalytics Inc., Fairfax, VA).

In order to determine if LPS injection resulted in a perturbation of Leydig cell $\Delta\Psi_m$, two in vivo TMRE staining protocols were followed. Two hours post-LPS or sham injection, testes were removed and crude interstitial cells were isolated following collagenase dispersion. In the first protocol (referred to as "pre-staining") crude isolated interstitial cells were incubated in 40 n*M* TMRE for 10 min at 37°C. Leydig cells were then purified as usual by metrizamide gradient, then plated in 24-well plates and examined immediately using an inverted microscope equipped for epi-fluorescence and photographed. In the second protocol (referred to as "post-staining") Leydig cells were purified by standard procedure, as described above for sham-injected and LPS-injected mice, plated in 24-well plates, incubated in 40 n*M* TMRE for 10 min at 37°C, and then analyzed immediately by epi-fluorescence microscopy as described above. An equal number of cells were examined in each field. Images of TMRE fluorescence are representative of data collected from 15 mice in each group.

For experiments utilizing reactive oxygen, primary Leydig cells were plated in 35 mm dishes and treated with 100 or 250 μ*M* hydrogen peroxide (H_2O_2) for 3 h. Cells were

then incubated with 40 n*M* TMRE for 10 min at 37°C and then examined immediately by fluorescent microscopy. Fluorescent images are representative of four independent experiments.

RIA

Blood was collected from control and LPS-injected mice by cardiac puncture. After clotting at room temperature for 1 h, the clot was loosened from the edge of the tube and centrifuged at 1000*g* for 20 min at 4°C. The serum was removed to a new tube and stored at –20°C prior to assaying. Media from primary Leydig cell cultures were boiled for 5 min and centrifuged at 2000*g* for 20 min at 4°C prior to assaying. Testosterone concentrations were determined with Coat-a-Count RIA kits (Diagnostic Products Corp., Los Angeles, CA).

Statistical Analysis

Data are represented as means ± SEM of three or more independent experiments. For independent data point comparisons, Student's *t* test was performed using Instat, version 3.0 software package for statistical data analysis (Graph Pad Software Inc., San Diego, CA). For group comparisons, one-way ANOVA followed by Student–Newman–Keuls multiple-range test were performed using the Instat, version 3.0. Differences were considered significant at $p < 0.05$.

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