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Antisteroidogenic Actions of Hydrogen Peroxide on Rat Leydig Cells

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Abstract It has been well known that reactive oxygen species (ROS) are produced in the steroidogenic pathway and spermatozoa. H_2O_2, one of ROS produced by spermatozoa, appears to be a primary toxic agent. In the present study, we examined the effects of H_2O_2 on the basal and evoked-testosterone release from primary Leydig cells, the protein expressions of cytochrome P450 side chain cleavage enzyme (P450scc) and steroidogenic acute regulatory (StAR) protein were also investigated. Our preparation was found to contain approximately 87% Leydig cells and very few macrophages. The results demonstrated that H_2O_2 ($>1 \times 10^{-4}$ M) significantly inhibited the basal and hCG-stimulated testosterone release. H_2O_2 abolished forskolin- or 8-Br-cAMP-evoked testosterone release. In the presence of pregnenolone, progesterone, or androstenedione, the inhibitory effect of H_2O_2 on testosterone release was prevented. H_2O_2 also inhibited pregnenolone production in the presence of trilostane (an inhibitor of 3β-hydroxysteroid dehydrogenase), therefore diminished the activity of P450scc in Leydig cells. In addition to the inhibition of hormone secretion, H_2O_2 also regulated steroidogenesis by diminishing protein expression of StAR. These results suggest that H_2O_2 acts directly on rat Leydig cells to diminish testosterone production by inhibiting P450scc activity and StAR protein expression. J. Cell. Biochem. 90: 1276–1286, 2003. © 2003 Wiley-Liss, Inc.

Key words: H_2O_2; testosterone; StAR protein; P450scc; Leydig cells

Reactive oxygen species (ROS), such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), are produced continuously in normal cellular metabolism. In human atrial myocardium, free radicals are released through the Fenton reaction from H_2O_2 and evoked marked intracellular acidosis [Chao et al., 2002]. A precedent for endocrine regulation of H_2O_2 production is in the thyroid, where H_2O_2 plays an important role in thyroid hormone synthesis [Nunez and Pommier, 1982]. Previous studies demonstrated that H_2O_2 is rapidly generated by prostaglandin $F_{2\alpha}$ during luteolysis in rat corpus luteum [Riley and Behrman, 1991]. In rat luteal and granulosa cells, H_2O_2 inhibits activation of adenylyl cyclase by receptor-bound gonadotropin and blocks basal and evoked progesterone production [Behrman and Preston, 1989; Margolin et al., 1990; Behrman and Aten, 1991; Endo et al., 1993].

During steroidogenesis in rat testes, luteinizing hormone (LH) causes lipid peroxidation and maintains high activities of peroxide-metabolizing enzymes [Peltola et al., 1996]. Because ROS play a role in infertility, the testicular production of free radicals and the function of the antioxidative defense system need further studies. Human spermatozoa were susceptible to lipid peroxidation [Johns et al., 1979]. H_2O_2 was also shown to decrease sperm motility and viability [MacLeod, 1943]. Steroidogenic cytochrome P450 enzymes in Leydig cells also

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produce ROS as a by-product of their catalytic reaction mechanism [Quinn and Payne, 1984, 1985]. Evidence has suggested that steroidogenic cells in culture are susceptible to O_2-mediated free radical damage, and reduction in O_2 tension results in an increased steroidogenic capacity of cultured Leydig cells, probably by protecting the steroidogenic enzymes from free radical inactivation [Myers and Abney, 1988]. However, only few studies in the effects of ROS on steroidogenesis have been reported.

It has been shown that the macrophage is another major cellular population in the testicular interstitium (approximately 20–30%) besides Leydig cells [Niemi et al., 1986]. Macrophages have been acknowledged to be a major source of cytokines that may interact with other cells in the testis. Several studies indicated that macrophages play an important role in regulating the steroidogenesis in Leydig cells [Gaytan et al., 1994, 1995]. Previous studies have shown that cytokines including INF-γ, TNF-α, and GM-CSF significantly increased production of H_2O_2 [Yuan et al., 1993; Lee and Shin, 1994]. Since our Leydig cell preparation contains approximately 87% Leydig cells and very few macrophages [Chiao et al., 2002], this study shows a direct effect of H_2O_2 on Percoll-purified Leydig cells via a macrophage–independent mechanism.

It has been indicated that the biosynthesis of steroid hormones by Leydig cells requires the sequential enzymes to convert cholesterol into various steroid classes [Payne and O'Shaughnessy, 1996; Tsai et al., 1997]. Earlier reports have demonstrated that the rate-limiting step in the production of steroids in steroidogenic tissues is the transfer of cholesterol from cellular stores to the inner mitochondrial membrane, where the cytochrome P450 side-chain cleavage (P450scc) catalyzes the first side chain cleavage of cholesterol to yield pregnenolone [Behrman and Aten, 1991]. Another protein identified as involved in the acute regulation of steroid production in steroidogenic tissues is steroidogenic acute regulatory protein (StAR protein). Based on the observation from the regulated expression of StAR protein and the corresponding increase in steroid biosynthesis [Clark et al., 1994; Stocco and Clark, 1996], it is proposed that StAR protein is rapidly synthesized in the cytosol in response to hormone stimulation and is quickly targeted to the mitochondria via a specific receptor on the mitochondrial outer membrane. After transferring cholesterol into mitochondria, StAR is catabolized. The cytochrome P450 enzymes in steroidogenic pathway are the most likely sites of free radical generation, because cytochrome P450 enzymes use molecular oxygen and electron transferred from NADPH to hydroxylate the substrate [Peltola et al., 1996]. However, the direct effect of H_2O_2 on the activity and expression of steroidogenic enzymes is still unclear.

Therefore, it is interesting to examine if the treatment of Leydig cells with H_2O_2 led to an inhibition of testosterone production and if an adverse effect on the activity and protein expression of steroidogenic enzymes might be observed. We have demonstrated that H_2O_2 induces a significant inhibition on the production of testosterone at least through a mechanism involving the decrease of the activity of cytochrome P450scc and the protein expression of StAR protein.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats weighing 300–350 g were housed in a temperature-controlled room ($22 \pm 1^{\circ}C$) with 14 h of artificial illumination daily (0600–2000 h). Food and water were available ad libitum.

Materials

Bovine serum albumin, HEPES, Hank's balanced salt solution, medium 199, sodium bicarbonate, penicillin-G, streptomycin sulfate, heparin, collagenase, hydrogen peroxide (H_2O_2), hCG (human chorionic gonadotropin), forskolin, 8-bromo-adenosine 3′:5′-cyclic monophosphate (8-Br-cAMP), 25-hydroxycholesterol (25-OH-Cholesterol), pregnenolone, progesterone, androstenedione, and testosterone were purchased from Sigma Chemical Co. (St. Louis, MO). Trilostane (4,5-epoxy-17-hydroxy-3-oxoandrostane-2-carbonitrile, an inhibitor of 3β-HSD), was provided by Sanofi-Synthelabo, Inc. (Malvern, PA). Anti-P450scc antibody was provided by Dr. Bon-Chu Chung (Academia Sinica, Taipei, Taiwan, ROC), and anti-StAR antibody was provided by Dr. D.M. Stocco (Department of Cell Biology and Biochemistry, Texas Tech University Health Sciences Center, Lubbock, TX). [^{3}H]-testosterone and [^{3}H]-pregnenolone were obtained from Amersham Pharmacia Biotech (Bucks, UK). The doses of

drugs are expressed as the final molar concentrations in the flask.

Preparation of Rat Leydig Cells

Animals were sacrificed and the testes were collected and decapsulated. The testicular interstitial cells were isolated with the collagenase dispersion method as previously described [Tsai et al., 1997]. The procedure used for the preparation of Leydig cells has been described elsewhere [Huang et al., 2001; Chiao et al., 2002]. The testicular interstitial cells were centrifuged at 4°C, 200*g* for 10 min. The cell pellet volume was suspended in the incubation medium (1% bovine serum albumin in Medium 199, with 25 mM HEPES, 2.2 g/ml $NaHCO_3$, 100 IU/ml penicillin-G, 50 μg/ml streptomycin sulfate, 2550 USP K U/L heparin, pH 7.4, and aerated with 95% O_2 and 5% CO_2) to 5 ml and then added gently to the upper layer of the continuous Percoll gradient. The continuous Percoll gradient (20 ml/dispersion) was made by adding of 9 parts of Percoll to 11 parts of 1.8 × concentrated incubation medium before centrifugation at 4°C, 20,000*g* for 60 min. The mixture of testicular interstitial cells was loaded onto the Percoll gradient and centrifuged at 4°C, 800*g* for 20 min. The Leydig cells were located at 3–7 ml layer from the bottom. The Leydig cells layer was collected and diluted to 10 ml in incubation medium and then centrifuged at room temperature, 200*g* for 10 min. After repeating the washing steps, the cell pellet was suspended to 10 ml in incubation medium. The cell concentration (2×10^5 cells/ml) and viability (over 95%) were determined using a hemocytometer and the trypan blue method. To measure the abundance of Leydig cells in our preparation, the 3β-hydroxysteroid dehydrogenase (3β-HSD) staining method was used [Krummen et al., 1994; Tsai et al., 1997]. The cells (2×10^5 cells/ml) were incubated with a solution containing 0.2 mg/ml nitro blue tetrazolium (Sigma) in 0.05 M PBS, pH 7.4 at 34°C for 90 min. Upon development of the blue formazan deposit sites of 3β-HSD activity, the abundance of Leydig cells was determined using a hemocytometer. Macrophages were determined by flowcytometry with FITC-labeled monoclonal antibody (ED1, IgG 1, Biosource, Camarillo, CA). Our preparation was found to contain approximately 87% Leydig cells and very few macrophages [Huang et al., 2001; Chiao et al., 2002].

Effects of H_2O_2 on Testosterone Release by Rat Leydig Cells

Cells at a concentration of 1×10^5 cells/ml were preincubated at 34°C for 1 h under a controlled atmosphere (95% O_2 and 5% CO_2), shaken at 100 cycles/min. The supernatant fluid was decanted after centrifugation of the tubes at 200*g* for 10 min. The cells were then incubated with H_2O_2 (0, $4 \times 10^{-5} \sim 4 \times 10^{-4}$ M) or human chorionic gonadotropin (hCG, 0.05 IU/ml) in 200 μl fresh medium. Following 1 h of incubation, 0.5 ml ice-cold PBSG (0.1% gelatin-phosphate buffer saline, pH 7.5) was added to stop the incubation. The medium was centrifuged at 200*g* for 10 min and the supernatant was stored at −20°C until analyzed for testosterone by radioimmunoassay (RIA) [Tsai et al., 1997; Huang et al., 2001; Chiao et al., 2002].

The cells (1×10^5 cells) were challenged with H_2O_2 in the presence of forskolin (an adenylyl cyclase activator, 10^{-5} and 10^{-4} M) or 8-Br-cAMP (a membrane permeable analog of cyclic AMP, 10^{-4} M) in 200 μl fresh medium. At the end of incubation, the media were collected for testosterone RIA, as previously described [Tsai et al., 1997; Huang et al., 2001; Chiao et al., 2002].

Effects of H_2O_2 on the Activities of Steroidogenic Enzymes in Rat Leydig Cells

Rat Leydig cells were incubated for 1 h with or without H_2O_2 at 200 μM in the presence of four steroidogenic precursors ($10^{-7} \sim 10^{-5}$ M). These precursors included 25-OH-cholesterol (a membrane-permeable cholesterol), pregnenolone, progesterone, and androstenedione. At the end of incubation, the media were collected for testosterone RIA, as previously described.

Effects of H_2O_2 on 25-OH-Cholesterol-Stimulated Pregnenolone Production in Rat Leydig Cells

Leydig cells (1×10^5 cells/ml) were preincubated with incubation medium for 1 h at 34°C. Cells were primed with trilostane (4,5-epoxy-17-hydroxy-3-oxoandrostane-2-carbonitrile, an inhibitor of 3β-HSD, 10^{-5} M) for 30 min and then incubated for 1 h with trilostane (10^{-5} M) or trilostane plus 200 μM H_2O_2 in the absence or presence of 25-OH-cholesterol (10^{-5} M). At the end of incubation, the media were collected for pregnenolone RIA [Chen et al., 2002].

Hormone RIAs

The concentrations of testosterone in media were determined by RIA as described previously

[Tsai et al., 1997; Huang et al., 2001; Chiao et al., 2002]. The sensitivity of testosterone RIA with anti-testosterone serum (No. W8) was 2 pg per assay tube. The intra- and interassay coefficients of variation (CV) were 4.1% (n = 6) and 4.7% (n = 10), respectively.

The concentrations of pregnenolone were determined by RIA as described previously [Chen et al., 2002; Chiao et al., 2002]. The sensitivity of the pregnenolone RIA was 16 pg per assay tube. The intra- and interassay coefficients of variation (CV) were 2.3% (n = 6) and 3.7% (n = 4), respectively.

Western Blot Analysis

The method of Western blotting has been described elsewhere [Chen et al., 2002; Chiao et al., 2002]. Leydig cells (1×10^6 cells/ml) were incubated with or without H_2O_2 at 0, $4 \times 10^{-5} \sim 2 \times 10^{-4}$ M for 1 h. At the end of incubation, cells were washed twice with ice-cold saline, and homogenized in 50 µl RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 mM Na-orthovanadate, 1 mM NaF). Aliquots (10-µg protein) of cell lysates were electrophoresis on a 12% mini gel by standard SDS–PAGE procedures, and electrotransferred to polyvinylidene difluoride (PVDF) membranes (NEN Life Science Products, Boston, MA) by a semi-dry transfer cell (Bio-Rad) as previously described. The membrane was washed in TBS-T buffer (0.8% NaCl, 0.02 M Tris base, 0.1% Tween 20, pH 7.6) for 5 min and blocked by a 2-h incubation in blocking buffer (TBS-T buffer containing 5% non-fat dry milk). Then the membrane was incubated overnight with anti-StAR (1:1,000) [Lin et al., 1998], anti-P450scc (1:2,000) [Hu et al., 1991], and anti-β-actin (1:8,000). After three washes for 5 min each with TBS-T buffer, the membranes were incubated for 2 h with horseradish peroxidase-conjugated secondary antibody (1:6,000). Specific signals were detected by chemiluminescence (ECL™ Western blotting detection reagents, Amersham Pharmacia Biotech).

Statistical Analysis

All values are given as mean ± standard error of the mean (SEM). The treatment means were tested for homogeneity by a one-way analysis of variance (ANOVA) and the differences between specific means were tested for significance by Duncan's multiple-range test [Steel and Torrie, 1960]. A difference between two means was considered statistically significant when $P < 0.05$.

RESULTS

Effects of H_2O_2 on Testosterone Secretion

As compared with the control group, H_2O_2 ($1 \times 10^{-4} \sim 4 \times 10^{-4}$ M) produced a significant inhibition of testosterone release from Leydig cells ($P < 0.01$, upper panel of Fig. 1). Incubation of Leydig cells with hCG for 1 h increased the level of testosterone release, and this acts as a positive control for this study ($P < 0.01$, lower panel of Fig. 1). A combination of hCG with H_2O_2 of $1 \times 10^{-4} \sim 4 \times 10^{-4}$ M resulted in a significant inhibition of the hCG-stimulated release of testosterone ($P < 0.05$ or <0.01).

In Figure 2, following priming with forskolin, levels of testosterone in the medium were significantly ($P < 0.01$) higher than those of the corresponding forskolin-free controls. H_2O_2 at $1 \times 10^{-4} \sim 4 \times 10^{-4}$ M resulted in a significant inhibition of forskolin-stimulated release of testosterone ($P < 0.05$ or <0.01).

Administration of 8-Br-cAMP stimulated testosterone release significantly ($P < 0.01$).

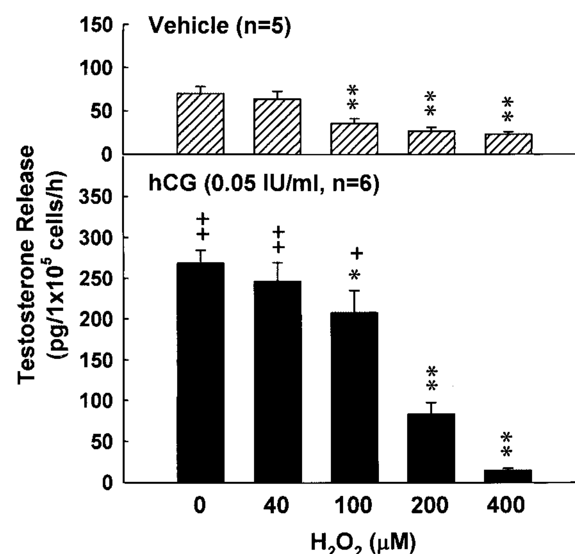

Fig. 1. Effects of different doses of H_2O_2 on the release of testosterone in the absence or presence of hCG (0.05 IU/ml). $*P < 0.05$, $**P < 0.01$ as compared with the value at $H_2O_2 = 0$ M, respectively. $^{+}P < 0.05$, $^{++}P < 0.01$ as compared with vehicle group. Each column represents mean ± SEM.

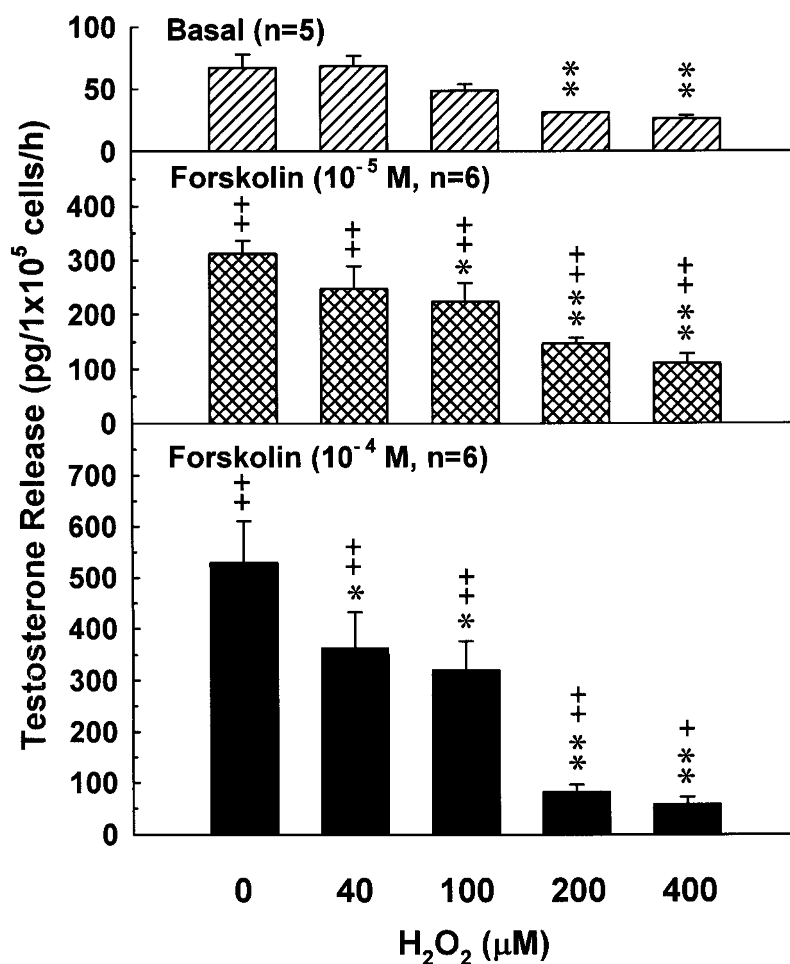

Fig. 2. Effects of different doses of H_2O_2 on the release of testosterone in response to forskolin (10^{-5} and 10^{-4} M). $^*P < 0.05$, $^{**}P < 0.01$ as compared with the value at $H_2O_2 = 0$ M, respectively. $^+P < 0.05$, $^{++}P < 0.01$ as compared with basal group. Each column represents mean ± SEM.

H_2O_2 (100 or 200 μM) inhibited 8-Br-cAMP-stimulated testosterone ($P < 0.01$) (Fig. 3).

Effects of H_2O_2 on Steroidogenesis in Leydig Cells

For investigating the effects of H_2O_2 on steroidogenesis, several steroidogenic precursors were used to challenge rat Leydig cells (Figs. 4 and 5). The precursors include 25-OH-cholesterol (substrate of P450scc), pregnenolone (substrate of 3β-HSD), progesterone (substrate of 17α-hydroxylase), and androstenedione (substrate of 17β-HSD). Three doses (10^{-7}, 10^{-6}, and 10^{-5} M) of each precursor were employed. Administration of all precursors at 10^{-5} M resulted in a significant ($P < 0.01$) and maximum testosterone release. H_2O_2 at 200 μM decreased not only the basal release of testosterone but also the evoked release of testosterone in response to 25-OH-cholesterol (10^{-7} and 10^{-5} M, $P < 0.05$ or < 0.01, Fig. 4). We also calculated the percent inhibition of H_2O_2 effects in the presence of different precursors. H_2O_2 at 200 μM decreased the basal release of testosterone by 75 ± 7%. In the presence of 25-OH-cholesterol at 10^{-7} M, 10^{-6} M, and 10^{-5} M, H_2O_2 decreased testosterone release by 58 ± 10%, 48 ± 9%, and 35 ± 8%, respectively.

H_2O_2 (200 μM) also decreased the stimulatory effects of pregnenolone, progesterone, or androstenedione on testosterone production (10^{-7} and 10^{-6} M; $P < 0.05$ and < 0.01). The administration of pregnenolone, progesterone, or androstenedione at 10^{-5} M abolished the inhibitory effects of H_2O_2 on testosterone release in rat Leydig cells. The mean values of percent

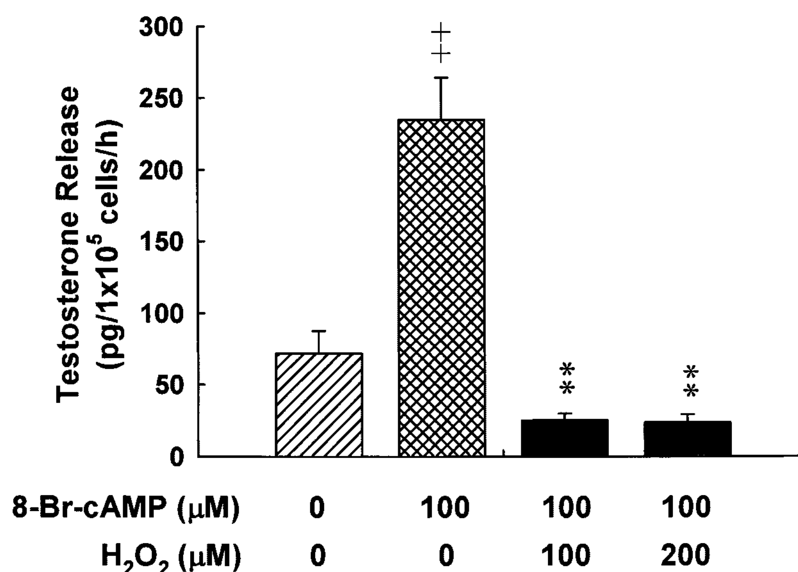

Fig. 3. Effects of different doses of H_2O_2 on the release of testosterone in the absence or presence of 8-Br-cAMP (10^{-4} M). $^{**}P < 0.01$ as compared with the value at 8-Br-cAMP treated only, respectively. $^{++}P < 0.01$ as compared with 8-Br-cAMP = 0 M. Each column represents mean ± SEM.

inhibition compared to respective vehicle of testosterone production were marked at corresponding columns (Fig. 4).

Effects of H_2O_2 on Pregnenolone Production

Pregnenolone is the product of P450scc by conversion from cholesterol. Intracellular pregnenolone is metabolized by 3β-HSD to produce progesterone. For investigating the pregnenolone accumulation, trilostane (10^{-5} M) was used to inhibit 3β-HSD. Rat Leydig cells were incubated for 1 h in the medium with trilostane plus 25-OH-cholesterol, combined with or without H_2O_2. In Figure 5, administration of 25-OH-cholesterol increased accumulation of pregnenolone ($P < 0.01$); H_2O_2 (200 μM) significantly decreased basal and 25-OH-cholesterol-induced accumulation of pregnenolone by 57 ± 3% and 28 ± 3%, respectively ($P < 0.01$ and <0.05).

Protein Expression of StAR and P450scc

Bands at 54 kDa (P450scc) and 30 kDa (StAR) molecular weight were detected in rat Leydig cells (Fig. 6). Signal of β-actin (45 kDa) was used as an internal control. Cells were incubated with or without H_2O_2 ($4 \times 10^{-5} \sim 2 \times 10^{-4}$ M) for 1 h before extraction of the proteins. Western blot analysis was employed to measure the expression of the StAR and P450scc proteins. Several independent experiments were repeated with similar results (upper panel, Fig. 6), the ratios of the protein expression were shown in lower panel (Fig. 6). The protein expression of neither StAR protein nor P450scc was altered by hCG. Interestingly, after the Leydig cells were challenged with 200 μM H_2O_2, the ratio of StAR/β-actin markedly dropped to 76% ($P < 0.01$). Meanwhile, H_2O_2 did not alter the protein expression of P450scc.

DISCUSSION

The present results demonstrate that H_2O_2 inhibits the spontaneous and hCG-stimulated steroidogenesis in normal rat Leydig cells, at least in part, via attenuation of the activities of cytochrome P450scc and expression of StAR protein by acting directly on rat Leydig cells.

Since steroidogenesis has been shown to generate ROS [Peltola et al., 1996] and steroidogenic cells and human spermatozoa are known susceptible to ROS [Aitken and Clarkson, 1987; Myers and Abney, 1988], we suggested that ROS, such as H_2O_2, might be involved in the regulation of testosterone production in Leydig cells. The mechanism of H_2O_2 action on testosterone secretion was investigated by a well-established in vitro cellular model, using 8–Br-cAMP and forskolin to mimic the action of hCG on Leydig cell to induce intracellular cAMP production [Tsai et al., 1997; Chiao et al., 2002]. Our unpublished data

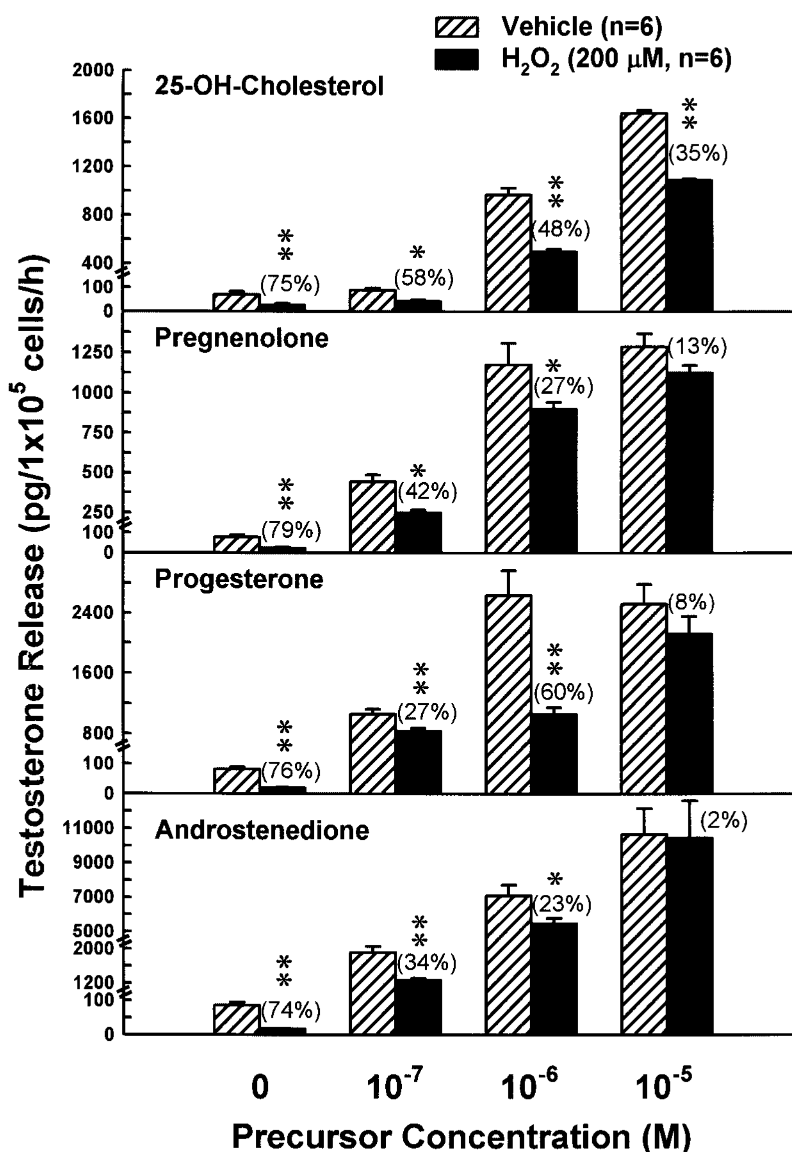

Fig. 4. Effect of H_2O_2 (200 μM) on steroidogenic precursors-stimulated testosterone release. The precursors include 25-hydroxycholesterol (25-OH-cholesterol), pregnenolone, progesterone, and androstenedione. All the precursors stimulated testosterone release significantly ($P < 0.01$). The mean values of percent inhibition of testosterone production, compared to respective vehicle, were marked above each black bar. $^{*}P < 0.05$ and $^{**}P < 0.01$ as compared with vehicle group. Each column represents mean ± SEM.

have shown that exposure of rat Leydig cells to H_2O_2 at 4×10^{-4} M did not affect the viability of Leydig cells. H_2O_2 inhibits not only basal but also hCG-, forskolin-, and 8-Br-cAMP-stimulated testosterone release, which strongly supports our hypothesis that H_2O_2 acts directly on Leydig cells to regulate testosterone production at a point distal to the formation of cAMP.

Previous studies and our present data have shown that exposure of rat Leydig cells to H_2O_2 in vitro, diminishes basal and evoked testosterone secretion [Stocco et al., 1993]. This evidence indicates that the effect of H_2O_2 on testis is independent of gonadotropin. It has been shown that the testicular macrophage is another major cellular population in the testicular interstitium (approximately 20–30%) besides Leydig cells [Niemi et al., 1986]. Macrophages produce cytokines to modulate physiological activities of other cells in the testis. Several reports have indicated that macrophages play an important role in regulating the steroidogenesis of Leydig cells [Gaytan et al., 1994, 1995]. The present study shows an inhibitory effect of H_2O_2 on

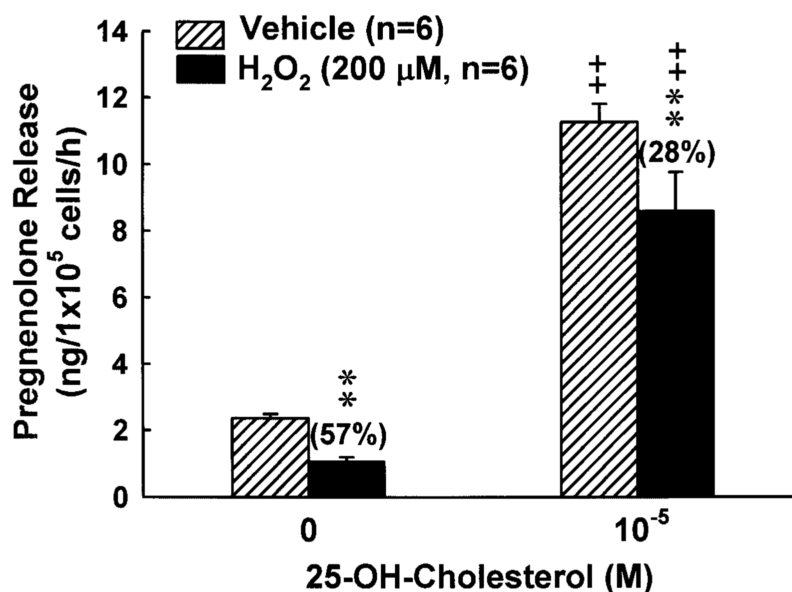

Fig. 5. Effects of H_2O_2 (200 μM) on pregnenolone production in rat Leydig cells. The cells were primed with trilostane (10^{-5} M) for 30 min and then incubated for 1 h with trilostane or trilostane plus 200 μM H_2O_2 in the absence or presence of 25-OH-cholesterol (10^{-5} M). The mean values of percent inhibition of pregnenolone production, compared to respective vehicle, were showed above each black bar. $^{**}P < 0.01$ as compared with 25-OH-cholesterol = 0 M. Each column represents mean ± SEM.

Percoll-purified rat Leydig cells. Since our Leydig cell preparation contained approximately 87% Leydig cells and very few macrophages [Chiao et al., 2002], H_2O_2 should act directly on the Leydig cells via a macrophage-independent mechanism.

In Leydig cells, testosterone is synthesized by several metabolic steps called "steroidogenesis." First, cholesterol is transformed to pregnenolone by P450scc, the rate-limiting enzyme. In Leydig cells, pregnenolone is then dehydrogenated to progesterone by 3β-HSD, and progesterone is converted to androstenedione by P450C17. Finally, androstenedione is reduced to testosterone by 17β-HSD [Payne and O'Shaughnessy, 1996]. In our study, Leydig cells were incubated in vitro with the precursor of each metabolic step. Cholesterol was replaced by 25-OH-cholesterol because the latter is membrane-permeable. The higher concentration (10^{-5} M) was used to obviate the interference of any endogenous precursor. Since, we observed the ending product, testosterone, the activities of steroidogenic enzymes must be examined firstly from the last enzyme, 17β-HSD. The inhibitory effect of H_2O_2 was detected at lower concentration (10^{-7} and 10^{-6} M) of androstenedione but not at higher concentration (10^{-5} M). This indicates that the activity of 17β-HSD might not be affected by H_2O_2. A similar effect was found for the challenges with progesterone and pregnenolone. These observations support the idea that neither 3β-HSD nor P450c17 activity is affected by H_2O_2.

However, 25-OH-cholesterol-stimulated testosterone production was inhibited by H_2O_2 suggesting that the activity of P450scc might be reduced by H_2O_2. In order to confirm whether the P450scc activity is altered by H_2O_2, trilostane was used to inhibit the activity of 3β-HSD and the accumulation of pregnenolone in rat Leydig cells was examined. We found that 25-OH-cholesterol-stimulated pregnenolone production was inhibited by H_2O_2, which reflected a reduction in the activity of P450scc. After examination by Western blot, the protein expression of P450scc was not altered by H_2O_2. These results demonstrated that the acute inhibitory effects of H_2O_2 on testosterone production might be via an inhibition of P450scc activity, rather than an effect on P450scc protein expression.

In all species, the rate-limiting step in androgen biosynthesis includes transferring of cholesterol by StAR protein from outer to inner mitochondria membrane and conversion of cholesterol to pregnenolone by P450scc. Some earlier studies of the effects of oxidative stress on steroidogenesis [Behrman and Aten, 1991; Stocco et al., 1993] did not investigate the changes of StAR protein. StAR protein is cAMP-dependent, and is also regulated by

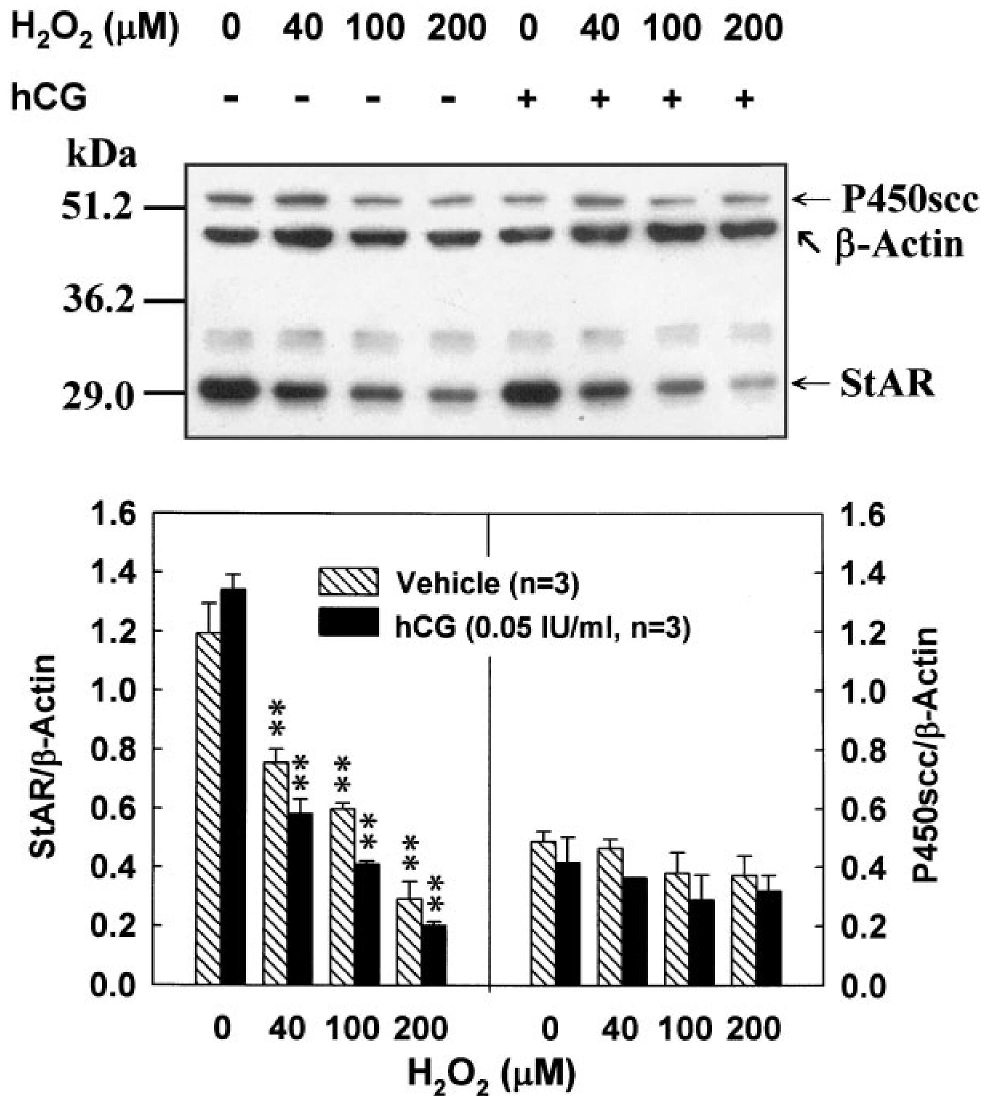

Fig. 6. Effects of H_2O_2 on the protein expression of cytochrome P450scc and StAR protein in rat Leydig cells. Rat Leydig cells were incubated with H_2O_2 in the absence or presence of hCG (0.05 IU/ml) at 34°C for 1 h. Then, the cells were collected and analyzed by Western blotting. Each lane was loaded with a sample of 10 μg protein. $^{**}P < 0.01$ as compared with H_2O_2 at 0 M. Each column represents mean ± SEM.

upstream trophic signal, such as follicle-stimulating hormone (FSH) and hCG. In the ovarian cells, the expression of StAR mRNA was elevated by incubated with FSH for 1 h, and the expression of StAR protein was elevated at least 2–h incubation [Silverman et al., 1999]. During 6-h incubation, treatment of MA-10 cells with 1 mM $(Bu)_2cAMP$ (an analog of cAMP) increased the expression of StAR transcript at 1, 3, and 6 h, and increased the expression of StAR protein at 3 and 6 h [Murphy et al., 2001]. The protein expression of StAR protein was not altered by hCG, but inhibited by H_2O_2 during 1-h incubation. Therefore, we suggested that the unchanged protein expression of StAR protein in response to hCG was due to short period of incubation. Inasmuch as H_2O_2 inhibits StAR protein level, we suggest that H_2O_2 might inhibit the transfer of cholesterol into mitochondria and prevent the translocation of StAR to mitochondria, which induce catabolism of StAR protein.

StAR protein is a nuclear-encoded, mitochondria-targeted protein. StAR protein are translated as a larger molecular mass (37 kDa) containing mitochondria targeting precursor in the amino terminus. Targeting sequences of mitochondrial matrix interact with the trans-

location complexes on the outer and inner membranes of mitochondria, and enter the matrix to produce the intermediate 32- and mature 30-kDa forms. The final intramitochondrial processing step to produce the 30-kDa mature form is the off-step in the cholesterol transfer process [Stocco, 2001]. Besides, defective mitochondria may generate more ROS via electron leak, oxidative stress and oxidative damage in tissues and culture cells are increased in elderly subjects and patients with mitochondria disease [Wei et al., 2001]. In this study, we did not exclude the possibility that post-transcriptional process of StAR protein might be inhibited through ROS-damaged mitochondrial function, and the influences of ROS production during steroidogenesis on StAR protein and spermatozoa need to be further examined.

In summary, the present results demonstrate that H_2O_2 can act directly on rat Leydig cells to inhibit testosterone production. H_2O_2 diminished the activity of P450scc and the protein expression of StAR protein rapidly and then modulated the steroidogenesis of testosterone.

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