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Induction of Lipid Peroxidation during Steroidogenesis in the Rat Testis*

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ABSTRACT

Free radical production and lipid peroxidation are potentially important mediators in testicular physiology and toxicology. The cytochrome P450 enzymes of the steroidogenic pathway are known to produce free radicals. The present study was conducted to elucidate *in vivo* the gonadotropin regulation of free radical-mediated lipid peroxidation and the antioxidative defense system in the rat testis.

GnRH antagonist (Org 30276; 1 mg/kg BW) and testosterone [40-mm SILASTIC brand (Dow-Corning) capsules] treatments were used to suppress serum gonadotropin levels. As expected, serum LH decreased to a very low level, whereas serum FSH decreased only slightly. Testosterone treatment for 8 days decreased the levels of the peroxide-metabolizing enzymes, catalase, glutathione peroxidase (GSH-Px), and glutathione transferase (−44%, −24%, and −31%, respectively; $P < 0.01$ for all). These changes predominately reflect the interstitial tissue, in which catalase and GSH-Px activities were much higher than in the seminiferous tubules. Testicular CuZn or Mn superoxide dismutase activities, which were high in the seminiferous tubules, were not affected by gonadotropin suppression. The total peroxyl radical-trapping capacity of the testis, or its components, vitamin E and ubiquinol 9, were not affected either. Lipid peroxidation was decreased after 8-day testosterone treatment, as detected by diminished formation of conjugated dienes and fluorescent chromolipids (−30% and −19%, respectively; $P < 0.05$ for both). Similar results of decreasing catalase and GSH-Px activities were found after gonadotropin suppression with GnRH antagonist treatment for 2 days or testosterone treatment for 5 days. Substitution with hCG, alone or in combination with recombinant human FSH, reversed the changes in enzyme activities, whereas FSH alone had no effect. After 5-day testosterone treatment, catalase messenger RNA expression was studied by Northern hybridization, and it was observed to parallel the changes in enzyme activity. The site of free radical production was studied by separating interstitial tissue and seminiferous tubules 5 h after hCG injection. GSH-Px was induced by hCG only in the interstitial tissue (+28%; $P < 0.01$), supporting the hypothesis of free radical production during steroidogenesis. Aminoglutethimide, an inhibitor of the P450 cholesterol side-chain cleavage enzyme, induced extensive lipid peroxidation in the testis. Presumably, aminoglutethimide leads to leakage of free radicals from the P450 enzyme when substrate oxygenation is prevented.

In conclusion, the present study suggests that physiological LH action in the rat testis causes lipid peroxidation and maintains high activities of peroxide-metabolizing enzymes in the interstitial tissue. The steroidogenic steps regulated by the P450 enzymes are the most likely sites of free radical generation. (*Endocrinology* **137:** 105–112, 1996)

REACTIVE OXYGEN species are produced in normal cellular metabolism. Testicular metabolism is under endocrine control; LH stimulates Leydig cell steroidogenesis, and FSH stimulates Sertoli cell functions and thereby, indirectly, spermatogenesis. The testicular production of free radicals and the function of the antioxidative defense system require further studies, because there is evidence of reactive oxygen species having a role in infertility caused by defective sperm function (1–4) and in testicular damage in cryptorchidism (5) or upon exposure to toxic chemicals (6–8).

The cytochrome P450 enzymes of the steroidogenic pathway use molecular oxygen and electrons transferred from NADPH to hydroxylate the substrate (9). In this process, superoxide anion or other oxygen free radicals can be produced as a result of electron leakage in normal reactions or due to interaction of steroid products or other pseudosubstrates with the enzyme (9–12). The inability of the pseudosubstrate to be oxygenated promotes the release of reactive oxygen species. The production of oxygen free radicals in steroidogenesis is suggested also by the high amounts of antioxidant vitamins, such as vitamin E, β-carotene, and ascorbate, in the steroidogenic organs (testis, ovary, and adrenal cortex) (11, 13–15). Moreover, the rat ovarian antioxidants are regulated by LH action *in vivo* (13).

Antioxidation enzymes form an essential part of the cellular defense against reactive oxygen species. Superoxide dismutase (SOD; Mn SOD in mitochondria and CuZn SOD in cytosol) reduces the superoxide anion to hydrogen peroxide, and its activity in the testis is high (16). The activities of the peroxide-metabolizing enzymes, catalase and glutathione peroxidase (GSH-Px), are very low in germ cells (17); therefore, the levels in whole testis samples are low compared to those in liver (16). Promotion of follicle growth in female rats by equine CG increases the expression of secreted SOD and Mn SOD messenger RNA (mRNA), and FSH-mediated suppression of follicular apoptosis has been suggested to involve enhanced protection from reactive oxygen species (18). Induction of SOD has also been observed in mouse Leydig cells (19) and rat ovary (20) after LH injections.

As stated above, mainly *in vitro* and ovarian studies propose testicular free radical metabolism to be affected by the

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action of LH. In addition to the effects of LH on steroidogenesis and those of testosterone on spermatogenesis, oxygen free radicals could be produced in FSH-stimulated metabolism in Sertoli cells or in differentiating germ cells under stimulation by Sertoli cells. The present study elucidates *in vivo* gonadotropin regulation of free radical-mediated lipid peroxidation and the antioxidative defense system in the rat testis. The results show that the production of free radicals during steroidogenesis is capable of inducing lipid peroxidation and peroxide-metabolizing enzymes.

Materials and Methods

Hormones, reagents, and complementary DNA (cDNA) probes

Partially purified hCG (Pregnyl; 3000 IU/mg) was purchased from Organon (Oss, The Netherlands), and recombinant human FSH (Org 32489) was donated by Organon. Testosterone was purchased from Sigma Chemical Co. (St. Louis, MO). The GnRH antagonist used, Ac-D-pClPhe-D-pClPhe-D-Trp-Ser-Tyr-D-Arg-Leu-Arg-Pro-D-Ala-NH_2CH_3COOH (Org 30276), was donated by Organon. Testosterone antiserum was donated by Prof. R. Vihko (University of Oulu, Oulu, Finland). ^{125}I-Labeled testosterone was purchased from Farmos Diagnostica (Oulunsalo, Finland). RIA reagents for rat FSH were supplied by the National Hormone and Pituitary Distribution Program (NIDDK, NIH, Bethesda, MD).

Aminoglutethimide (AMG), 1-chloro-2,4-dinitrobentsene, cumene hydroperoxide, hydrogen peroxide (H_2O_2), linoleic acid, NADPH, xanthine, and the enzymes, catalase (bovine liver), glutathione reductase (baker's yeast), SOD (Cu/Zn-form, bovine erythrocytes), and xanthine oxidase (buttermilk), were purchased from Sigma Chemical Co. Lucigenin (bis-*N*-methylacridinium nitrate) and luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) were purchased from Bio-Orbit (Turku, Finland). Reduced glutathione was purchased from Boehringer (Mannheim, Germany). 2,2′-Azobis-(2,4-dimethylvaleronitrile) (AMVN) was purchased from Polysciences (Warrington, PA), and D-α-tocopherol was obtained from Eastman Kodak Co. (Rochester, NY).

The cDNAs for rat CuZn SOD (21) and Mn SOD (22) were provided by Dr. Y.-S. Ho (Wayne State University., Detroit, MI), and the cDNA for rat catalase (23) was supplied by Dr. S. Furuta (Shinshu University School of Medicine, Matsumoto, Japan). Hybridization with a 1.3-kilobase (kb) fraction of the cDNA clone pI-19 for mouse 28s RNA (24) was used as a loading control.

Animals and treatments

Adult male Sprague-Dawley rats were used. The GnRH antagonist treatments were carried out at the University of Tampere, and the rats used were produced in the departmental vivarium. The other rats used were produced at the vivarium of University of Turku or obtained from B & K Universal (Sollentuna, Sweden; for hCG with or without AMG treatments). The rats were kept under constant environmental conditions and fed standard laboratory rat chow and water *ad libitum*. In the first experiment, testosterone treatment for 8 days was administered in SILASTIC brand capsules (Dow Corning, Midland, MI; id, 1.98 mm; od, 3.18 mm; length, 40 mm). The capsules were kept in 0.01 mol/liter PBS (pH 7.4) at room temperature for 24 h before implantation under Hypnorm (fluanisone plus fentanyl; Janssen, Beerse, Belgium) and Dormicum (midazolame; Hoffman LaRoche, Basel, Switzerland) anesthesia. Control rats were identically implanted with empty capsules. The 8-day treatment period was approximated to be long enough to reveal the hypothesized changes in lipid peroxidation products. In the second experiment, rats were treated for 2 days with vehicles (sun flower oil and saline), GnRH antagonist (1 mg/kg BW), or GnRH antagonist plus hCG (10 IU/kg BW) plus recombinant human FSH (35 IU/kg BW). Injections were given sc once daily. The GnRH antagonist treatment was carried out for only 2 days to avoid possible secondary changes caused by testicular atrophy during gonadotropin suppression. In the third experiment, rats were implanted with SILASTIC capsules containing testosterone, and control rats were implanted with empty capsules. Groups of testosterone-treated rats were injected sc with vehicle (saline), hCG (10 IU/kg BW), FSH (20 IU/kg BW), or hCG plus FSH. hCG injections were given once daily for the entire 5-day experiment, whereas FSH injections were given once daily for the last 2 days only. Control rats received daily injections of vehicle. The last experiment included four different treatments: vehicle (saline), hCG (400 IU/kg BW), AMG (190 mg/kg BW), and hCG plus AMG. The AMG was administered in two ip doses each of 95 mg/kg BW in saline, pH 3.0, to obtain maximal inhibition of steroidogenesis (25). The first injection was given 1 h before the sc injection of hCG (or vehicle), and the other dose was given 4 h after the first injection. The control rats were injected with the vehicle according to the same protocol. The rats were killed 5 h after hCG (or vehicle) injection. Light carbon dioxide anesthesia was induced before decapitation. All animal studies were approved by the committees on animal care and use of the University of Tampere or Turku.

Tissue preparation

After decapitation, trunk blood was collected, and serum was prepared by centrifugation. The testes were dissected out and weighed. For total peroxyl radical-trapping capacity (TRAP), vitamin E, and ubiquinol measurements, the other testis was frozen in liquid nitrogen and homogenized with Mikro-Dismembrator (B. Braun, Melsungen, Germany). For lipid peroxidation and enzyme activity measurements, a 33% (wt/vol) testicular homogenate was prepared in 0.15 mol/liter potassium chloride using a Potter-Elvehjem glass-Teflon homogenizer at 0 C. A part of the homogenate was centrifuged (10,000 × *g* for 10 min at 4 C), and the supernatant was saved. An aliquot of the other testis was used for RNA isolation. In the last experiment, seminiferous tubules were isolated under a stereomicroscope in PBS, pH 7.4. Care was taken to avoid interstitial cells in the tubule preparation. Isolated tubules were rinsed and homogenized in PBS with a Teflon homogenizer. The dispersed interstitial cells were separated from tubule fragments, centrifuged, and homogenized in PBS with a Teflon homogenizer. For determination of GSH-Px activity, a 10,000 × *g* supernatant was prepared from the interstitial tissue and seminiferous tubule homogenates.

Lipid peroxidation measurements

The level of lipid peroxidation was determined by assays of conjugated diene double bonds in the supernatant of centrifuged testicular homogenate and fluorescent chromolipids in crude testis homogenate (see above). In the assay of diene conjugation, lipids extracted by chloroform-methanol were dried under nitrogen, then redissolved in cyclohexane immediately before spectrophotometrical analysis at 233 nm (26). Fluorescent chromolipids were assayed in testicular homogenates essentially as described by Esterbauer *et al.* (27). The samples were eluted with chloroform-methanol (2:1, vol/vol) and centrifuged at 2000 × *g* for 10 min. The organic phase was evaporated under nitrogen to dryness and redissolved in chloroform-methanol (10:1), and its fluorescence was measured at 360 nm (excitation)/430 nm (emission). Results were expressed per unit weight of protein, which was determined by the method of Lowry *et al.* (28).

Enzyme assays

The activity of SOD was measured by inhibition of xanthine-xanthine oxidase-dependent chemiluminescence enhanced by lucigenin and linoleate (29). The assay conditions were as described previously (30), except for a higher concentration of lucigenin (100 μmol/liter in cuvette) and a lower concentration of linoleic acid (4 mmol/liter in cuvette). CuZn SOD was measured at pH 10.0 and Mn SOD at pH 7.4 with 6 mmol/liter KCN in the reaction mixture. The activity of catalase was determined by measuring the rate of disappearance of 15 mmol/liter H_2O_2 at 240 nm (31). SOD and catalase activities were expressed as units of enzyme activity per mg protein with reference to standard samples of a purified enzyme preparation. GSH-Px was assayed by measuring the oxidation of $NADPH^+$ at 340 nm, with cumene hydroperoxide as the substrate (32). In the assay of glutathione transferase (GSH-Tr), 1-chloro-dinitrobenzene was used as the substrate, and the absorbance change was recorded at 340 nm (33). The results of GSH-Px and GSH-Tr assays

were expressed as micromoles of product per min/mg protein. SOD and catalase activities were determined in whole testis, interstitial tissue, or seminiferous tubule homogenates, whereas the supernatant of centrifuged homogenate was used in GSH-Px and GSH-Tr assays.

Northern blot analysis

Total RNA was isolated using the acid guanidinium thiocyanate-phenol-chloroform extraction method (34) and quantified by absorbance at 260 nm. RNA samples (20 μg) were resolved on 1% denaturing agarose gels, transferred onto nylon membranes (Hybond-N, Amersham International, Aylesbury, UK), and UV immobilized. Membranes were prehybridized at 42 C for 6 h in 50% formamide containing prehybridization solution made according to the recommendation of the membrane manufacturer (Amersham). The cDNA inserts were labeled with ^{32}P using the Multiprime DNA labeling system (Amersham). The labeled probe was added to the prehybridization solution, and hybridization was carried out at 42 C overnight. The membranes were washed under high stringency conditions and autoradiographed at −50 C on Kodak XAR-5 film (Eastman Kodak). Densitometric analysis of the autoradiograms was performed with an LKB UltroScan XL 2222–020 densitometer and an LKB 2400 GelScan XL software package (LKB Produkter, Bromma, Sweden). Results were expressed as arbitrary densitometric units; the mean of control values was taken as 100%. Calculations of the densitometric intensities per hybridization signal of 28S cDNA did not affect the results.

Hormone measurements

Serum FSH was measured by a double antibody RIA with NIDDK reagents. Iodinations with Na[^{125}I]iodide of the purified hormone preparation were performed using the chloramine-T method (35). The assay sensitivity for FSH was 0.15–0.3 ng/tube. The intra- and interassay variations were below 8% and 15%, respectively. A supersensitive immunofluorometric assay was used for serum LH measurements (36). The method is based on use of two monoclonal antibodies, one to human and the other to bovine LH, and on time-resolved fluorescence in signal detection (Delfia, Wallac Oy, Turku, Finland). The sensitivity of this assay is 0.75 pg/tube: the intra- and interassay variations were 2.5% and 7.5%, respectively. Testosterone was measured from diethylether extracts of serum by RIA as described previously (37).

Determinations of TRAP, vitamin E, and ubiquinol

The tissue homogenates were extracted with chloroform-methanol (1:1) and divided for determinations of vitamin E (D-α-tocopherol) and ubiquinol and for a chemiluminescent assay of chain-breaking antioxidants. In the TRAP assay, the sample or standard was exposed to peroxyl radicals produced by the thermal decomposition of AMVN at 32 C. The rate of peroxyl radical production in the decomposition of AMVN was followed by the luminol-enhanced chemiluminescence. The addition of an antioxidant to the reaction mixture extinguishes the chemiluminescence, and its duration has a linear correlation to the radical-trapping capability of the sample. The TRAP assay mixture contained 625 μl chloroform-methanol (3:2), 50 μl AMVN (25 mmol/liter) and 25 μl luminol (10 mmol/liter) in borate buffer (40 mmol/liter; pH 9.0). D-α-Tocopherol was used as a standard. The duration of the extinction caused by 1 nmol D-α-tocopherol was 1344 sec. D-α-Tocopherol is known to trap two peroxyl radicals (38). As the TRAP curve of testicular samples was biphasic, we measured the extinction times for both of the two phases (see Fig. 2). Vitamin E, ubiquinol 9, and, in some samples, ubiquinol 10 were determined by the HPLC method of Lang *et al.* (39). The concentration of ubiquinol 10 was less than 1 nmol/g testis, and its contribution to TRAP was negligible.

Statistical analysis

ANOVA followed by Duncan's test and two-tailed Student's *t* test for unpaired data were used in statistical analyses of the data. Statistical analyses of the hormone results were carried out after logarithmic transformation. $P < 0.05$ was considered statistically significant.

Results

Gonadotropin suppression by 8-day testosterone treatment

Testosterone treatment for 8 days decreased the serum LH concentration efficiently (−93%; $P < 0.01$), whereas the serum FSH level was decreased by only 17% ($P < 0.05$; Table 1). The dose of testosterone used did not increase the serum testosterone concentration over the level in the control rats (Table 1). Testis weights and protein concentration did not differ significantly from those in the control group (Table 1).

The activities of the lipid peroxide-metabolizing enzymes, catalase (Fig. 1a), GSH-Px (Fig. 1b), and GSH-Tr (Fig. 1c), were decreased in the testosterone-treated rats (−44%, −24%, and −36%, respectively; $P < 0.01$ for all). Testosterone treatment decreased the level of lipid peroxidation products in the testes; diene conjugates were lowered by 30% ($P < 0.05$; Fig. 1d) and fluorescent chromolipids by 19% ($P < 0.05$; Fig. 1e). The activities of CuZn SOD or Mn SOD were not affected by testosterone treatment (data not shown).

The testicular lipid phase TRAP curve was biphasic (Fig. 2). The contributions of vitamin E and ubiquinol 9 to TRAP were only 9% and 6%, respectively. Thus, the major part of testicular TRAP appeared to be composed of unidentified antioxidants. There was no difference between the control and treatment groups in the two phases of TRAP, total TRAP, or vitamin E or ubiquinol 9 concentrations (data not shown).

GnRH antagonist treatment for 2 days and gonadotropin replacement

As with testosterone treatment, GnRH antagonist decreased the serum LH concentration to a very low level (−80%; $P < 0.01$), but serum FSH was decreased only slightly (−26%; $P < 0.01$; Table 1). As the substituted recombinant human FSH cross-reacted in the rat FSH RIA, reliable quantitation of FSH was not possible from these samples. The serum testosterone concentration decreased after GnRH antagonist treatment (−91%; $P < 0.01$), and gonadotropin substitution returned it to the control level (Table 1). The testis weights and protein concentrations were not affected (Table 1).

Short term GnRH antagonist treatment decreased the activities of catalase (−20%; $P < 0.05$; Fig. 3a) and GSH-Px (−20%; $P < 0.01$; Fig. 3b). Gonadotropin replacement reversed the change in catalase activity, whereas GSH-Px activity remained below the control level. Changes in GSH-Tr activity were not statistically significant (Fig. 3c). In this experiment, lipid peroxidation was not significantly affected (Fig. 3, d and e). Testicular CuZn SOD or Mn SOD activities, TRAP, and vitamin E concentration were not affected by these treatments (data not shown).

LH and FSH substitution during gonadotropin suppression by testosterone treatment for 5 days

Testosterone treatment for 5 days decreased the serum LH concentration as expected (−84%; $P < 0.01$; Table 1). The decrease in the serum FSH concentration was not statistically significant. The serum testosterone concentration was not changed by testosterone treatment, but hCG substitution alone or in combination with FSH doubled the serum level

TABLE 1. Testis weights and protein concentrations and serum concentrations of LH, FSH, and testosterone in the different treatment groups of the experiments described in *Materials and Methods*

	Wt (g)	Protein (mg/g)	LH (μg/liter)	FSH (μg/liter)	Testosterone (nmol/liter)
Exp I					
Control	3.50 ± 0.05	67.4 ± 5.1	2.28 ± 0.34	15.4 ± 0.5	7.2 ± 1.5
T	3.42 ± 0.10	62.3 ± 4.7	0.16 ± 0.05[a]	12.8 ± 0.9[b]	9.2 ± 0.4
Exp II					
Control	3.87 ± 0.08	78.6 ± 2.4	1.13 ± 0.20	13.2 ± 0.5	13.4 ± 2.7
GnRH-ant.	3.76 ± 0.08	85.1 ± 1.3	0.22 ± 0.06[a]	9.7 ± 0.5[a]	1.2 ± 0.6[a]
GnRH-ant. +hCG+FSH	3.97 ± 0.10	83.1 ± 1.9	0.11 ± 0.04[a]	ND	16.8 ± 0.9
Exp III					
Control	3.42 ± 0.12	75.3 ± 2.8	1.17 ± 0.34	20.3 ± 1.7	20.4 ± 3.0
T	3.07 ± 0.09	77.7 ± 1.7	0.19 ± 0.02[a]	17.7 ± 0.8	19.6 ± 4.0
T+hCG	3.09 ± 0.14	76.7 ± 4.4	0.13 ± 0.01[a]	25.3 ± 3.2	48.9 ± 7.3[a]
T+FSH	3.10 ± 0.11	78.8 ± 0.8	0.15 ± 0.01[a]	ND	25.2 ± 3.4
T+hCG+FSH	3.14 ± 0.37	83.8 ± 2.9	0.11 ± 0.01[a]	ND	40.9 ± 5.2[a]
Exp IV					
Control	2.76 ± 0.09	84.6 ± 3.8	ND	ND	6.8 ± 1.6
hCG	2.73 ± 0.02	79.0 ± 5.4	ND	ND	24.6 ± 3.1[a]
AMG	2.51 ± 0.06[b]	80.3 ± 3.4	ND	ND	1.0 ± 0.4[a]
hCG+AMG	2.62 ± 0.06	78.7 ± 0.7	ND	ND	2.0 ± 0.4[a]

Values are the mean ± SEM of five or six rats per group. Durations of the treatments were 8 days, 2 days, 5 days, and 6 h in Exp I–IV, respectively. T, Testosterone-treated rats; GnRH-ant., GnRH-antagonist-treated rats; GnRH-ant.+hCG+FSH, GnRH antagonist-treated rats with gonadotropin replacement; T+hCG, testosterone-treated rats with hCG substitution; T+FSH, testosterone-treated rats with FSH substitution; T+hCG+FSH, testosterone-treated rats with hCG and FSH substitution. ND, Not determined.

[a] $P < 0.01$ *vs.* control.

[b] $P < 0.05$ *vs.* control.

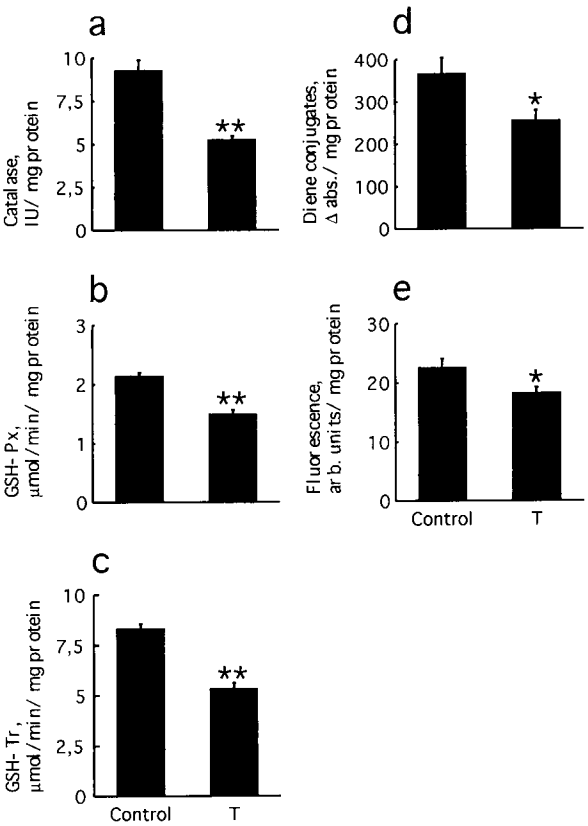

FIG. 1. Effects of 8-day testosterone treatment on the activities of catalase (a), GSH-Px (b), and GSH-Tr (c) and on levels of diene conjugates (d) and fluorescent chromolipids (e) in the rat testis. Values are the mean ± SEM (n = 5–6). T, Testosterone-treated rats. The *asterisks* indicate a significant difference from the control: *, $P < 0.05$; **, $P < 0.01$.

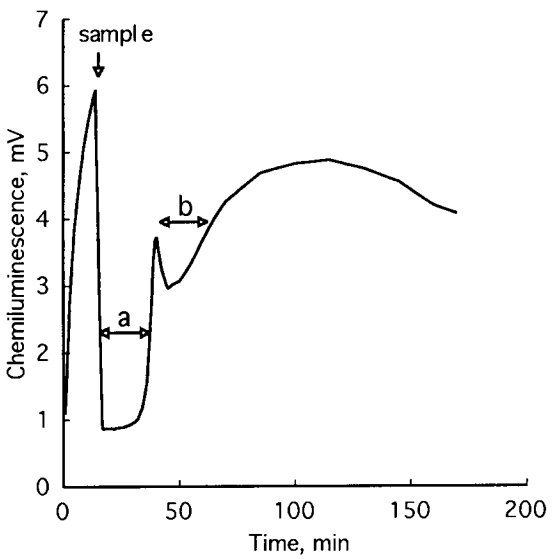

FIG. 2. An example of the biphasic testicular TRAP curve. The first (a) and second (b) phases of the chemiluminescence extinction caused by the sample added at 15 min are shown.

of testosterone ($P < 0.01$; Table 1). Testis weights and protein concentrations were not affected (Table 1).

Testosterone treatment decreased testicular catalase activity (−30%; $P < 0.01$; Fig. 4a). hCG substitution reversed the change, whereas FSH had no effect on catalase activity. The parallel decrease in testicular GSH-Px activity was observed after treatment with testosterone alone (−16%; not significant) or with substitution of FSH (−18%; $P < 0.05$; Fig. 4b). GSH-Tr activity showed only slight and not significant changes (Fig. 4c). The lipid peroxidation level was not low-

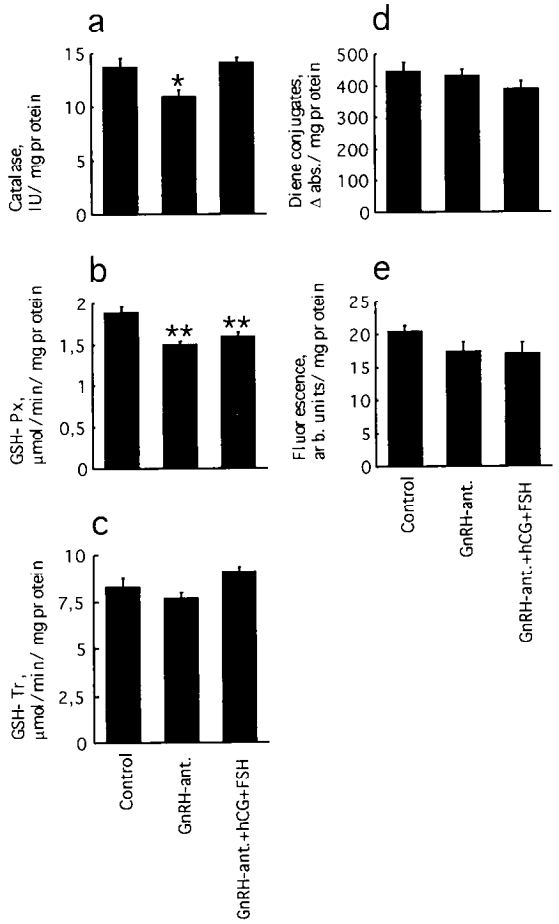

FIG. 3. Effects of 2-day GnRH antagonist treatment and gonadotropin replacement on the activities of catalase (a), GSH-Px (b), and GSH-Tr (c) and on the levels of diene conjugates (d) and fluorescent chromolipids (e) in the rat testis. Values are the mean ± SEM (n = 6). GnRH-ant., GnRH antagonist-treated rats; GnRH-ant.+hCG+FSH, GnRH-antagonist-treated rats with gonadotropin replacement. The *asterisks* indicate a significant difference from the control: *, $P < 0.05$; **, $P < 0.01$.

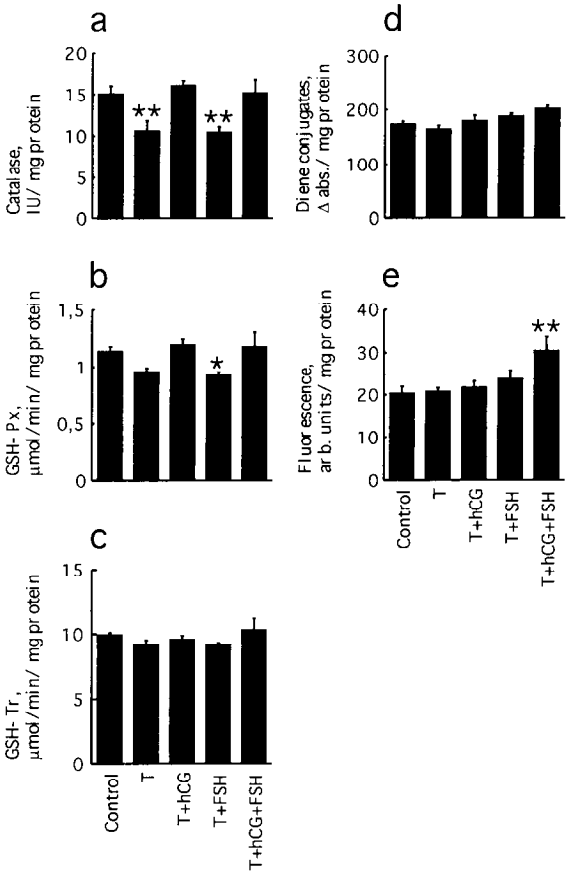

FIG. 4. Effects of 5-day testosterone treatment with substitution of hCG, FSH, or both on the activities of catalase (a), GSH-Px (b), and GSH-Tr (c) and on levels of diene conjugates (d) and fluorescent chromolipids (e) in the rat testis. Values are the mean ± SEM (n = 4–6). T, Testosterone-treated rats; T+hCG, testosterone-treated rats with hCG substitution; T+FSH, testosterone treated rats with FSH substitution; T+hCG+FSH, testosterone treated rats with hCG and FSH substitution. The *asterisks* indicate a significant difference from the control: *, $P < 0.05$; **, $P < 0.01$.

ered by 5-day testosterone treatment. It was not affected by hCG or FSH alone, but combined treatment with hCG and FSH increased testicular fluorescent chromolipids over the control level (+50%; $P < 0.01$; Fig. 4e). SOD activities were not affected in this experiment. Northern blot analysis showed a 2.7-kb catalase transcript, a 0.7- to 0.9-kb CuZn SOD band (which presumably reflects two transcripts of 0.77 and 0.94 kb), and multiple Mn SOD transcripts. The sizes of the RNA species detected are consistent with previously reported transcript sizes (18, 40). The changes in antioxidant enzyme gene expression were parallel with the results of enzyme activities; catalase mRNA was decreased by testosterone treatment (−38%; $P < 0.05$; Fig. 5) and returned to the control level by hCG substitution. No difference between the groups was observed in CuZn SOD or Mn SOD mRNAs (results not shown).

Effects of hCG and AMG injections

Five hours after hCG injection, the serum testosterone concentration was increased 3.6-fold ($P < 0.01$; Table 1). AMG treatment for 6 h decreased serum testosterone to 15% of the control level ($P < 0.01$). Combined treatment with hCG and AMG resulted in a serum testosterone concentration 29% of the control level ($P < 0.01$). Testis weight was 9% lower in the AMG-treated than in the control group ($P < 0.05$; Table 1). Testicular protein concentrations were similar in the different treatment groups.

AMG treatment increased the level of testicular diene conjugates over 2-fold with or without hCG ($P < 0.01$; Fig. 6a). Fluorescent chromolipids were increased over the control level only when hCG injection was combined with AMG treatment (+48%; $P < 0.05$; Fig. 6b). After treatment with hCG alone, the levels of diene conjugates and fluorescent chromolipids were slightly above the control values, but the changes were not statistically significant. hCG injection induced GSH-Px activity in the interstitial tissue (+28%; $P < 0.01$), whereas the activity in the seminiferous tubules was not affected (Fig. 7a). The interstitial tissue activities of CuZn SOD (Fig. 7b) and Mn SOD (Fig. 7c) also were higher in the testes of hCG-treated than in those of control rats, but the variation was great, and the changes

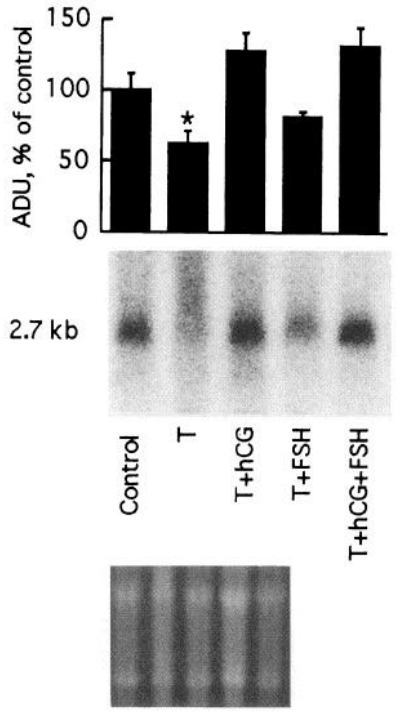

FIG. 5. Northern blot analysis of catalase gene expression in the rat testis after 5-day testosterone treatment with substitution of hCG, FSH, or both. Values are the mean ± SEM of densitometric analysis of the results (n = 3–4). The mean of the control group is taken as 100%. Representative bands and corresponding ethidium bromide staining of the gel are shown. T, Testosterone-treated rats; T+hCG, testosterone-treated rats with hCG substitution; T+FSH, testosterone-treated rats with FSH substitution; T+hCG+FSH, testosterone-treated rats with hCG and FSH substitution. The *asterisk* indicates a significant difference from the control ($P < 0.05$).

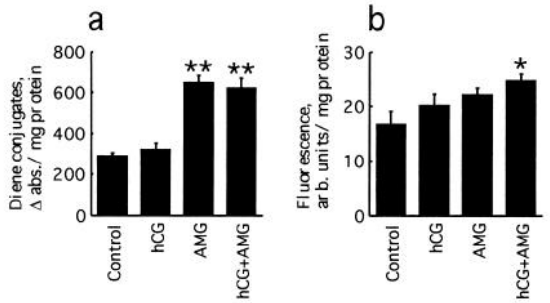

FIG. 6. Levels of diene conjugates (a) and fluorescent chromolipids (b) in the testes of rats treated with hCG, AMG, or both. Values are the mean ± SEM (n = 4–5). The *asterisks* indicate a significant difference from the control: *, $P < 0.05$; **, $P < 0.01$.

were not statistically significant. No difference was observed in catalase activity measured in testicular homogenate. Compared to the interstitial tissue, the antioxidant activities in the seminiferous tubules were approximately as follows: GSH-Px, 75% lower; CuZn SOD, 8-fold higher; and Mn SOD, the same level. Catalase activity in the seminiferous tubules was at or below the level of detection (0.7 IU/mg protein). Catalase activity could not be measured in the interstitial tissue samples because of large proportion of red blood cells in the samples.

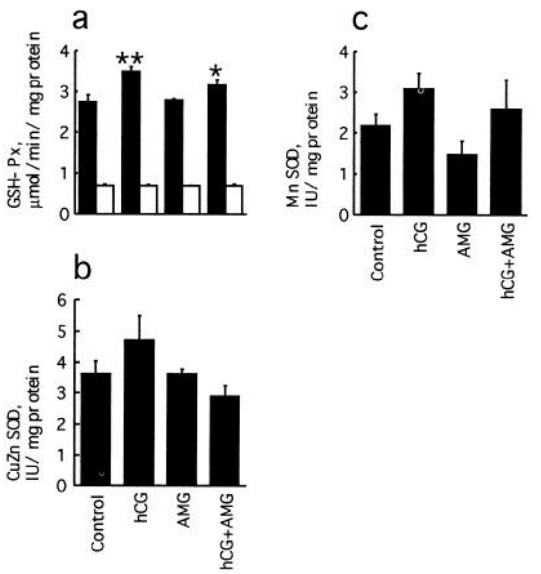

FIG. 7. GSH-Px activity in the interstitial tissue (■) and the seminiferous tubules (□; a) and CuZn SOD (b) and Mn SOD (c) activities in the interstitial tissue prepared from the testes of rats treated with hCG, AMG, or both. Values are the mean ± SEM (n = 4). The *asterisks* indicate a significant difference from the control: *, $P < 0.05$; **, $P < 0.01$.

Discussion

In the present study, the suppression of gonadotropins by either testosterone or GnRH antagonist treatment decreased the activities of the peroxide-metabolizing enzymes catalase and GSH-Px in the rat testis. GSH-Tr activity also decreased in the longest 8-day experiment. As the levels of catalase and GSH-Px activities were very low in the seminiferous tubules, the decrease in testicular catalase and GSH-Px activities during gonadotropin suppression predominately reflects a decrease in lipid peroxidation in the interstitial tissue. The changes induced by gonadotropin suppression were reversed by hCG substitution, whereas FSH had no effect on the enzyme activities. Parallel changes at the mRNA level support the findings of an effect of hCG on catalase activity. Thus, these experiments suggest that physiological LH action causes lipid peroxidation in the interstitial tissue of the rat testis. However, the levels of lipid peroxidation products were decreased by gonadotropin suppression only in the 8-day experiment. The measurements were performed in whole testis samples, and in the shorter experiments, the decrease in peroxidation products in the interstitial tissue was possibly not seen because of lipid peroxidation in the seminiferous tubules. As gonadotropin suppression causes germ cell atrophy, it may increase lipid peroxidation in the germ cells concomitantly with a decrease in Leydig cells.

The same phenomenon can explain the absent response of testicular TRAP, ubiquinol, and vitamin E; the possible changes in Leydig cells were concealed by concomitant germ cell effects. However, the biphasic shape of the testicular TRAP curve is of interest. Increasing radical production at the first increasing part of the curve seems to generate new antioxidative potential, which again lowers the level of peroxyl radicals. The total peroxyl radical-trapping capacity of the testicular lipid phase is high, but only a small proportion

of it is composed of vitamin E or ubiquinol, which are important TRAP components in plasma lipoproteins. The high TRAP may be due to the high content of steroids that contain phenolic hydrogroups (41).

The treatments used induced a drastic decline only in LH levels, whereas FSH was only slightly decreased, if at all. Similar maintenance of the serum FSH level in testosterone or GnRH antagonist treatments has been observed previously, and it has been demonstrated to be mainly caused by a direct stimulatory effect of androgens at the pituitary level (42, 43). Although FSH alone had no effect, substitution of hCG and FSH during 5-day testosterone treatment increased the testicular level of fluorescent chromolipids. Combined action of FSH-stimulated Sertoli cells and hCG-stimulated testosterone production may stimulate free radical-producing metabolism or differentiation processes in the germ cells. However, the present study does not provide an explanation for lipid peroxidation after treatment with FSH and hCG.

Reactive oxygen species produced in Leydig cell steroidogenesis arise presumably from the action of cytochrome P450 enzymes, which are known to produce free radicals at least *in vitro* (10, 44). Free radicals initiate the chain reaction of lipid peroxidation (Fig. 8). Lipid peroxides are capable of inactivating P450 enzymes (10), and in this way, free radical production has been suggested to control steroidogenesis in the adrenal cortex (11) and corpus luteum (45). Young *et al.* (46) have shown that a high level of the antioxidant β-carotene is needed to prevent inactivation of the mitochondrial P450 cholesterol side-chain cleavage enzyme by cross-linkage with adrenodoxin in bovine luteal cells. In cultured rat Leydig cells, Quinn and Payne (47) demonstrated inactivation of the microsomal P450 C_{21} steroid side-chain cleavage enzyme in desensitization of the cells after hCG stimulus to be free radical mediated and suggest a regulatory role for free radical production in Leydig cell steroidogenesis.

We used hCG treatment with or without AMG to study testicular steroidogenesis as the suggested origin of free radical production on LH action. In addition to stimulating steroidogenesis, a single dose of hCG induces leukocyte accumulation in the testis (48, 49), which is another potential source of free radical generation. In accordance with radical production during steroidogenesis and subsequent lipid peroxidation, hCG induced GSH-Px in the interstitial tissue, but not in the seminiferous tubules. Lipid peroxidation could not be measured in interstitial tissue samples because of the potential interfering effect of tissue handling during sample preparation (44). In whole testis samples, AMG was unexpectedly observed to induce abundant lipid peroxidation, seen as doubling of the level of diene conjugates. Fluorescent chromolipids are end products of lipid peroxidation, and the smaller increase in fluorescence measurements is probably due to the short treatment period. Antioxidant enzyme activities were not affected by AMG, which may be due to simultaneous induction of enzyme production and increased enzyme inactivation. However, the effect of AMG supports the view of the P450 enzymes of the steroidogenic pathway as producers of free radicals. AMG inhibits cholesterol side-chain cleavage by forming a ligand with the P450 enzyme (50, 51). The blocking action of AMG may be similar to the binding of a pseudosubstrate to the enzyme, leading to the production of free radicals instead of the product.

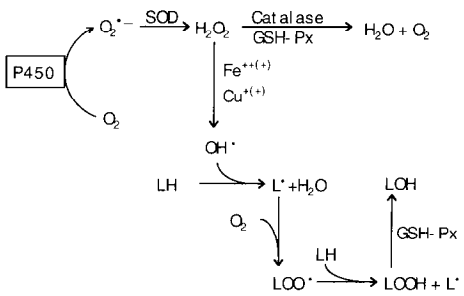

FIG. 8. The putative pathway for induction of lipid peroxidation during testicular steroidogenesis. The steroidogenic P450 enzymes generate superoxide ($O_2^{\cdot-}$), which is metabolized by SOD to hydrogen peroxide (H_2O_2). These products may generate more reactive species, such as hydroxyl radical ($OH^{\cdot}$), which can initiate peroxidation of membrane lipids (LH). The chain reaction of lipid peroxidation is propagated by the produced lipid radical ($L^{\cdot}$). The catalytic actions of catalase and GSH-Px are shown.

In the present study, lipid peroxidation occurred in normal steroidogenesis *in vivo*; therefore, a regulatory role for lipid peroxidation is plausible. However, the production of free radicals is potentially harmful. Although no extensive lipid peroxidation was seen in the whole testis after hCG injection, high doses of hCG apparently lead to oxidative damage of Leydig cells. Continuing lipid peroxidation and recruitment of Leydig cell antioxidative functions may also contribute to the vulnerability of the testis to external prooxidative attacks, such as exposure to environmental chemicals. This aspect should be studied in the future.

In conclusion, the present findings suggest that physiological LH action in the rat testis increases lipid peroxidation, observed mainly as a decrease in peroxide-metabolizing enzyme activities during gonadotropin suppression and reversibility of the changes with hCG substitution. Catalase mRNA expression paralleled the enzyme activity changes. The P450 enzymes of the steroidogenic pathway are the most likely sites of free radical generation. This view is supported by induction of GSH-Px activity after hCG injection in the interstitial tissue, but not in the seminiferous tubules. The P450 cholesterol side-chain cleavage enzyme-blocking agent, AMG, induced extensive lipid peroxidation in the testis, presumably by leakage of free radicals from the enzyme when substrate oxygenation was prevented.

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