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Reactive Oxygen Disrupts Mitochondria in MA-10 Tumor Leydig Cells and Inhibits Steroidogenic Acute Regulatory (StAR) Protein and Steroidogenesis

THORSTEN DIEMER, JOHN A. ALLEN, KAREN HELD HALES, AND DALE BUCHANAN HALES

Department of Physiology and Biophysics (T.D., J.A.A., K.H.H., D.B.H.), University of Illinois at Chicago, Chicago, Illinois 60612-7342; and Department of Urology (T.D.), University Hospital of the Justus-Liebig-University, 35392 Giessen, Germany

Reactive oxygen species (ROS) are involved in a variety of pathophysiological conditions of the testis, and oxidative stress is known to inhibit ovarian and testicular steroidogenesis. The site of ROS-mediated inhibition of steroidogenesis in the corpus luteum and MA-10 tumor Leydig cells was shown to be the hormone-sensitive mitochondrial cholesterol transfer step. The purpose of this study was to examine the effects of ROS on steroidogenic acute regulatory (StAR) protein in MA-10 cells and determine the extent to which MA-10 cell mitochondria are sensitive to oxidative stress. cAMP-stimulated progesterone production was inhibited in a dose-dependent manner in MA-10 cells exposed to H_2O_2. StAR protein, but not mRNA levels, was decreased in parallel to changes in progesterone production. Even at the highest concentrations of H_2O_2 tested, there was no effect on P450 side-chain cleavage enzyme protein levels. Oxidative stress from exposure to exogenous xanthine oxidase and xanthine resulted in the inhibition of both progesterone production and StAR protein expression. The mature 30- and 32-kDa intramitochondrial forms of StAR were decreased relative to the 37-kDa extramitochondrial precursor form of StAR, indicating that the ROS-mediated inhibition of StAR protein was due, in part, to the inhibition of mitochondrial import and processing. Vital staining with the fluorescent dye tetramethylrhodamine ethyl ester was used to visualize changes in the mitochondrial electrochemical gradient-dependent membrane potential ($\Delta\psi m$). ROS caused a significant dissipation of $\Delta\psi_m$ and time-dependent loss of tetramethylrhodamine ethyl ester fluorescence. The inhibitory effects of H_2O_2 were transient. There was no evidence for ROS-induced cell death, and following H_2O_2 removal in the presence of continuous treatment with 8-bromo-cAMP, StAR protein levels and progesterone production were restored. In addition, there was no loss of cell viability following treatment with H_2O_2 or xanthine/xanthine oxidase as determined by trypan blue exclusion. H_2O_2 did not cause a significant decrease in total cellular ATP levels. These data indicate that oxidative stress-mediated perturbation of the mitochondria and dissipation of $\Delta\psi_m$ results in the inhibition of StAR protein expression and its import, processing, and cholesterol transfer activity. These findings confirm earlier studies demonstrating the requirement for maintenance of an intact $\Delta\psi_m$ for StAR protein function in cholesterol transport. The significant reduction in the 32- to 30-kDa mature forms of StAR, cessation of cholesterol transport, and loss of $\Delta\psi_m$ are consistent with mitochondrial perturbation because of oxidative stress. This mechanism likely contributes to a host of pathophysiological events evident in testicular disorders such as infection, reperfusion injury, aging, cryptorchidism, and varicocele. (*Endocrinology* 144: 2882–2891, 2003)

REACTIVE OXYGEN SPECIES (ROS) are 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 UV radiation (1–11). Cumulative oxidative damage from prolonged exposure to ROS is thought to be one of the major causes of cellular aging (for review see Ref. 12). Leydig cells, which reside in the testicular interstitium, are particularly susceptible to extracellular sources of ROS because of their close proximity to testicular interstitial macrophages (13, 14). The release of ROS (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 (15). The release of ROS by activated macrophages not only affects invading microorganisms but also exposes adjacent tissues and cells, such as Leydig cells, to oxidative stress. Testicular macrophages are known to produce ROS during inflammation or infection (16). In addition, ROS are produced continuously in cells as the by-products of mitochondrial and microsomal electron transport reactions and other metabolic processes. Mitochondrial respiration consumes 85–90% of the oxygen used by cells and represents the greatest potential source of ROS within the cell. Steroidogenic cytochrome P450 enzymes in Leydig cells also produce ROS as a by-product of their catalytic reaction mechanism (17–20).

There have been relatively few studies on the effects of ROS on steroidogenesis. ROS has been implicated in the ovary in the process of luteolysis (21–23), and earlier studies indicated that ROS blocks the hormone-sensitive cholesterol transfer step (22). ROS have also been shown to inhibit steroidogenesis in MA-10 tumor Leydig cells, also at the level of cholesterol transfer (24). ROS have also been suggested to be important to the senescence of Leydig cell function during aging (25–27).

Oxidative damage is a consequence of excessive oxidative

Abbreviations: $\Delta\psi_m$, Mitochondrial membrane potential; 8-Br-cAMP, 8-bromo-cAMP; IOD, integrated OD; P450scc, P450 side-chain cleavage enzyme; ROS, reactive oxygen species; SFM, serum-free media; StAR, steroidogenic acute regulatory protein; TMRE, tetramethylrhodamine ethyl ester dye.

stress, insufficient antioxidant potential, or a combination of both. Oxidative damage induced by ROS is implicated as an important contributing factor in the pathogenesis of more than 100 conditions (2). ROS include oxygen radicals such as superoxide anion and hydroxyl radical, and reactive oxygen and nitrogen species such as hydrogen peroxide (H_2O_2), nitric oxide, and peroxynitrite anion, which are not radicals in nature but are capable of radical formation in cellular environments. ROS cause tissue damage by a variety of mechanisms including DNA damage, lipid peroxidation, protein oxidation, depletion of cellular thiols, and activation of proinflammatory cytokine release. ROS have short half-lives; they inflict damage by initiating free radical chain reactions. Several cellular antioxidant systems are present to help protect against free radical damage. These antioxidant systems include antioxidant molecules such as α-tocopherol, ascorbic acid, and glutathione and antioxidant enzymes such as glutathione peroxidase and superoxide dismutase. When the levels of ROS exceed the cellular antioxidant capacity, cells are subjected to oxidative stress, and oxidative damage occurs.

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 (4). Recently much attention has been paid to mitochondria because mutations of mitochondrial DNA are the basis of certain diseases, and perturbation of mitochondria is important to mechanisms of cell death.

Another significant role for mitochondria is as 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 inner mitochondrial space from the outer mitochondrial membrane to the inner mitochondrial membrane, a process now known to depend on the action of the steroidogenic acute regulatory protein (StAR) (28, 29). Testosterone production by Leydig cells is primarily under the control of LH, which acts via its intracellular second messenger cAMP to regulate testosterone production acutely at the level of cholesterol transport into the mitochondria via the action of StAR and chronically at the level of the steroidogenic enzyme gene transcription.

To analyze the effects of ROS on steroidogenesis, we examined the effects of H_2O_2 on progesterone production, and steroidogenic enzyme and StAR protein expression in MA-10 tumor Leydig cells. MA-10 cells are a convenient model to study the acute regulation of steroidogenesis because they are LH/human chorionic gonadotropin and cAMP responsive and express StAR, P450 side-chain cleavage enzyme (P450scc), and 3β-hydroxysteroid dehydrogenase. MA-10 cells do not express P450c17 or produce testosterone to a significant degree but instead produce primarily progesterone. The purpose of this study was to examine the effects of ROS on steroidogenesis in MA-10 cells and determine the extent to which MA-10 cell mitochondria are sensitive to oxidative stress.

Materials and Methods

Materials

Sodium dodecyl sulfate (SDS), Waymouth's MB752/1 medium, RPMI medium, penicillin, streptomycin, heat-inactivated horse serum, and ultrapure formamide were from Invitrogen (Gaithersburg, MD). 8-Bromo-cAMP (8-Br-cAMP), BSA, hydrogen peroxide (3% solution), and trypan blue (0.4% solution) were from Sigma (St. Louis, MO). Random primed labeling kit was from Roche Molecular Biochemicals (Mannheim, Germany). [α^{32}P]Deoxyguanidine triphospate was from ICN Biochemicals (Irvine, CA). Enhanced chemiluminescence Western blot detection kit was from Amersham Pharmacia Biotech (Piscataway, NJ). Tetramethylrhodamine ethyl ester dye (TMRE) was from Molecular Probes, Inc. (Eugene, OR). Xanthine and xanthine oxidase were from Calbiochem (San Diego, CA). ATP bioluminescent assay kit was from Sigma. Mouse StAR cDNA and CMV-StAR were a generous gift from Douglas Stocco (Lubbock, TX).

MA-10 cell culture and treatments

MA-10 cells derived from a transplantable Leydig cell tumor in mice were a kind gift from Dr. Mario Ascoli (University of Iowa College of Medicine, Iowa City, IA) and were maintained as described previously (30). MA-10 cells were cultured in Waymouth's complete medium MB752/1 (Invitrogen) containing 20 mM HEPES, 1.2 g/liter sodium bicarbonate, 15% heat-inactivated donor herd horse serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. MA-10 cells were grown in culture to approximately 75% confluency and were preincubated in serum-free media (SFM) for 1 h. Cells were then treated for 3 h in Waymouth's SFM that contained 1 mM 8-Br-cAMP or cAMP plus increasing concentrations of H_2O_2 (10–1000 μM). A 3% stock solution of H_2O_2 was used, and appropriate volumes were added to Waymouth's SFM just before cell treatments. Before treatment with xanthine oxidase, cells were preincubated with serum-free RPMI media for 1 h. Cells were then treated for 3 h in RPMI SFM that contained 1 mM 8-Br-cAMP or cAMP plus 100 μM xanthine plus increasing milliunit quantities of xanthine oxidase (1–100 mU). One milliunit xanthine oxidase will covert 1 μmol xanthine to uric acid per minute, yielding superoxide anion as a by-product. Cells were incubated at 37 C in 5% CO_2 in a humidified incubator.

Northern blotting and hybridization

Extraction and analysis of total RNA was performed after lysis of cells with a guanidine-isothiocyanate-based lysis buffer followed by phenol extractions under acidic conditions and isopropanol precipitation of nucleic acids (APGC method) as described previously (31–33). Ten micrograms total RNA from each sample were analyzed. Agarose gel electrophoresis of RNA and Northern blotting to nitrocellulose paper (Millipore Corp., Bedford, MA) membranes was performed as described previously (33, 34). Northern hybridization was performed by use of ^{32}P-labled random primed cDNA probes specific for StAR and cyclophilin (35–38). Hybridization signal from the radioactively labeled Northern blots were obtained after exposure to a phosphor screen for 24 h. Hybridization signals were quantified by phosphorimaging (Storm 860, Molecular Dynamics, Inc., Sunnyvale, CA) and computer software (Image QuaNT, Molecular Dynamics, Inc.). Expression data for mRNA are expressed as integrated OD (IOD). Expression of StAR mRNA IOD has been normalized to the expression of the constitutively expressed gene cyclophilin.

Western analysis and immunodetection of proteins

After treatments, total cellular protein was obtained by placing cells in lysis buffer (PBS/0.1% SDS) followed by brief sonication (ultrasonic processor GE 50 T (Sonics, Newtown, CT), 50% power for ~2 sec). Protein concentrations were determined by microBCA protein assay (Pierce Chemical Co., Rockford, IL). Thirty micrograms total cellular protein were separated by SDS-PAGE using 10% acrylamide/SDS separating gels and transferred to nitrocellulose paper membranes as described previously (33, 39). The preparation of the polyclonal antiserum to StAR and P450scc has been described previously (40, 41). Detection of bound antibody on the blot was assessed with a horseradish peroxidase-conjugated, goat antirabbit IgG antibody (Sigma) visualized by chemiluminescent detection (ECL Western blot detection kit, Amersham Pharmacia Biotech), and quantitated after densitometry (personal densitometer, Molecular Dynamics, Inc.) using Imagequant software (Molecular Dynamics, Inc.). Expression data for protein are expressed as IOD.

Progesterone RIA

After respective cell treatments, culture media were removed and stored at −20 C until later analysis. Before assaying, culture media were boiled for 5 min and centrifuged at 2000 × *g* for 20 min at 4 C. Concentrations of progesterone in the media samples were determined using Coat-a-Count RIA kits (Diagnostic Products, Los Angeles, CA). Progesterone concentration in each sample was measured in duplicate. To control for variability in cell number in each culture, progesterone concentrations were normalized to protein concentrations for each sample (nanograms per milligram protein).

Cell viability assay

Cell viability was assessed by trypan blue exclusion, according to the manufacturer's instructions. Briefly, cells were treated as described under MA-10 cell culture and treatments and in figure legends. After respective treatments, cells were trypsinized for 1 min at room temperature and then resuspended in 1× PBS. Equal volumes of resuspended cells and 0.4% trypan blue (Sigma) were combined, incubated at room temperature for 10 min, and counted using a standard hemocytometer (Fisher Scientific, Itasca, IL). Viable cells (unstained) and nonviable cells (stained) were counted, and the percent cell viability was calculated as follows: [% cell viability = total viable cells (unstained)/total cells (stained and unstained) × 100]. The viability assay was repeated for each treatment group, and the data are represented as mean ± SEM for three independent experiments.

Measurement of cellular ATP

After treatments, cells were briefly washed with cold PBS. Cells were then incubated for 15 min in a lysis/precipitation solution of 3% trichloroacetic acid and 2 mM EDTA. Cells were transferred to fresh tubes and subjected to brief sonication (ultrasonic processor GE 50 T, 50% power for approximately 2 sec, four times). Lysates were centrifuged at 3000 × *g* for 20 min at 4 C, and the supernatant containing total cellular ATP was collected. The supernatants containing ATP were adjusted to a pH of 7.8. Protein lysis buffer (PBS/0.1% SDS) was added to the pellet to obtain protein lysates for each sample. The lysates were briefly sonicated and protein concentrations were determined by microBCA protein assay (Pierce Chemical Co.). ATP was assayed using the ATP bioluminescent assay kit (Sigma), according to the manufacturer's instructions. Light intensity of the sample was measured using a luminometer (Lumat 9506, EG&G Berthold, Alliquippa, PA). Standard concentrations of ATP (1–500 pmol) were prepared and a standard curve was generated. ATP concentrations in each sample were determined by linear regression analysis using Instat, version 3.0, software package for statistical data analysis (GraphPad Software, Inc., San Diego, CA). To control for variability in original cell number, ATP concentrations were normalized to protein concentrations for each sample and expressed as pmol/μg protein.

Assessment of the mitochondrial membrane potential ($\Delta\psi_m$)

MA-10 cells were grown on poly-L-lysine-coated glass coverslips (Fisher Scientific, Fair Lawn, NJ) and prestained with 40 nM TMRE (Molecular Probes, Inc.) for 10 min at 37 C. Cells were then transferred to a temperature-controlled microscope stage and analyzed using time-lapse video fluorescent microscopy. A polarized, intact mitochondrial potential is necessary for the uptake and accumulation of TMRE dye in mitochondria. Thus, analysis of TMRE fluorescence by microscopy allowed the semiquantitative assessment of the $\Delta\psi_m$ as described (42). Fluorescence of the dye in Leydig cell mitochondria was visualized before treatment with H_2O_2. Cells were treated for 1 h with 250 μM H_2O_2 in SFM. At time 0 min, H_2O_2 was added to the cells, which were subsequently examined for 1 h using time-lapse video fluorescent microscopy. MA-10 cells in SFM alone were also examined to control for photo-bleaching artifacts. Fluorescent images were obtained using an inverted microscope equipped for fluorescent microscopy (Eclipse TE 300, 547 nm wavelength excitation, 579 nm emission, Nikon, via high pressure Xenon XBO 75 W lamp, Nikon); a digital camera (RTE/CCD-1300 Y/HS, Roper Scientific, Trenton, NJ; MicroMAX camera controller, Princeton Instruments, Trenton, NJ; λ 10–2 shutter, Sutter Instruments Co., Navato, CA), and image-processing software (IPLab, Scanalytics Inc., Fairfax, VA).

Statistical analysis

Data were presented as means ± SEM of three or more independent experiments. For group comparisons, one-way ANOVA followed by Student-Newman-Keuls multiple-range tests were performed using the Instat, version 3.0, software package for statistical data analysis (GraphPad Software, Inc.). Differences are considered significant at $P < 0.05$.

Results

Effect of H_2O_2 on cAMP-stimulated progesterone production in MA-10 cells

Stimulation of MA-10 cells with a maximal stimulating concentration of 8-Br-cAMP (1 mM) for 3 h results in an increase in progesterone production of more than 60-fold above the basal production rate in controls (control *vs.* 8-Br-cAMP, 3 h: 8.7 ± 4 *vs.* 527.9 ± 105 ng/mg protein) (Fig. 1). Treatment of 8-Br-cAMP-stimulated MA-10 cells with H_2O_2 caused a dose-dependent decrease in progesterone production. Treatment of MA-10 cells with 100, 250, and 500 μM H_2O_2 caused significant decrease in cAMP-stimulated progesterone production, compared with cells treated with cAMP alone, by 44%, 68%, and 71%, respectively, to 294.3 ± 54, 169.0 ± 42, and 151.3 ± 21 ng/mg protein (n = 4, $P < 0.05$ for all groups).

Effect of H_2O_2 on StAR and steroidogenic enzyme protein levels

To identify which components of the steroidogenic pathway were decreased by ROS treatment, changes in steroidogenic proteins were analyzed by Western blot (Fig. 2). Exposure of MA-10 cells to H_2O_2 caused a significant decrease in cAMP-stimulated StAR protein expression. StAR protein was virtually undetectable in MA-10 cells from control cultures. Treatment with 1 mM 8-Br-cAMP for 3 h results in a significant 50-fold increase in StAR protein levels. Treatment of cAMP-stimulated MA-10 cells with H_2O_2 caused a dose-dependent decrease in StAR protein. Treatment of MA-10 cells with 100, 250, and 500 μM H_2O_2 reduced 30-/32-kDa StAR protein by 22%, 89%, and 100%, respectively,

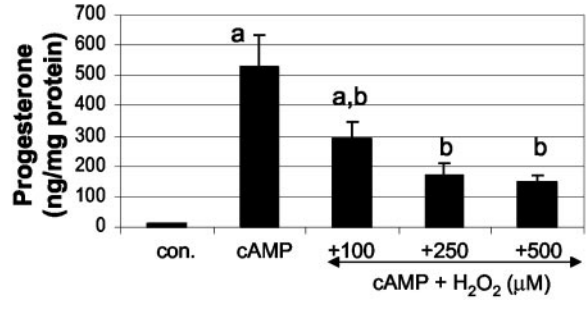

FIG. 1. Effect of H_2O_2 on cAMP-stimulated progesterone production in MA-10 cells. MA-10 cells were grown in culture to 75% confluency, preincubated in SFM for 1 h and treated for 3 h in SFM that contained 1 mM 8-Br-cAMP or cAMP plus increasing concentrations of H_2O_2. Media were collected and subjected to progesterone RIA as described in *Materials and Methods*. Progesterone concentrations were normalized to protein concentrations for each sample and are represented as mean ± SEM for four independent experiments (n = 4) (a, $P < 0.05$ *vs.* control; b, $P < 0.05$ *vs.* cAMP).

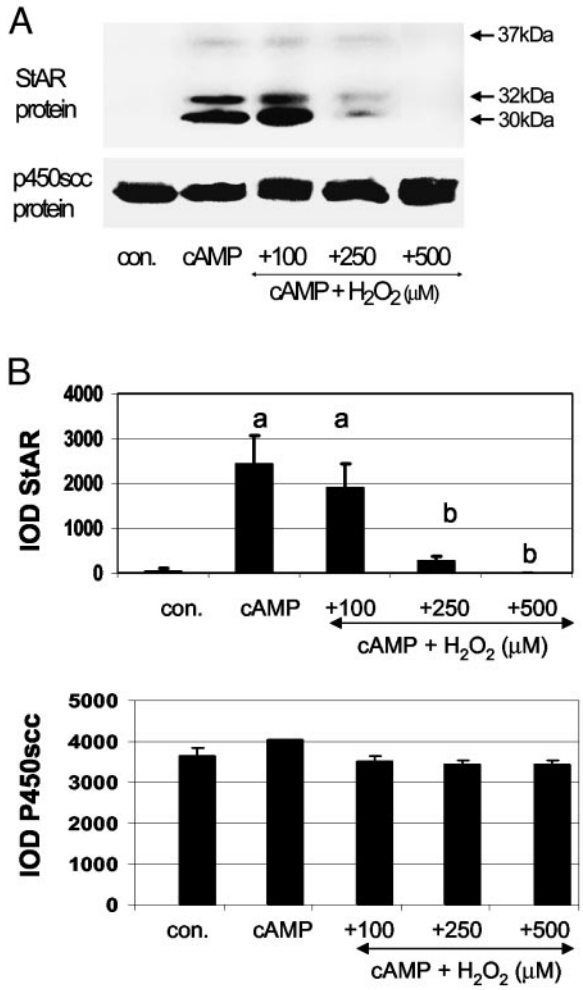

FIG. 2. Effect of H_2O_2 on StAR and steroidogenic enzyme protein levels. MA-10 cells were grown in culture and treated for 3 h with 1 mM 8-Br-cAMP or cAMP plus increasing concentrations of H_2O_2 as described in Fig. 1. After treatment, cell preparations from Fig. 1 were lysed and subjected to Western blot analysis as described in *Materials and Methods.* A, Representative Western blots of StAR and P450scc proteins. B, Quantitation of protein levels by scanning densitometry. Both intramitochondrial forms of StAR (30 and 32 kDa) were included in the quantitation. Data are represented as mean ± SEM for three independent experiments (n = 3) (a, $P < 0.05$ *vs.* control; b, $P < 0.05$ *vs.* cAMP).

compared with cells treated with cAMP alone. Only the decreases observed with 250 and 500 μM H_2O_2 were significant ($P < 0.05$, n = 3). Although H_2O_2 caused a significant decrease in 30/32 kDa StAR, the 37-kDa precursor form of StAR did not decrease appreciably, suggesting that the intramitochondrial mature forms were affected to a greater extent (Fig. 2). In contrast, treatment of MA-10 cells with 1 mM 8-Br-cAMP or cAMP plus increasing concentrations of H_2O_2 for 3 h had no effect on P450scc protein levels (Fig. 2B).

Effect of H_2O_2 on StAR mRNA

A decrease in StAR protein level was the most significant component of the steroidogenic pathway in which ROS exerted its inhibitory effects. To determine whether decreased StAR protein was due to decreased StAR mRNA levels, StAR mRNA transcripts were quantitated by Northern blot (Fig. 3). There was no difference observed in the relative expression levels of the three StAR transcripts (3.4, 2.9, and 1.9 kb). Data are presented as the ratio of the 3.4-kb StAR transcript to cyclophilin mRNA. Treatment with 1 mM 8-Br-cAMP for 3 h results in a significant 65-fold increase in StAR mRNA. In marked contrast to the effects of H_2O_2 on StAR protein levels, treatment of cAMP-stimulated MA-10 cells with H_2O_2 had no effect on StAR mRNA levels. Only treatment of MA-10 cells with cAMP plus 1000 μM H_2O_2 significantly reduced StAR transcript levels by 97%, compared with cells treated with cAMP alone. It is apparent that the RNA isolated from cells exposed to 1000 μM H_2O_2 was degraded by this concentration of H_2O_2 as indicated by the appearance of cyclophilin and StAR mRNA transcripts. These data indicate that StAR protein, but not mRNA, is decreased during oxidative stress, suggesting that ROS acts posttranscriptionally to inhibit StAR protein expression.

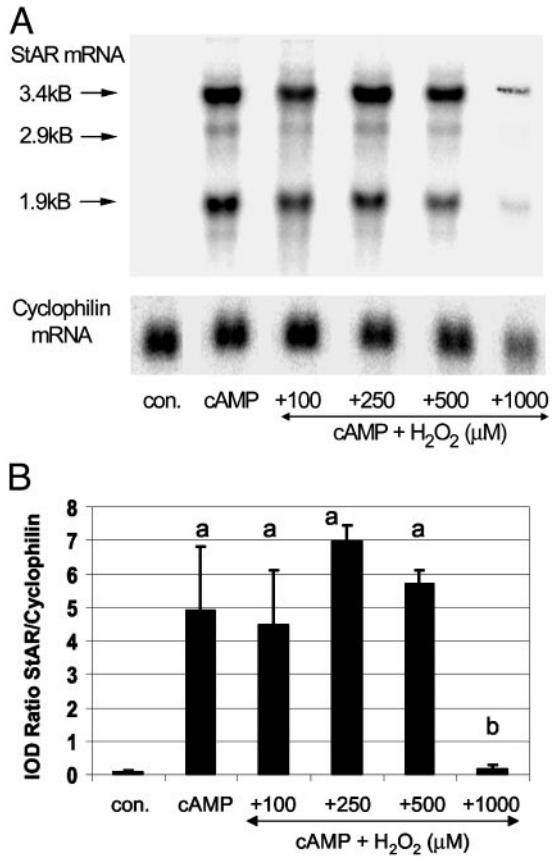

FIG. 3. Effect of H_2O_2 on StAR mRNA. MA-10 cells were grown in culture and treated for 3 h with 1 mM 8-Br-cAMP or cAMP plus increasing concentrations of H_2O_2 as described in Fig. 1. Total RNA was extracted, and a Northern blot analysis was performed as described in *Materials and Methods.* A, Representative northern blots of MA-10 cell mRNA for StAR and cyclophilin. Blot was probed sequentially with ^{32}P-labeled cDNA for StAR and cyclophilin. B, Quantitation of StAR mRNA levels by phosphorimaging. All three StAR mRNA transcripts were included in the quantitation. StAR mRNA levels were normalized to that of cyclophilin and are expressed as the ratio of 3.4 kb StAR transcript to cyclophilin. Data are represented as mean ± SEM for three independent experiments (n = 3) (a, $P < 0.05$ *vs.* control; b, $P < 0.05$ *vs.* cAMP).

Recovery of StAR protein levels after H_2O_2 treatment

To determine whether the inhibitory effects of H_2O_2 on StAR protein were reversible, MA-10 cells were treated with 1 mM 8-Br-cAMP or cAMP plus 250 μM H_2O_2 for 3 h, and then H_2O_2 was removed and treatment was continued for an additional 3 h with cAMP alone (Fig. 4). Compared with untreated controls, cAMP treatment for 3 h resulted in a significant increase in 30/32 kDa StAR protein of greater than 50-fold, similar to the results shown in Fig. 2. Treatment of cells with cAMP plus H_2O_2 for 3 h caused a significant decrease in StAR protein levels by 70% ($P < 0.5$, n = 3). Three hours after removal of H_2O_2 with continued treatment with cAMP alone, StAR protein levels recovered by 37%, compared with cells treated with cAMP plus H_2O_2 for 3 h ($P < 0.05$, n = 3). Progesterone concentrations were analyzed, and there was a parallel recovery of progesterone production (data not shown). When cells were exposed to H_2O_2 continuously for 6 h in the presence of cAMP, StAR protein levels also began to return to the level of StAR protein in cultures treated continuously with cAMP alone (data not shown).

Effect of xanthine oxidase on cAMP-stimulated progesterone production in MA-10 cells

To determine whether other forms of ROS in addition to H_2O_2 inhibit steroidogenesis and StAR protein expression the effects of the xanthine oxidase enzyme system were tested. Xanthine oxidase uses oxygen as an electron acceptor to generate superoxide anion. The substrates for xanthine oxidase include xanthine or hypoxanthine. Waymouths MB 752/1, the medium conventionally used for culturing MA-10 cells, was supplemented with 50 mM hypoxanthine. Thus, experiments in which the effects of exogenous xanthine oxidase were tested were performed exactly as described and in media identical to that used in other experiments, except RPMI 1640 medium replaced Waymouth's MB 752/1. MA-10 cells were grown to 75% confluency and then preincubated in serum-free RPMI media for 1 h. Cells were then treated for 3 h with media that contained 1 mM 8-Br-cAMP or cAMP plus 100 μM xanthine and increasing concentrations of xanthine oxidase. cAMP-stimulated progesterone production was inhibited in a dose-dependent manner by xanthine oxidase (Fig. 5). Treatment with 10, 50, and 100 mU xanthine oxidase, in the presence of 100 μM xanthine, resulted in a 29%, 31%, and 79% decrease in progesterone production, respectively.

Effect of xanthine oxidase on StAR protein levels

In parallel to measuring progesterone production, as shown in Fig. 5, StAR protein was quantitated in cell preparations. The mature, intramitochondrial forms of StAR protein (30/32 kDa) decreased in a similar manner to changes in progesterone from the same cultures (Fig. 6). Exposure to 10, 50, and 100 mU xanthine oxidase, in the presence of 100 μM xanthine, resulted in a decrease in 30/32 kDa StAR protein by 87, 90, and 99%, respectively. In contrast, the 37-kDa extramitochondrial precursor form of StAR did not decrease as a result of xanthine oxidase treatment but was significantly increased relative to the intramitochondrial 30/32-kDa forms of StAR. As shown in Fig. 6B, treatment with 10, 50, and 100 mU xanthine oxidase, in the presence of 100 μM xanthine, resulted in a change in the 37-kDa form of StAR of 120%, 210%, and 55%, relative to cells treated with cAMP alone. When the ratio of 37 to 30/32 kDa StAR is plotted (Fig. 6C), the increase in 37 kDa relative to 30/32 kDa, as a result of exposure to xanthine oxidase, is more apparent: 10, 50, and 100 mU xanthine oxidase result in an increase in 37 to 30/32

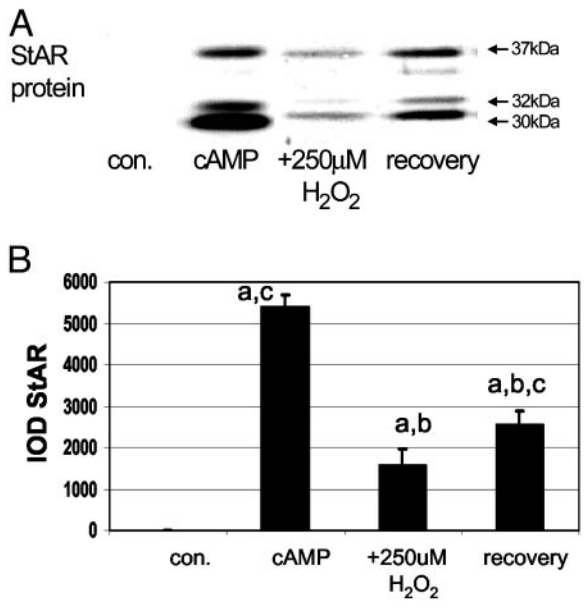

FIG. 4. Recovery of StAR protein levels after H_2O_2 treatment. MA-10 cells were treated for 3 h with 1 mM 8-Br-cAMP or cAMP plus 250 μM H_2O_2. The recovery groups were first treated with cAMP plus 250 μM H_2O_2 for 3 h and then with cAMP alone for 3 h. Cells were lysed and subjected to Western blot analysis and quantitated as described in *Materials and Methods.* A, Representative Western blot of StAR protein. B, Quantitation of StAR protein levels by scanning densitometry. Data are represented as mean ± SEM for three independent experiments (n = 3) (a, $P < 0.05$ *vs.* control; b, $P < 0.05$ *vs.* cAMP; c, $P < 0.05$ *vs.* +250 μM H_2O_2).

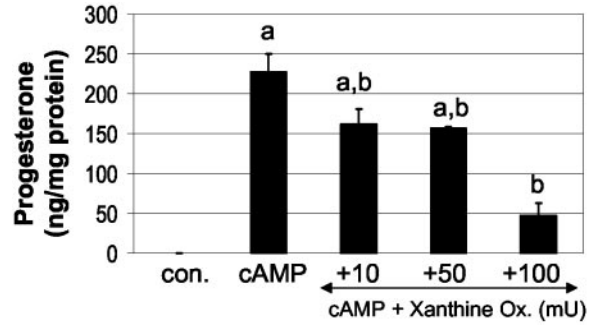

FIG. 5. Effect of xanthine oxidase on cAMP-stimulated progesterone production in MA-10 cells. MA-10 cells were grown in culture to 75% confluency and preincubated in SFM for 1 h. Cells were then treated for 3 h in SFM that contained 1 mM 8-Br-cAMP or cAMP plus 100 μM of the substrate xanthine. The enzyme xanthine oxidase was added to the cultures in increasing milliunit quantities of 10 mU, 50 mU, and 100 mU. Media were collected and subjected to progesterone RIA as described in *Materials and Methods.* Progesterone concentrations were normalized to protein concentrations for each sample and are represented as mean ± SEM for three independent experiments (n = 3) (a, $P < 0.05$ *vs.* control; b, $P < 0.05$ *vs.* cAMP).

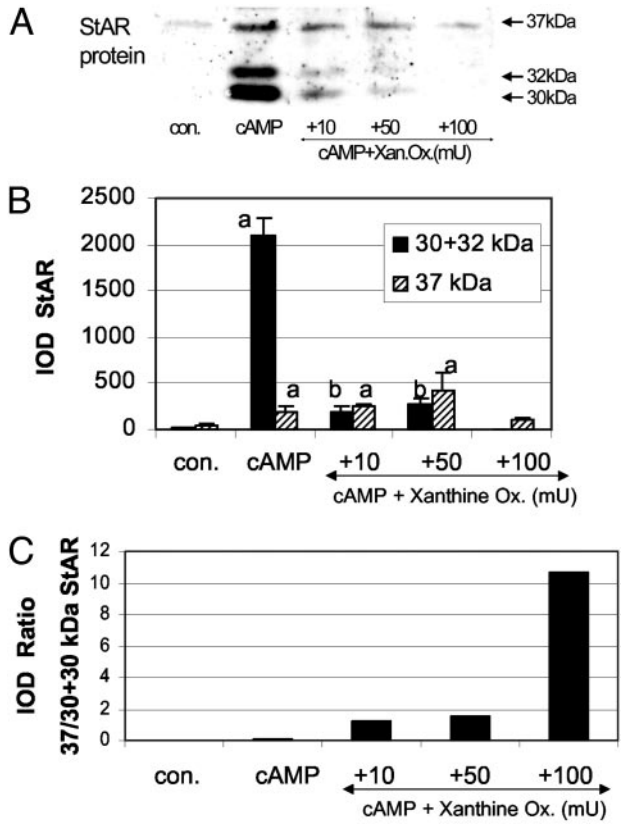

FIG. 6. Effect of xanthine oxidase on StAR protein levels. MA-10 cells were grown in culture and treated for 3 h with 1 mM 8-Br-cAMP or cAMP plus increasing milliunit quantities of the enzyme xanthine oxidase as described in Fig. 5. After treatment, cell preparations from Fig. 5 were lysed and subjected to Western blot analysis as described in *Materials and Methods.* A, Representative Western blot for StAR protein. B, Quantitation of StAR protein levels by scanning densitometry. Levels of the 37-kDa precursor and the 30 + 32 kDa processed forms of StAR were quantitated as described in *Materials and Methods.* Data are represented as mean ± SEM for three independent experiments (n = 3) (a, $P < 0.05$ *vs.* control; b, $P < 0.05$ *vs.* cAMP). C, Ratio of 37/30 + 32 kDa StAR quantitated by densitometry. Levels of 37 kDa and 30 + 32 kDa StAR determined in B are expressed as the ratio of the two forms.

kDa StAR of 13-, 16-, and 111-fold, in xanthine oxidase-treated cells, compared with cells treated with cAMP only. The sizes of the 37-, 32-, and 30-kDa forms of StAR were deduced from molecular mass markers and confirmed by comparison to ^{35}S-methione labeling COS-7 cells that were transfected with CMV-StAR expression vector, immunoprecipitating overexpressed StAR protein, and subjecting it to SDS-PAGE analysis in parallel to gels used for Western blot analysis (data not shown; see Ref. 40).

Effect of H_2O_2 and xanthine oxidase on cellular viability

To assess the effect of reactive oxygen exposure on cell viability, trypan blue dye exclusion assays were performed. MA-10 cells were treated for 3 h control medium, 1 mM 8-Br-cAMP, cAMP plus 250 μM H_2O_2, or cAMP plus 100 μM xanthine/10 mU xanthine oxidase. As shown in Fig. 7, exposure to any of the treatments did not alter the viability of MA-10 cells. The percent of viable cells ± SEM was similar in control and cAMP- and ROS-treated cells (control: 94.8% ± 1.2%; cAMP: 91.4% ± 1.3%; cAMP+250 μM H_2O_2: 92.2% ± 1.3%; cAMP+10 mU XanOx: 89.8% ± 0.5%; Fig. 7).

Effect of H_2O_2 and xanthine oxidase on cellular ATP levels

ATP was quantitated to determine whether exposing cells to ROS donors for 3 h affects total cellular ATP levels (Fig. 8). MA-10 cells were treated with control media or media that contained 1 mM 8-Br-cAMP, cAMP plus 250 μM H_2O_2, or cAMP plus xanthine (100 μM) and xanthine oxidase 10 mU. ATP was determined in cell lysates by bioluminescence assay as described in *Materials and Methods,* and data normalized to protein concentrations for each sample. Treatment of the cells with cAMP alone or cAMP plus xanthine/xanthine oxidase resulted in significant decrease in cellular ATP levels, compared with control (n = 3, $P < 0.05$). Control and cAMP- and cAMP plus xanthine/xanthine oxidase-treated cells had 34.9 ± 4.0, 21.8 ± 2.1, and 16.1 ± 2.8 pmol ATP/μg protein ± SEM, respectively. In cells exposed to cAMP plus H_2O_2, there was 28.7 ± 6.3 pmol ATP/μg protein.

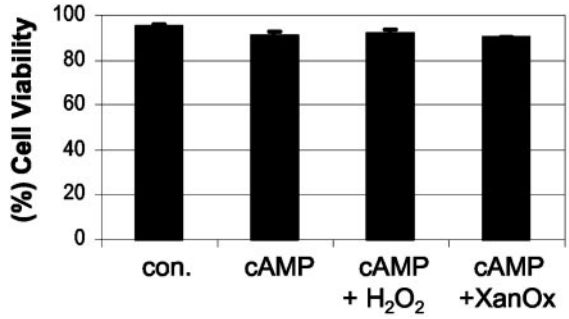

FIG. 7. Cell viability assay of MA-10 cells. MA-10 cells were grown to 75% confluency in culture and then preincubated with SFM for 1 h. Cells were then treated for 3 h in SFM that contained 1 mM 8-Br-cAMP, cAMP plus 250 μM H_2O_2, or cAMP plus xanthine (100 μM) and xanthine oxidase (10 mU). After treatment, cells were incubated with 0.4% trypan blue solution and cell viability staining was determined by light microscopy as described in *Materials and Methods.* Data are represented as mean ± SEM for three independent experiments.

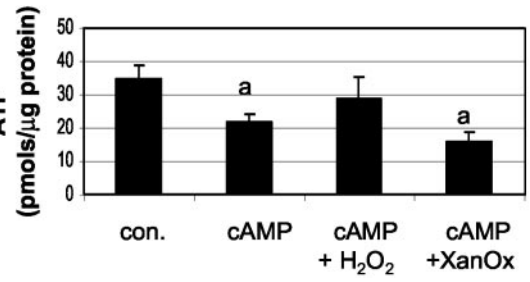

FIG. 8. Effect of H_2O_2 and xanthine oxidase on cellular ATP levels. MA-10 cells were grown to 75% confluency in culture and then preincubated with SFM for 1 h. Cells were then treated for 3 h in SFM that contained 1 mM 8-Br-cAMP, cAMP plus 250 μM H_2O_2, or cAMP plus xanthine (100 μM) and xanthine oxidase (10 mU). After treatment, cells were lysed and cellular extracts were subjected to an ATP bioluminescence assay as described in *Materials and Methods.* ATP levels were normalized to protein concentrations for each sample and are represented as mean ± SEM for three independent experiments (n = 3) (a, $P < 0.05$ *vs.* control).

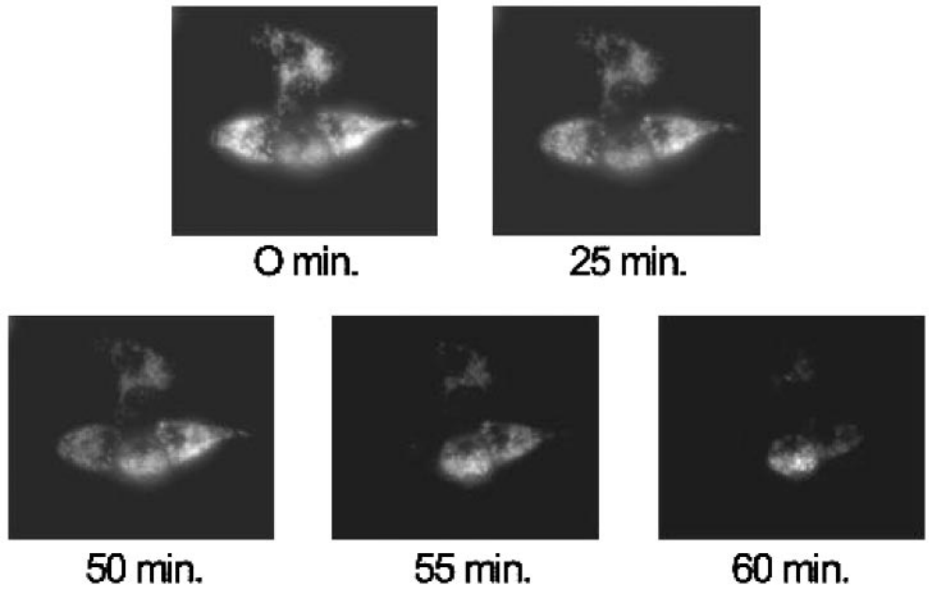

FIG. 9. Time-course effect of H_2O_2 on $\Delta\psi_m$. MA-10 cells were incubated in SFM for 1 h. Cells were then preincubated with 40 nM TMRE for 30 min. At time 0, SFM plus 250 μM H_2O_2 was added to the cells. The cells were then examined for TMRE fluorescence over a 1-h period by video capture time-lapse fluorescent microscopy as described in *Materials and Methods*. Fluorescent images shown are from the indicated times post addition of H_2O_2.

Time-course effect of H_2O_2 on $\Delta\psi_m$

To determine whether ROS perturbs the mitochondria of MA-10 cells, changes in the mitochondrial transmembrane potential $\Delta\psi_m$ were assessed by fluorescence microscopy using the lipophilic cationic dye TMRE. Lipophilic fluorochrome cations such as TMRE accumulate in the mitochondrial matrix, driven by the electrochemical gradient following the Nernst equation, and thus are useful to measure changes in $\Delta\psi_m$ (43). MA-10 cells were grown on poly-L-lysine-coated coverslips, and then were preincubated with 40 nM TMRE for 30 min at 37 C. Cells were transferred to the temperature-controlled stage of a microscope equipped with epi-fluorescence. At time 0, 250 μM H_2O_2 were added to the cells and images of the cells were captured for 300 msec every 5 min for 1 h and recorded by time-lapse video capture. Select frames are shown from images captured at 0, 25, 50, 55, and 60 min. The cluster of cells shown in Fig. 9 reveal intense punctuate mitochondrial fluorescence that does not appreciably decrease for the first 25 min following addition of H_2O_2. Starting at 50 min post addition of H_2O_2, there is a rapid and extensive loss of mitochondrial fluorescence indicating that by 60 min, $\Delta\psi_m$ has been completely dissipated. In control experiments, cell were labeled with TMRE and subjected to video capture time-lapse fluorescent microscopy without the addition of H_2O_2. There was no loss of fluorescence signal because of the effects of photobleaching (not shown). Mitochondrial potentials of MA-10 cells were markedly decreased over a similar time course with 100 μM H_2O_2. Treatment with other more lipophilic H_2O_2 derivatives such as t-butyl-H_2O_2 and cumene-H_2O_2 at concentrations of 50–100 μM resulted in an even more severe disruption of $\Delta\psi_m$ (not shown).

Discussion

In this study, we examined the effect of reactive oxygen on steroidogenesis in MA-10 tumor Leydig cells. Our data show that cAMP-stimulated progesterone production is inhibited by exposure to exogenous sources of ROS and cAMP induction of StAR protein is inhibited following exposure to ROS. This inhibition of StAR protein is posttranscriptional because there is no decrease in StAR mRNA levels. Exposure to ROS does not inhibit the expression of P450scc, suggesting that StAR protein is particularly sensitive to oxidative stress. Removal of H_2O_2 causes the partial restoration of StAR protein expression and progesterone production, indicating that MA-10 cells were not irreversibly damaged by exposure to ROS. There was slight but significant decrease in total cellular ATP levels in cells exposed to cAMP or cAMP plus xanthine/xanthine oxidase but not cAMP plus H_2O_2. There was no change in cell viability with any of the treatments, as assessed by trypan blue exclusion. These observations indicated that there was no overt cellular toxicity during ROS exposure. However, treatment with H_2O_2 resulted in a loss of $\Delta\psi_m$. These data suggest that StAR protein expression and function depend on an intact $\Delta\psi_m$, and perturbation of the mitochondria causes a block to StAR protein expression. The 37-kDa precursor of StAR remains at a constant level during incubation with xanthine oxidase and its substrate xanthine, but the intramitochondrial 32- and 30-kDa mature forms of StAR decrease, suggesting that StAR protein expression is blocked in part at the level of mitochondrial import and processing.

Previous studies of the effects of oxidative stress on steroidogenesis predicted that the main effect of ROS on steroidogenic cells is the inhibition of StAR protein function. At the time Behrman and Aten (22) and Stocco *et al.* (24) assessed the effects of H_2O_2 on steroidogenesis, the molecular identity of StAR had not yet been defined. However, in these earlier studies, the first hormonally regulated step in steroid biosynthesis, the transfer of cholesterol to the inner mitochondrial membrane, was shown to be the step in which ROS primarily exerted its effect. The studies reported herein confirm and extend these earlier observations and demonstrate that the inhibition of StAR protein by ROS is a major target

of reactive oxygen attack. Our data indicate that ROS perturbation of the mitochondria and dissipation of the electrochemical gradient is likely what causes the inhibition of StAR protein expression and, consequently, the inhibition of progesterone synthesis.

Our data indicate that StAR is posttranscriptionally inhibited by ROS. There also appears to be a constant level of the 37 kDa relative to the decreased 30- and 32-kDa forms (see especially Fig. 6, A and B). This finding suggests that processing and translocation of StAR at the mitochondria is disrupted by reactive oxygen treatment, which results in dissipation of $\Delta\psi_m$. How StAR facilitates cholesterol transfer across the mitochondrial intermembrane space is not completely understood. StAR is a nuclear-encoded, mitochondrial-targeted protein. Typical of most matrix-targeted proteins, StAR is translated as a larger molecular mass (37 kDa) precursor that contains mitochondrial targeting sequences in its amino terminus. Matrix targeting sequences are composed of a series of largely cationic amino acids that interact with the mitochondrial translocation complexes on the outer and inner mitochondrial membranes. The acidic nature of these sequences provides an ionic driving force that helps propel proteins bearing these sequences into the electronegative matrix. After StAR enters the matrix, it is proteolytically processed by matrix metaloproteases to the intermediate 32- and mature 30-kDa forms. Considerable evidence has been presented over the past several years in support of the hypothesis that the newly synthesized 37-kDa precursor form of StAR is the active form involved in cholesterol transfer (28, 44–46). The role of import and processing of StAR and its importance in cholesterol transfer remains controversial. However, it is generally accepted that the final intramitochondrial processing step rendering StAR as the 30-kDa mature form is the off step in the cholesterol transfer process. The cholesterol transfer activity of StAR has an absolute requirement for an intact $\Delta\psi_m$; indeed, the importance of $\Delta\psi_m$ to StAR function and cholesterol transfer activity is unequivocal (44, 47–53). Our current study reports that oxidative stress causes the depolarization of the mitochondria and dissipation of $\Delta\psi_m$, resulting in concomitant inhibition of StAR protein expression and the inhibition of steroidogenesis, which confirms and extends these previous findings.

Disruption of the mitochondria and dissipation of the membrane potential often is associated with irreversible changes in mitochondria permeability that trigger a series of catabolic reactions resulting in apoptotic cell death (43). In this study, MA-10 cells exposed to concentrations of H_2O_2 as high as 250 μM, however, did not appear to undergo irreversible changes. There was no morphological evidence for apoptosis. Even though treatment with ROS resulted in dissipation of $\Delta\psi_m$, there was no appreciable loss of cell viability observed (Fig. 7). The trypan blue dye exclusion assay depends on intact cellular membranes as a measure of viability. These data indicate exposure to reactive oxygen treatments did not cause significant alterations in the plasma membrane. Lipid peroxidation is a well-established mechanism of nonspecific cellular injury leading to the production of lipid peroxides and their by-products such as malondialdehyde and 4-hydroxy-2(E)-nonenal. Measurement of malondialdehyde and 4-hydroxy-2(E)-nonenal provides a convenient index of lipid peroxidation. There was no apparent increase in the level of these lipid peroxidation products following exposure to ROS, as determined using the lipid peroxidation assay kit (Calbiochem, data not shown). Because there was no significant change in these lipid peroxidation products further indicates that overt damage to cellular membranes did not occur. Another widely used measure of cell viability is based on determination of cellular ATP levels (*e.g.* CellTiter-Glo viability assay, Promega Corp.; ApoSensor viability assay, BioVision, Mountain View, CA). As shown in Fig. 8, treatment with H_2O_2 did not affect cellular ATP levels, further supporting the conclusion that the effects of H_2O_2 were not due to cellular toxicity.

Removal of H_2O_2 and continued treatment with cAMP resulted in the partial restoration of StAR protein and progesterone production. Moreover, in cells exposed to prolonged treatment with H_2O_2 (for up to 6 h), we observed that steroidogenic function began to return to normal. These observations suggest that the effects of H_2O_2 are transient and the perturbation of the mitochondria resulted in a temporary loss of membrane potential. Considering that the effects of H_2O_2 were transient, it is even more striking how sensitive StAR protein is to loss of $\Delta\psi_m$ that results from oxidative stress to the cells. Another consequence of long-term perturbation of $\Delta\psi_m$ would be the inhibition of ATP production and decrease in cellular ATP levels.

In the present study, there was not a significant decrease in total cellular ATP observed as a result of H_2O_2 exposure (see Fig. 8). There is a significant decrease in ATP in cells treated with cAMP alone or cAMP plus xanthine/xanthine oxidase. These data contradict the study by Margolin *et al.* (23), who reported that treatment of rat granulosa cells with H_2O_2 resulted in a rapid and significant decrease in total cellular ATP levels. However, these investigators determined that inhibition of DNA repair activity during H_2O_2 exposure with 3-aminobenzamide prevented the H_2O_2-induced decrease in ATP levels. They concluded that changes in cellular ATP levels did not account for the inhibitory effects of H_2O_2 on granulosa cell FSH-stimulated progesterone production. Subsequently, they demonstrated that the effects of H_2O_2 were due to inhibition of the hormone-sensitive cholesterol transport into the mitochondria of rat luteal cells (22). The data we present here confirm these observations. First, because there is no significant decrease in total ATP levels, then depletion of total cellular ATP pools must not account for the inhibition of StAR. Second, our data demonstrate that the primary effect of H_2O_2 on the mitochondrial components of the steroidogenic machinery is on StAR protein because there is no change in P450scc protein. The earlier studies on the effects of oxidative stress on steroidogenesis from Behrman and Aten (22) and Stocco *et al.* (24), did not directly examine changes in StAR protein, and they did not assess changes in mitochondrial function.

The data we present in this study indicate that the dissipation of $\Delta\psi_m$ that accompanies oxidative stress results in the inhibition of StAR protein expression and progesterone biosynthesis. Although total cellular ATP levels were not significantly altered during the time of ROS exposure in this study, it is likely that intramitochondrial levels of ATP were significantly diminished. To test this possibility, ATP levels

in mitochondria isolated from oxidatively stressed MA-10 cells will need to be quantitated. Another possible consequence of dissipating $\Delta\psi_m$ would be depletion of intramitochondrial Ca^{2+} levels. Mitochondrial [Ca^{2+}] is regulated primarily by pump-leak mechanisms that depend on an intact electrochemical gradient for activity (54). A compelling body of work from Capponi and co-workers (55–59) has demonstrated a role for intramitochondrial Ca^{2+} in the regulation of StAR-mediated adrenal steroidogenesis. Investigations are currently underway to assess the relative contributions of intramitochondrial ATP and Ca^{2+} to the control of steroidogenesis in MA-10 cells.

Oxidative damage induced by ROS is implicated as an important contributing factor in the pathogenesis of more than 100 conditions (2). ROS-mediated perturbation of Leydig cell mitochondria resulting in inhibition of StAR protein expression and function may well be an important contributing factor to the decrease in testosterone associated with a host of pathological conditions, including inflammation, infection, ischemia-reperfusion injury, cryptorchidism, alcohol abuse, diabetes, and aging.

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Address all correspondence and requests for reprints to: Dale Buchanan Hales, Ph.D., Department of Physiology and Biophysics, University of Illinois at Chicago, 835 South Wolcott Avenue, Chicago, Illinois 60612-7342. E-mail: dbhale@uic.edu.

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