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Effect of Glutathione Redox State on Leydig Cell Susceptibility to Acute Oxidative Stress*

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Abstract

The free radical, or oxidative stress, theory posits that imbalance in cells between prooxidants and antioxidants results in an altered redox state and, over time, an accumulation of oxidative damage. We hypothesized herein that cells with an increasingly prooxidant intracellular environment also might be particularly susceptible to *acute* oxidative stress. To test this hypothesis, MA-10 cells were used as a model because of their well-defined, measurable function, namely progesterone production. We first experimentally altered the redox environment of the cells by their incubation with buthionine sulfoximine (BSO) or diethyl maleate (DEM) so as to deplete glutathione (GSH), and then exposed the GSH-depleted cells acutely to the prooxidant tert-butyl hydroperoxide (t-BuOOH). Neither BSO or DEM by themselves affected progesterone production. However, when the GSH-depleted cells subsequently were exposed acutely to t-BuOOH, intracellular reactive oxygen species concentration was significantly increased, and this was accompanied by significant reductions in progesterone production. In striking contrast, treatment of control cells with t-BuOOH had no effect. Depletion of GSH and subsequent treatment of the cells with t-BuOOH-induced the phosphorylation of each of ERK1/2, JNK and p38, members of the MAPK family. Inhibition of p38 phosphorylation largely prevented the t-BuOOH-induced down-regulation of progesterone production in GSH-depleted cells. These results suggest that, as hypothesized, alteration of the intracellular GSH redox environment results in the increased sensitivity of MA-10 cells to oxidative stress, and that this is mediated by activation of one or more redox-sensitive MAPK members.

Keywords

Glutathione; Oxidative Stress; Steroidogenesis

INTRODUCTION

Imbalance between prooxidants and antioxidants in cells can result in an altered redox state and an accumulation of oxidative damage to a variety of intracellular macromolecules (Ames et al., 1993; Finkel and Holbrook, 2000). An altered redox environment is characteristic of the aging of many cell types, and may contribute to functional deficits that accompany aging

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(Rebrin and Sohal, 2008). Changes in redox environment, particularly the glutathione (GSH) redox state, also have been shown to be associated with the etiology and progression of neurodegenerative, cardiovascular, pulmonary, inflammatory and immune system diseases, and with cystic fibrosis, cancer and diabetes (Ballatori et al., 2009). An increasingly prooxidant environment, and particularly changes in the GSH redox state, also are reported to have consequences for how organisms and cells respond to environmental stressors (Biswas and Rahman, 2009).

It is established that there are age-related increased susceptibilities to both internal and external insults (Hagen et al., 2000; Palomero et al., 2001; Najjar et al., 2005). For example, the skin of older individuals is particularly susceptible to such extrinsic factors as photodamage (Farage et al., 2008), and older athletes have greater susceptibility to exercise-induced skeletal-muscle damage (Fell and Williams, 2008). As yet, the mechanisms that are responsible for age-dependent increased susceptibilities to stress are far from clear.

The studies that are presented herein were designed to examine the relationship between the redox environment and the susceptibility of cells to acute oxidative stress. The experiments are based on the hypothesis that cells with an altered GSH redox environment may have increased susceptibility to external, acute oxidative stress. To test this hypothesis, we examined the effect of GSH depletion in MA-10 Leydig cells on the susceptibility of the cells to a lipid prooxidant, tert-butyl hydroperoxide (t-BuOOH). MA-10 cells were used because of their well-defined, measurable function, namely progesterone production. We show that cells with a reduced GSH pool were far more sensitive to additional, acute oxidative stress than cells with normal or increased GSH content, and that the increased susceptibility to oxidative stress is mediated by redox-sensitive MAPK signaling pathways.

MATERIALS AND METHODS

Chemicals

Dibutyl cAMP (dbcAMP), L-buthionine-sulfoximine (BSO), glutathione ethyl ester (GSHEE), 22-hydroxycholesterol (22-HC), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), diethyl maleate (DEM), Waymouth's MB/752 medium, poly-L-lysine, horse serum, N-acetyl-cysteine (NAC), SB202190, PD169316 and tert-butyl hydroperoxide (t-BuOOH) were from Sigma-Aldrich (St. Louis, MO). L-Stereoisomer was from Enzo Life Sciences International, Inc. (Plymouth Meeting, PA). 1,2-dithiole-3-thione (D3T) was kindly provided by Dr. Rex Munday (Ruakura Agricultural Research Centre, Hamilton, New Zealand). SP600125, and 2',7'-dichlorodihydrofluorescein diacetate (DCF) was from Invitrogen (Carlsbad, CA). [1,2,6,7-³H(N)]-progesterone was from Perkin Elmer Life Sciences, Inc (Boston, MA). All antibodies for phosphorylated and total mitogen-activated protein kinase (MAPK) proteins (ERK1/2, JNK and p38) were from Cell Signaling Tech (Danvers, MA). Progesterone antibody was from ICN (Costa Mesa, CA). StAR antibody was a generous gift from Dr. Dale B. Hales (University of Illinois at Chicago, Chicago, Illinois). P450scc antibody was from Chemicon International (Temecula, CA). The HRP-conjugated anti-rabbit donkey IgG and [³H]-cAMP assay kits were from GE Healthcare Bio-Sciences Corp (Piscataway, NJ). Bovine LH (USDA-bLH-B-6) was provided by USDA Animal Hormone Program (Beltsville, MD). Pregnenolone and progesterone were from Steraloids (Newport, RI).

MA-10 cell culture

The MA-10 mouse Leydig tumor cell line was a generous gift from Dr. Mario Ascoli (University of Iowa, Iowa City, IA). The cells were cultured in Waymouth's MB/752 medium containing 15% horse serum and 5% CO₂ at 34 °C (Ascoli, 1981). To deplete or replete

intracellular GSH concentration, cells with 70% confluence were treated with BSO (100 μ M), DEM (100 μ M), or D3T (up to 400 μ M) for up to 24 hours. Although Waymouth's MB/752 medium contains a relatively high concentration of GSH, BSO and DEM treatments depleted intracellular GSH. For subsequent treatment with the prooxidant t-BuOOH, the cells were switched to serum-free M-199 medium, a cholesterol-containing medium that contains low GSH. After treatment with t-BuOOH (up to 400 μ M) and LH (100 ng/ml) or other steroidogenic stimulators (see below) for up to 2 hours, the medium was frozen for progesterone assay and the cells were used for Western blot analysis of StAR and P450_{scc} proteins. To inhibit MAPK activities, cells were preincubated with JNK inhibitors (SP600125, 10 μ M; or L-Stereoisomer, 2 μ M) or p38 inhibitors (SB202190, 10 μ M; or PD169316, 10 μ M) for 30 min before and during t-BuOOH (100 μ M, 2h) treatment. Control cells were incubated with vehicle (0.1% DMSO) before and during t-BuOOH treatment. Progesterone production was assayed after incubation of the cells with LH for an additional hour after t-BuOOH treatment. The viability of the cells was estimated by MTT assay (see below).

Progesterone and cAMP assays

For progesterone assays, the cells were cultured in a 24-well plate and treated with t-BuOOH (100 μ M) for 2 hours with LH (100 ng/ml), dbcAMP (2 mM), 22-HC (25 μ M), or pregnenolone (25 μ M). The medium was collected at the end of each experiment and stored at -80°C . Progesterone concentration in the medium was determined by radioimmunoassay and expressed as micrograms per milligram cellular protein. For cAMP assays, the cells were cultured in a 24-well plate with t-BuOOH for 2 hours, then switched to phenol red-free M-199 medium containing LH (100 ng/ml) and isobutyl-methylxanthine (IBMX, 100 μ M) for 30 minutes. At the end of the incubation, an equal volume of Tris buffer (0.05 M, pH 7.5) containing 4 mM EDTA and 2 mg/ml theophylline was added to the culture medium to block cAMP degradation. The plates were frozen on dry ice and stored at -80°C before cAMP and progesterone assays. cAMP levels were measured using the [^3H]cAMP assay system from GE Healthcare Bio-Sciences Corp (Piscataway, NJ) according to the manufacturer's instructions. The sensitivity of the assay was 0.05 pmoles per assay tube.

GSH assay

GSH was measured as previously described (Hissin and Hilf, 1976) with minor modification. In brief, cells were lysed in 5% metaphosphoric acid and sonicated. Protein was precipitated by centrifugation at 13,000g for 30 minutes. The supernatant was diluted 10 times with sodium phosphate buffer (0.1 M, pH 8.0, with 5 mM EDTA). Diluted sample (10 μ l) was incubated with 10 μ l of o-phthalaldehyde (in methanol) and 180 μ l of phosphate buffer for 15 minutes at room temperature. Fluorescence was read with a BioRad luminescence spectrometer at an excitation wavelength of 350 nm and an emission wavelength of 420 nm. The cellular GSH content was calculated using a concurrently run GSH standard curve.

Analysis of intracellular ROS concentrations

Intracellular ROS concentrations were assessed by measuring the ability of the cells to oxidize the redox sensitive dye 2',7'-dichlorodihydrofluorescein diacetate (DCF). In brief, the cells were cultured in black 96-well fluorescence assay plates (BD Falcon, Franklin Lakes, NJ). After being treated with BSO overnight or DEM for 30 minutes, the cells were switched to serum-free M199 medium and incubated with t-BuOOH (up to 100 μ M) for 1 hour. Some of the BSO treated cells were incubated with GSH ethyl ester (GSHEE, 6 mM) for 2 hours to restore the GSH concentration, or with the antioxidant NAC (10 mM) for 30 minutes before and during t-BuOOH treatment. The cells were then incubated in 200 μ l M199 medium containing 20 μ M DCF for 30 minutes. After washing 3 times with PBS, 50 μ l PBS was added to the plates, and the plates were read by a DTX800 Multimode Detector (Beckman Coulter, Fullerton, CA).

with excitation of 485 nm and emission of 535 nm. DCF fluorescence also was examined by fluorescence microscopy. The cells were cultured in poly-L-lysine-coated six well chamber slides, treated with t-BuOOH, and then incubated with DCF (as above). Images were obtained with a Nikon Eclipse 800 microscope equipped with a Princeton Instruments 5-Mhz cooled CCD camera, custom CRI color filter, and IP-Lab digital image analysis software (Scanalytics Inc., Fairfax, VA). All photos were taken at the same magnification and with the same exposure times.

MTT assay

Cell viability was estimated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay, which measures the reduction of MTT (a yellow tetrazolium salt) to blue formazan in viable cells (Mosmann, 1983). After their treatment with BSO and t-BuOOH (up to 100 μ M), cells were incubated with MTT (0.5 mg/ml) for one hour. The medium was then removed and the reduced formazan was dissolved in 100 μ l acidified (0.04 N HCl) isopropanol at room temperature for 20 minutes. The dissolved formazan was then transferred to a new 96-well plate and read by a DTX800 Multimode Detector (Beckman, Coulter, Inc., Fullerton, CA) at 562 nm wavelength. Control (blank) wells contained isopropanol without MTT. Cells from three different experiments were analyzed for each treatment.

Western blot analysis

For StAR and P450scc analysis, GSH-depleted cells (BSO 100 μ M, overnight) were further treated with t-BuOOH and with LH, dbcAMP, 22-HC or pregnenolone for 2 hours. To examine MAPK phosphorylation, cells with or without BSO (100 μ M) pre-treatment were incubated with t-BuOOH (0–100 μ M) for 1 hour, then lysed with Tris-SDS buffer (62.5 mM Tris, 2% SDS, 50 mM dithiothreitol, pH 6.8) and sonicated on ice. Lysates were mixed with 3X SDS loading buffer (New England BioLab, Ipswich, MA). Equal amounts of protein (30 μ g) were separated by 10% SDS-PAGE, and then transferred onto a nitrocellulose membrane. After incubation with primary antibody (1:1,000) and horseradish peroxidase (HRP)-conjugated secondary antibody (1:5,000), the signals were detected by the enhanced chemiluminescence Western blot kit from Pierce (Rockford, IL). The bound antibodies (StAR) on the membranes were stripped by Restore Western Blot Stripping Buffer (Pierce, Rockford, IL), and the membranes were re-probed with P450 side-chain cleavage enzyme (P450scc) antibody. For the MAPK experiments, the membranes were stripped and re-probed with antibodies in the sequence: p38, total p38, p-JNK, total JNK, p-ERK1/2 and total ERK1/2.

Statistical analyses

Data are expressed as the mean $\pm$ standard error of the mean (SEM). Group means were evaluated by one-way ANOVA. If group differences were revealed by ANOVA ($P < 0.05$), differences between individual groups were determined with the Student-Neuman-Kuels-test, using SigmaStat software (Systat Software Inc., Richmond, CA). Values were considered significant at $P < 0.05$.

RESULTS

Effect of GSH depletion on steroidogenesis in MA-10 Leydig cells

The biosynthesis of GSH is mediated by two ATP-dependent enzymes, γ -glutamylcysteine ligase (the rate-limiting enzyme) and glutathione synthetase (Griffith and Meister, 1979; Anderson, 1998). Buthionine sulfoximine (BSO), a specific γ -glutamylcysteine ligase inhibitor, can block the rate-limiting step of GSH biosynthesis, and in doing so is able to deplete the intracellular GSH pool in both cultured cells and in whole animals (Griffith and Meister, 1979; Anderson, 1998). Diethyl maleate (DEM), another GSH depleting reagent, can reduce

intracellular GSH concentration by forming glutathione conjugates in reactions catalyzed by glutathione S-transferase (Esposito et al., 2001). Treatment of cells with 1,2-dithiole-3-thione (D3T), on the other hand, can increase intracellular GSH concentration by increasing γ -glutamylcysteine ligase (Cao et al., 2004). Incubation of MA-10 Leydig cells with 100 μ M BSO decreased intracellular GSH concentration by more than 80% by 24 hours (Fig. 1A). Incubation of the cells with 100 μ M DEM also depleted the GSH concentration, but much more quickly (by about 70% in 30 minutes) (Fig. 1A). Incubation of MA-10 cells with BSO for 24 hours or with DEM for 30 minutes did not affect the steroidogenic function (production of progesterone) of the cells significantly in either case (Fig 1B). However, when BSO or DEM-treated cells were incubated with the oxidative stressor tert-butyl hydroperoxide (t-BuOOH) for 2 hours, progesterone production decreased significantly in response to t-BuOOH concentration. With both BSO- and DEM-treated cells, progesterone production was significantly reduced at t-BuOOH concentrations as low as 12.5 μ M.

The effect of glutathione depletion and t-BuOOH treatment on specific steps of the steroidogenic pathway of MA-10 cells was determined by providing the stimulator/precursor for particular steps and measuring products. To examine the effect of BSO plus t-BuOOH on cAMP production by MA-10 cells, the cells were incubated overnight with BSO, then treated with t-BuOOH (100 μ M, 2 hours) and during the last 30 minutes incubated with LH. To examine the rest of the steroidogenic pathway, cells were incubated overnight with BSO and then treated with t-BuOOH plus: dbcAMP to by-pass the LH receptor/G-protein-adenylyl cyclase cascade; 22-hydroxycholesterol (22HC) to by-pass StAR-stimulated cholesterol transport into mitochondria; or pregnenolone (P5), the immediate substrate of the last enzyme in the pathway (3β -hydroxysteroid dehydrogenase, 3β -HSD). When intracellular GSH was depleted by BSO overnight, incubation of the cells with t-BuOOH resulted in decreases in LH-stimulated cAMP production as a function of t-BuOOH dose that were comparable in magnitude to the decreases seen in progesterone production (Fig. 2A). This suggests detrimental effects of t-BuOOH on the LH receptor/G-protein-adenylyl cyclase cascade. Incubation of the GSH-depleted, t-BuOOH treated cells with dbcAMP also resulted in low progesterone production (15% of control cells) (Fig. 2B). When the cells were incubated with 22-HC (the mitochondrial membrane-permeable substrate of the P450_{scc} enzyme), there was a lesser reduction in progesterone production in response to t-BuOOH than by cells treated with LH or dbcAMP. These results suggest detrimental effects of t-BuOOH on cholesterol transport into mitochondria and/or on P450_{scc} enzyme function. When the BSO/t-BuOOH cells were incubated with pregnenolone, progesterone production was at control levels, suggesting that the last enzyme in the pathway, 3β -HSD, was unaffected by BSO/t-BuOOH. Western blots (Fig. 2C) showed that stimulation of control cells with LH or dbcAMP, but not with 22-HC or pregnenolone, resulted in increases of the mature form (30 kD) of StAR. However, when cells were treated with BSO/t-BuOOH, incubation with LH or dbcAMP did not result in StAR protein stimulation. Although no changes were seen in protein levels of the mitochondrial enzyme P450_{scc}, the possibility that t-BuOOH affects P450_{scc} activity was not ruled out.

Effect of GSH depletion on steroidogenesis in MA-10 Leydig cells

We reasoned that if depletion of the intracellular GSH pool indeed results in MA-10 cells becoming more susceptible to oxidative stress, increase in the GSH pool might increase the resistance of the cells to stress. To address this, 1,2-dithiole-3-thione (D3T), which increases intracellular GSH by inducing the key enzyme in the GSH biosynthesis pathway, γ -glutamylcysteine ligase, was provided to the cells 24 hours before they were exposed to t-BuOOH. Figure 3A shows that the GSH concentration increased in a dose-dependent manner in cells treated with D3T; at the highest concentration (400 μ M), D3T treatment resulted in a GSH pool that was almost three times that of the control cells. When incubated with LH plus t-BuOOH at high concentration (400 μ M), the cells produced 40% of the progesterone of LH-

incubated control cells (Fig. 3B). However, preincubation of the cells with increasing concentrations of D3T (25–400 μ M) for 24 hours protected the cells from 400 μ M t-BuOOH as a function of D3T dose (Fig. 3B). When cells were provided with pregnenolone (P5) rather than stimulated by LH, progesterone production was maintained at control levels even in the presence of 400 μ M t-BuOOH (Fig. 3B). To rule out the possibility that t-BuOOH might affect LH-induced steroidogenic function by affecting cell viability, the MTT assay was used to measure cell viability. As seen in Figure 3C, incubation of cells with t-BuOOH (400 μ M) or D3T (25–400 μ M), alone or in combination, did not affect cell viability, suggesting that D3T increases the resistance of the cells to t-BuOOH through increasing cellular GSH content.

To further address this issue, the cells were treated with a combination of BSO (100 μ M) and D3T (400 μ M) for 24 hours before their exposure to 50 μ M t-BuOOH. As seen in Figure 4A, incubation of the cells with BSO alone reduced intracellular GSH by 80% compared to controls, and incubation with D3T increased GSH three-fold. Incubation of the cells with a combination of the two completely abolished the D3T-induced GSH accumulation in the cells. Figure 4B shows that whereas progesterone production was high in D3T-incubated cells subsequently treated with t-BuOOH, cells treated with a combination of BSO plus D3T produced low levels of progesterone, supporting the conclusion that D3T increases the resistance of MA-10 cells to t-BuOOH through increasing cellular GSH content. Taken together, these observations indicate that the GSH pool, or the GSH redox, is an important defense mechanism that protects steroidogenic function in MA-10 Leydig cells.

Effect of GSH depletion on reactive oxygen species (ROS) production in MA-10 Leydig cells

Having shown that steroidogenic function in MA-10 cells becomes vulnerable to t-BuOOH-induced stress when the cellular GSH pool is reduced, we wished to determine whether t-BuOOH's effect on steroidogenic function is through elevated ROS within the cells. To address this, ROS concentration was assayed in cells with 2, 7-dichlorodihydrofluorescein diacetate (DCF) (20 μ M, 30 min), a dye that fluoresces upon its oxidation by ROS. For these studies, cells were treated with BSO or DEM, t-BuOOH, and then DCF. Control cells were not exposed either to BSO or t-BuOOH. Figure 5A shows control and treated cells as seen under fluorescence microscopy. Untreated cells produced little fluorescence when incubated with DCF. Fluorescence intensity did not increase appreciably upon t-BuOOH treatment of the control cells. However, when the cells were pretreated with BSO (100 μ M, 24hr), ROS production was significantly increased by t-BuOOH (100 μ M). Treatment with the antioxidant NAC reduced the fluorescence of BSO/t-BuOOH-treated cells to the level of the controls. The results seen by fluorescence microscopy were confirmed and extended by quantification of fluorescence (Fig. 5B). Each group of cells (control, BSO, BSO+GSHEE, BSO+NAC, DEM, DEM+NAC) was exposed to t-BuOOH. t-BuOOH treatment of control cells did not elicit increased ROS production. Fluorescence increased significantly with t-BuOOH treatment of BSO and DEM pretreated cells (50 and 100 μ M). When the cell membrane-permeable glutathione ethyl ester (GSHEE, 6 mM) or the antioxidant NAC (10 mM) was provided to the cells, t-BuOOH-induced ROS production by the BSO- or DEM- pretreated cells was prevented. As seen in Figure 5C, cell viability, measured with the MTT assay, was not affected by conditions that decreased progesterone production and increased ROS production.

Effect of GSH depletion on oxidative stress induced MAPK phosphorylation

To begin to address the mechanism by which depletion of GSH affects the sensitivity of cells to the acute oxidative stress elicited by t-BuOOH, we tested the possibility that oxidative stress might exert its negative effects on steroidogenesis through modulation of oxidant-sensitive MAPK signaling pathways (Abidi et al., 2008a; Abidi et al., 2008b). To do so, we examined the phosphorylation of the three major MAPK families ERK1/2, JNK and p38 after incubating MA-10 cells with BSO to deplete GSH and subsequently with increasing concentrations of t-

BuOOH (0–100 μ M, 1h) (Fig. 6). Without GSH depletion, t-BuOOH (up to 100 μ M) did not affect the phosphorylation of ERK1/2, JNK or p38 significantly. However, in the case of cells with reduced GSH, t-BuOOH increased the phosphorylation of all three MAPK families in a dose-dependent manner, with effects seen at t-BuOOH concentration as low as 25 μ M. To examine whether the two most affected MAPK members, JNK and p38, might be involved in the oxidative stress-induced down-regulation of steroidogenesis, GSH depleted cells were pre-incubated with the JNK inhibitors SP600125 or L-Stereoisomer (referred to as JNK1 and JNK2, respectively, in Fig. 7) or the p38 inhibitors SB202190 or PD169316 (referred to as P38-1 and P38-2, respectively) for 30 min before and during t-BuOOH treatment. Cells incubated with vehicle (DMSO) before and during t-BuOOH treatment served as control. As seen in Figures 7A and 7B, blocking the JNK pathway had no effect on the t-BuOOH-induced down-regulation of progesterone production, whereas blocking the p38 pathway had significantly inhibited the t-BuOOH-induced down-regulation of progesterone production, though did not prevent it entirely.

DISCUSSION

Glutathione (GSH), a highly prevalent antioxidant molecule in cells, plays a central role in the maintenance of the thiol redox balance in cells. Thiol homeostasis is a critical cellular defense system against internal (ROS) and external (environmental) insults (Anderson, 1998; Jones, 2006; Kemp et al., 2008; Biswas and Rahman, 2009). The GSH cycle within the cell, involving the reversible conversion of reduced glutathione (GSH) to oxidized glutathione (GSSG), is an important intracellular pathway for removing hydroxyl groups from oxidized lipids and proteins (Deneke, 2000).

In the present study, we hypothesized, using MA-10 Leydig cells as the experimental model, that changes in the redox environment may affect the susceptibility of the cells to acute oxidative stress. Under the experimental conditions that we used, depletion of GSH or treatment of the cells with the prooxidant t-BuOOH (up to 100 μ M), by themselves, had little effect on the steroidogenic function of the cells. However, MA-10 cells with a decreased intracellular GSH concentration that resulted from BSO or DEM pre-treatment were found to become far more sensitive to the oxidative stress subsequently caused by t-BuOOH. Thus, after GSH depletion, progesterone production was reduced significantly at a t-BuOOH concentration of only 12.5 μ M; and at 100 μ M, the reduction was by more than 80%. Increasing the GSH concentration with D3T resulted in the cells becoming increasingly resistant to subsequent t-BuOOH treatment. When the intracellular GSH pool was increased 3-fold by overnight D3T treatment, the negative effect of t-BuOOH (400 μ M) was almost completely prevented. The fact that complete protection was not achieved might be explained by the inability of D3T to elevate GSH enough, or by the inability of D3T to induce other antioxidant molecules that are also required to completely overcome the negative effect of t-BuOOH.

It is well known that in addition to its affect on GSH, D3T might induce other antioxidant molecules and phase II metabolizing proteins (Cao et al., 2004). However, incubation of cells with D3T plus BSO was found to abolish the rise in GSH achieved with D3T and also its protective effect on progesterone production. This strongly supports the conclusion that D3T protects MA-10 cells through modifying the GSH pool. Taken together, these results indicate that modification of the GSH pool and thus alteration of the redox environment of MA-10 cells affects the susceptibility of the cells to oxidant-induced functional deficits.

In recent studies, evidence was presented that the negative effects of oxidative stress on steroidogenic function by adrenal cells is mediated by oxidant-sensitive MAPK signaling pathways (Abidi et al., 2008a; Abidi et al., 2008b), with the latter in essence serving as redox sensors (McCubrey et al., 2006; Matsuzawa and Ichijo, 2008). Our studies of MA-10 cells

similarly tie the MAPK pathway to the effects of acute oxidative stress. Each of JNK, p38, and ERK was activated in response to the intracellular redox state of MA-10 cells depleted of GSH and then incubated with the prooxidant t-BuOOH. To examine possible cause-effect relationships between activation of MAPK signaling molecules and reduction in steroidogenic function, the MA-10 cells were incubated with inhibitors that specifically target JNK or p38, the two pathways that were mostly affected by BSO/t-BuOOH. Inhibition of p38, but not JNK, significantly, though only partially, suppressed the reduced steroidogenesis resulting from the t-BuOOH-induced oxidative stress, suggesting that the p38 pathway might be involved in the GSH redox dependent, oxidative stress-induced down-regulation of steroidogenic function. Taken together, the present studies of MA-10 cells and the previous observations of adrenal cells (Abidi et al., 2008a; Abidi et al., 2008b) suggest the involvement of MAPK signaling in the increased susceptibility of cells with an altered redox environment to oxidative stress, and in particular suggest a unique role for p38 MAPK.

How might activation of p38 MAPK inhibit steroidogenesis? One possibility could be through the effect of activated p38 on cyclooxygenase 2 (COX-2) activation, as suggested for adrenal cells (Abidi et al., 2008b). COX-2 can be induced by oxidative stress (Nakamura and Sakamoto, 2001; Kumgai et al., 2004; Yang et al., 2005), and p38 MAPK signaling is an important regulator of COX-2 expression (Guan et al., 1998; Lasa et al., 2000; Hendrickx et al., 2003; N'Guessan et al., 2006; Yang et al., 2006; Park et al., 2007; Zarrouki et al., 2007; Sun et al., 2008; Ulivi et al., 2008). The inhibitory effect of COX-2 on steroidogenesis has been shown both in MA-10 cells (Wang et al., 2003) and in primary Leydig cells (Wang et al., 2005; Chen et al., 2007). Additionally, up-regulation of COX-2 in aged Leydig cells has been shown to be associated with reduced StAR and testosterone production, and its inhibition in vitro or in vivo to partially reverse these reductions (Wang et al., 2005; Chen et al., 2007). These results suggest the possibility of a regulatory network involving oxidative stress, p38 MAPK, COX-2 and steroidogenesis.

In both humans and experimental animals, aging has been shown to be associated with significant reductions of GSH content in brain, heart, liver and Leydig cells (Cao et al., 2004; Liu et al., 2004; Suh et al., 2004; Luo et al., 2006; Jones, 2006; Rebrin et al., 2007; Rebrin and Sohal, 2008). A number of studies have suggested that antioxidant defenses may not keep pace with age-related increases in ROS production, resulting in a progressive, age-related shift towards a more prooxidant state (Rebrin and Sohal, 2008). The results presented herein indicate that depletion of the intracellular GSH pool, and thus a more prooxidant environment, can significantly increase the susceptibility of MA-10 cells to additional oxidative stress, leading to altered function. These results thus have implications for aging cells. If an altered redox environment leads to increased susceptibility to acute oxidative stress, cellular age may be a significant risk factor for the effects of toxicants. If this is true, future studies of environmental toxicants should address not only effects on cells during the developmental, peripubertal and/or adult periods as now, but also effects on aged cells. Such an approach would represent a paradigm shift in how potential environmental chemicals are evaluated.

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References

- Abidi P, Leers-Sucheta S, Cortez Y, Han J, Azhar S. Evidence that age-related changes in p38 MAP kinase contribute to the decreased steroid production by the adrenocortical cells from old rats. *Aging Cell* 2008;7:168–178. [PubMed: 18241324]
- Abidi P, Zhang H, Zaidi SM, Shen WJ, Leers-Sucheta S, Cortez Y, Han J, Azhar S. Oxidative stress-induced inhibition of adrenal steroidogenesis requires participation of p38 mitogen-activated protein kinase signaling pathway. *J Endocrinol* 2008;198:193–207. [PubMed: 18417530]
- Ames BN, Shigenaga MK, Hagen TM. Oxidants, antioxidants, and the degenerative diseases of aging. *Proc Natl Acad Sci USA* 1993;90:7915–7922. [PubMed: 8367443]
- Anderson ME. Glutathione: an overview of biosynthesis and modulation. *Chem Biol Interact* 1998;111:1–14. [PubMed: 9679538]
- Ascoli M. Characterization of several clonal lines of cultured Leydig tumor cells: gonadotropin receptors and steroidogenic responses. *Endocrinology* 1981;108:88–95. [PubMed: 6257492]
- Ballatori N, Krance SM, Notenboom S, Shi S, Tieu K, Hammond CL. Glutathione dysregulation and the etiology and progression of human diseases. *Biol, Chem* 2009;390:191–214. [PubMed: 19166318]
- Biswas SK, Rahman I. Environmental toxicity, redox signaling and lung inflammation: the role of glutathione. *Mol Aspects Med* 2009;30:60–76. [PubMed: 18760298]
- Cao Z, Hallur S, Qiu HZ, Peng X, Li Y. Induction of endogenous glutathione by the chemoprotective agent, 3H-1,2-dithiole-3-thione, in human neuroblastoma SH-SY5Y cells affords protection against peroxynitrite-induced cytotoxicity. *Biochem Biophys Res Commun* 2004;316:1043–1049. [PubMed: 15044090]
- Cao L, Leers-Sucheta S, Azhar S. Aging alters the functional expression of enzymatic and non-enzymatic anti-oxidant defense systems in testicular rat Leydig cells. *J Steroid Biochem Mol Biol* 2004;88:61–67. [PubMed: 15026084]
- Chen H, Luo L, Liu J, Zirkin BR. Cyclooxygenases in rat Leydig cells: effects of luteinizing hormone and aging. *Endocrinology* 2007;148:735–742. [PubMed: 17068133]
- Deneke SM. Thiol-based antioxidants. *Curr Top Cell Regul* 2000;36:151–180. [PubMed: 10842751]
- Esposito F, Russo L, Chirico G, Ammendola R, Russo T, Cimino F. Regulation of p21waf1/cip1 expression by intracellular redox conditions. *IUBMB Life* 2001;52:67–70. [PubMed: 11795596]
- Farage MA, Miller KW, Elsner P, Maibach HI. Functional and physiological characteristics of the aging skin. *Aging Clin Exp Res* 2008;20:195–200. [PubMed: 18594185]
- Fell J, Williams D. The effect of aging on skeletal-muscle recovery from exercise: possible implications for aging athletes. *J Aging Phys Act* 2008;16:97–115. [PubMed: 18268815]
- Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. *Nature* 2000;408:239–247. [PubMed: 11089981]
- Griffith OW, Meister A. Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (S-n-butyl homocysteine sulfoximine). *J Biol Chem* 1979;254:7558–7560. [PubMed: 38242]
- Guan Z, Buckman SY, Miller BW, Springer LD, Morrison AR. Interleukin-1- α induced cyclooxygenase-2 expression requires activation of both c-Jun NH2-terminal kinase and p38 MAPK signal pathways in rat renal mesangial cells. *J Biol Chem* 1998;273:28670–28676. [PubMed: 9786861]
- Hagen TM, Vinarsky V, Wehr CM, Ames BN. (R)- α -lipoic acid reverses the age-associated increase in susceptibility of hepatocytes to tert-butylhydroperoxide both in vitro and in vivo. *Antioxid Redox Signal* 2000;2:473–483. [PubMed: 11229361]
- Hendrickx N, Volanti C, Moens U, Seternes OM, de White P, Vandenheede JR, Piette J, Agostinis P. Up-regulation of cyclooxygenase-2 and apoptosis resistance by p38 MAPK in hypericin-mediated photodynamic therapy of human cancer cells. *J Biol Chem* 2003;278:52231–52239. [PubMed: 14557269]
- Hissin PJ, Hilf R. A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Anal Biochem* 1976;74:214–226. [PubMed: 962076]
- Jones DP. Redefining oxidative stress. *Antioxid Redox Signal* 2006;8:1865–1879. [PubMed: 16987039]
- Kemp M, Go YM, Jones DP. Nonequilibrium thermodynamics of thiol/disulfide redox systems: a perspective on redox systems biology. *Free Radic Biol Med* 2008;44:921–937. [PubMed: 18155672]

- Kumagai T, Matsukawa N, Kaneko Y, Kusumi Y, Mitsumata M, Uchida K. A lipid peroxidation-derived inflammatory mediator: identification of 4-hydroxy-2-nonenal as a potential inducer of cyclooxygenase-2 in macrophages. *J Biol Chem* 2004;279:48389–48396. [PubMed: 15355999]
- Lasa M, Mahtani KR, Finch A, Brewer G, Saklatvala J, Clark AR. Regulation of cyclooxygenase 2 mRNA stability by the mitogenactivated protein kinase p38 signaling cascade. *Mol Cell Biol* 2000;20:4265–4274. [PubMed: 10825190]
- Liu H, Wang H, Shenvi S, Hagen TM, Liu RM. Glutathione metabolism during aging and in Alzheimer disease. *Ann NY Acad Sci* 2004;1019:346–349. [PubMed: 15247041]
- Luo L, Chen H, Trush MA, Show MD, Anway MD, Zirkin BR. Aging and the brown Norway rat Leydig cell antioxidant defense system. *J Androl* 2006;27:240–247. [PubMed: 16304208]
- Matsuzawa A, Ichijo H. Redox control of cell fate by MAP kinase: physiological roles of ASK1-MAP kinase pathway in stress signaling. *Biochim Biophys Acta* 2008;1780:1325–1336. [PubMed: 18206122]
- McCubrey JA, Lahair MM, Franklin RA. Reactive oxygen species-induced activation of the MAP kinase signaling pathways. *Antioxid Redox Signal* 2006;8:1775–1789. [PubMed: 16987031]
- Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983;65:55–63. [PubMed: 6606682]
- Najjar SS, Scuteri A, Lakatta EG. Arterial aging: is it an immutable cardiovascular risk factor? *Hypertension* 2005;46:454–462. [PubMed: 16103272]
- Nakamura T, Sakamoto K. Reactive oxygen species up-regulates cyclooxygenase-2, p53, and Bax mRNA expression in bovine luteal cells. *Biochem Biophys Res Commun* 2001;284:203–210. [PubMed: 11374891]
- N'Guessan PD, Hippenstiel S, Etouem MO, Zahlten J, Beermann W, Lindner D, Opitz B, Witzernath M, Rosseau S, Suttrop N, Schmeck B. *Streptococcus pneumoniae* induced p38 MAPK- and NF-kappaB-dependent COX-2 expression in human lung epithelium. *Am J Physiol Lung Cell Mol Physiol* 2006;290:L1131–L1138. [PubMed: 16414978]
- Palomero J, Galán AI, Muñoz ME, Tuñón MJ, González-Gallego J, Jiménez R. Effects of aging on the susceptibility to the toxic effects of cyclosporin A in rats. Changes in liver glutathione and antioxidant enzymes. *Free Radic Biol Med* 2001;30:836–845. [PubMed: 11295526]
- Park YS, Kim J, Misonou Y, Takamiya R, Takahashi M, Freeman MR, Taniguchi N. Acrolein induces cyclooxygenase-2 and prostaglandin production in human umbilical vein endothelial cells: roles of p38 MAP kinase. *Arterioscler Thromb Vasc Biol* 2007;27:1319–1325. [PubMed: 17363696]
- Rebrin I, Forster MJ, Sohal RS. Effects of age and caloric intake on glutathione redox state in different brain regions of C57BL/6 and DBA/2 mice. *Brain Res* 2007;1127:10–18. [PubMed: 17113050]
- Rebrin I, Sohal RS. Pro-oxidant shift in glutathione redox state during aging. *Adv Drug Deliv Rev* 2008;60:1545–1552. [PubMed: 18652861]
- Suh JH, Shenvi SV, Dixon BM, Liu H, Jaiswal AK, Liu RM, Hagen TM. Decline in transcriptional activity of Nrf2 causes age-related loss of glutathione synthesis, which is reversible with lipoic acid. *Proc Natl Acad Sci USA* 2004;101:3381–3386. [PubMed: 14985508]
- Sun H, Xu B, Inoue H, Chen QM. P38 MAPK mediates COX-2 gene expression by corticosterone in cardiomyocytes. *Cell Signal* 2008;20:1952–1959. [PubMed: 18657608]
- Uli V, Giannoni P, Gentili C, Cancedda R, Descalzi F. p38/NF-kB-dependent expression of COX-2 during differentiation and inflammatory response of chondrocytes. *J Cell Biochem* 2008;104:1393–1406. [PubMed: 18286508]
- Wang X, Dyson MT, Jo Y, Stocco DM. Inhibition of cyclooxygenase-2 activity enhances steroidogenesis and steroidogenic acute regulatory gene expression in MA-10 mouse Leydig cells. *Endocrinology* 2003;144:3368–3375. [PubMed: 12865315]
- Wang X, Shen CL, Dyson MT, Eimerl S, Orly J, Hutson JC, Stocco DM. Cyclooxygenase-2 regulation of the age-related decline in testosterone biosynthesis. *Endocrinology* 2005;146:4202–4208. [PubMed: 16002525]
- Yang T, Zhang A, Honeggar M, Kohan DE, Mizel D, Sanders K, Hoidal JR, Briggs JP, Schnermann JB. Hypertonic induction of COX-2 in collecting duct cells by reactive oxygen species of mitochondrial origin. *J Biol Chem* 2005;280:34966–34973. [PubMed: 16024921]

- Yang T, Zhang A, Pasumarthy A, Zhang L, Warnock Z, Schnermann JB. Nitric oxide stimulates COX-2 expression in cultured collecting duct cells through MAP kinases and superoxide but not cGMP. *Am J Physiol Renal Physiol* 2006;291:F891–F895. [PubMed: 16705145]
- Zarrouki B, Soares AF, Guichardant M, Lagarde M, Gélœn A. The lipid peroxidation end-product 4-HNE induces COX-2 expression through p38 MAPK activation in 3T3-L1 adipose cell. *FEBS Lett* 2007;581:2394–2400. [PubMed: 17481611]

Figure 1.

GSH depletion (A) and its effect on the cellular susceptibility to t-BuOOH-induced oxidative stress (B). A) Cells were incubated with BSO (100 μM, 24 h) or DEM (100 μM, 30 min), after which intracellular GSH was assayed. B) The GSH depleted cells were treated with increasing concentrations of t-BuOOH (0–100 μM) plus LH (100 ng/ml) for two hours. Progesterone was assayed in the culture medium. The data are expressed as mean ± SEM of 4 experiments. All t-BuOOH doses above 6.25 μM resulted in significantly decreased progesterone production compared to the controls, $P < 0.05$.

Figure 2.

Effect of GSH depletion on t-BuOOH induced changes in cAMP production and on downstream components of the steroidogenic pathway of MA-10 Leydig cells. A) Dose-dependent effects of t-BuOOH on LH stimulated cAMP and progesterone production by BSO-treated cells. The cells were treated with t-BuOOH for 2 hours, then switched to phenol red-free M-199 medium containing LH (100 ng/ml) and isobutyl-methylxanthine (IBMX, 100 μM) for 30 min. B) Progesterone production by cells treated with BSO plus t-BuOOH and with LH, dbcAMP, 22HC or progesterone (P5) for 2 hr. Control cells were not incubated with either BSO or t-BuOOH. C) StAR and P450scc proteins analyzed by Western blots after cells were treated with BSO/t-BuOOH. Data are expressed as mean ± SEM of 4 experiments. *, Significantly different from cells cultured without BSO at $P < 0.05$.

Figure 3.

Effect of increased intracellular GSH concentration on cellular resistance to t-BuOOH. A) MA-10 Leydig cells were incubated with D3T from 0–400 μ M for 24 hours to induce γ -glutamylcysteine ligase and thus increase GSH concentration in the cells. B) After 24 h of D3T treatment, cells were incubated with a high concentration of t-BuOOH (400 μ M) and LH (100 ng/ml) or pregnenolone (P5; 25 μ M) for 2h, and progesterone production was assayed in the medium. C) After the cells were treated with t-BuOOH (400 μ M) for 2h, they were provided with MTT for an additional hour. The reduction of the MTT dye by the viable cells was read on plate reader at 562 nm wavelength. The data are expressed as mean $\pm$ SEM of 3 experiments. *, Significantly different from controls (400 μ M t-BuOOH, Fig. 4B), $P < 0.05$.

Figure 4.

Effect of co-treatment of cells with BSO and D3T on cellular resistance to t-BuOOH. MA-10 Leydig cells were incubated with BSO (100 μM), D3T (400 μM) or a combination of the two for 24 h. A) GSH was assayed in the cells immediately after the 24 h BSO/D3T treatments. B) After 24 h BSO/D3T treatments, the cells were incubated with t-BuOOH (50 μM) and LH (100 ng/ml) for 2h, and progesterone production was assayed in the medium. The data are expressed as mean ± SEM of 3 experiments. *, Significantly different from cells without any treatment (C), $P < 0.05$.

Figure 5.

Effect of GSH depletion and supplementation on t-BuOOH-induced ROS production (A, B) and on cell viability (C). Cells were treated with BSO (100 μ M, 24h) or DEM (100 μ M, 30min), then with t-BuOOH (0–100 μ M, 1h), and finally with DCF. Some of the BSO-treated cells were incubated with GSHEE (6 mM) for 2 hours to restore the GSH concentration, or with the antioxidant NAC (10 mM) for 30 min, before and during t-BuOOH treatment. A) Cells as seen by fluorescence microscopy. The conditions are indicated for each of the four panels. Control cells were not treated either with BSO or t-BuOOH. B) ROS production, as assayed by fluorescence plate reader. C) After the cells were treated with t-BuOOH (0–100 μ M) for two hr, they were provided with MTT for an additional hour. The reduction of the MTT dye by the viable cells was read on plate reader at 562 nm wavelength. The data are expressed as mean $\pm$ SEM of 3 experiments. *, Significantly different from control cells, $P < 0.05$.

Figure 6.

Effect of GSH depletion on the cellular susceptibility to t-BuOOH-induced MAPK phosphorylation. MA-10 cells without (–) or with (+) GSH depletion (100 μ M BSO, 24 h) were treated with increasing concentrations of t-BuOOH (0–100 μ M) for one hour. Phosphorylated (P) and total (T) MAPK proteins (ERK1/2, JNK and p38) were examined by Western blots. The experiments were repeated three times with similar results.

Figure 7.

Effect of MAPK inhibition on t-BuOOH-induced down-regulation of steroidogenesis. GSH-depleted cells (100 μ M BSO, 24 h) were pre-incubated with JNK inhibitors (JNK1: SP600125, 10 μ M; or JNK2: L-Stereoisomer, 2 μ M) or p38 inhibitors (P38-1: SB202190, 10 μ M; or P38-2: PD169316, 10 μ M) for 30 min before and during t-BuOOH (100 μ M, 2h) treatment. Control cells (DMSO) were incubated with vehicle (0.1% DMSO) before and during t-BuOOH treatment. Progesterone production was assayed by incubating the cells with LH (100 ng/ml) for an additional hour after t-BuOOH. A) Progesterone production by unstressed (C) and t-BuOOH-treated cells in response to JNK and p38 inhibitors. B) Progesterone production as in (A) but expressed as percentage of unstressed cells. The data are expressed as mean $\pm$ SEM of 3 experiments. *, Significantly different from control cells, $P < 0.05$.