Oxidative stress-induced inhibition of adrenal steroidogenesis requires participation of p38 mitogen-activated protein kinase signaling pathway

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Abstract

Previous studies from this laboratory identified excessive oxidative stress as an important mediator of age-related decline in steroid hormone production. Here, we investigated whether oxidative stress exerts its antisteroidogenic action through modulation of oxidant-sensitive mitogen-activated protein kinase (MAPK) signaling pathways. To accomplish these studies, we employed a highly responsive mouse adrenocortical cell line, Y1-BS1 cells that secrete large quantities of steroids when stimulated with lipoprotein plus hormone. Treatment of these cells with superoxide, H₂O₂ or 4-hydroxy-2-nonenal (HNE) significantly inhibited steroid production and increased phosphorylation and activation of p38 MAPK. None of the treatments altered the phosphorylation of either extracellular signal-regulated kinases or c-Jun N-terminal kinases (JNKs). Pretreatment of Y1-BS1 cells with MnTMPyP, a cell-permeable superoxide-dismutase/catalase mimetic reactive oxygen species (ROS scavenger), completely prevented the superoxide- and H₂O₂-mediated inhibition of steroid production. Likewise, antioxidant N-acetylcysteine completely blocked the HNE-induced loss of steroidogenic response. Incubation of Y1-BS1 cells with either MnTMPyP or NAC also upregulated Bt₂cAMP and

Bt₂cAMP + hHDL₃-stimulated steroid synthesis, indicating that endogenously produced ROS can inhibit steroidogenesis. Inhibition of p38 MAPK with SB203580 or SB202190 upregulated the basal steroid production and also prevented the oxidant-mediated inhibition of steroid production. mRNA measurements by qPCR indicated that Y1-BS1 adrenal cells predominantly express p38 MAPK α isoform, along with relatively low-level expression of p38 MAPK γ . By contrast, little or no expression was detected for p38 MAPK β and p38 MAPK δ isoforms in these cells. Transfection of Y1-BS1 cells with either caMKK3 or caMMK6 construct, the upstream p38 MAPK activators, decreased steroidogenesis, whereas transfection with dnMKK3 or dnMKK6 plasmid DNA increased steroidogenesis. Similarly, transfection of cells with a dnp38 MAPK α or dnp38 MAPK β construct also increased steroid hormone production; however, the effect was less pronounced after expression of either dnp38 MAPK γ or dnp38 MAPK δ construct. These results indicate that activated p38 MAPK mediates oxidant (excessive oxidative stress)-induced inhibition of adrenal steroidogenesis.

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Introduction

Advancing age in humans (Shifren & Schiff 2000, Burger *et al.* 2002, Arlt 2004, Dharia & Parker 2004, Harman 2005, Kaufman & Vermeulen 2005) and experimental animals (Zirkin & Chen 2000, Medicherla *et al.* 2002, Wang & Stocco 2005) is associated with profound changes in the synthesis and secretion of steroid hormones. In rats, this steroidogenic decline is found in adrenocortical (Malamed & Carsia 1983, Popplewell *et al.* 1986, Cheng *et al.* 1990) and testicular

Leydig cells (Bethea & Walker 1979, Tsitouras *et al.* 1979, Liao *et al.* 1993, Chen *et al.* 1994) of several strains and is a progressive defect increasing in impact as the rat ages from 5 months to 12, 18, and 24 months (Malamed & Carsia 1983, Popplewell *et al.* 1986, Liao *et al.* 1993, Medicherla *et al.* 2002). The major functional alteration occurs distal to sites of tropic hormone interaction and cAMP generation (Tsitouras *et al.* 1979, Popplewell *et al.* 1986, Liao *et al.* 1993) and is primarily related to a defect in the transport of free cholesterol to CYP11A1 (P450_{scc}) sites within the mitochondria where

the conversion of cholesterol to pregnenolone (parent steroid) begins with side-chain cleavage (Poppellwell & Azhar 1987, Liao *et al.* 1993).

Although the cellular and molecular mechanisms controlling this aging defect (i.e. impaired cholesterol transport to mitochondria) have not been completely defined, considerable evidence from this laboratory points to excessive free radical formation and oxidative damage (especially from lifelong continued processing of cholesterol for steroid production (Azhar *et al.* 1995, Hanukoglu 2006)) to the cell machinery regulating the functional expression of key proteins involved in mitochondrial cholesterol transport (Azhar *et al.* 1995, Leers-Sucheta *et al.* 1999, Abidi *et al.* 2004, Cao *et al.* 2004). For example, previous studies from this laboratory have shown that age-induced loss of steroidogenic function is accompanied by a significant alteration in oxidative status of rat adrenal and testicular tissues including enhanced oxidative stress, loss of enzymatic and non-enzymatic antioxidants and increased membrane lipid peroxidation (Azhar *et al.* 1995, Cao *et al.* 2004). In addition, there is evidence that aging leads to alterations in the expression of two oxidant-sensitive transcription factors, AP-1 and NF- κ B (Medicherla *et al.* 2001, 2002). Additional studies further demonstrated that expression of two cellular cholesterol transport/delivery proteins, StAR (Stocco & Clark 1996) and peripheral-type benzodiazepine receptor (PBR) (now renamed translocator protein (18 kDa) (TSPO) (Papadopoulos *et al.* 2006)), that assist in the intracellular delivery of cholesterol to mitochondrial CYP11A1 are also reduced during aging (Leers-Sucheta *et al.* 1999, Luo *et al.* 2001, Culty *et al.* 2002). However, the underlying mechanism(s) by which excessive oxidative stress leads to loss of steroid production during aging remain unclear. Oxidative stress has been implicated in the development of various pathophysiological changes that occur during aging. One important consequence of oxidative stress is the altered phosphorylation and activation of MAP kinases (Lewis *et al.* 1998, Martindale & Holbrook 2002, Matsuzawa & Ichijo 2005, McCubrey *et al.* 2006).

Mitogen-activated protein kinase (MAPK) signal transduction pathways are well-characterized signaling networks that regulate a variety of cellular processes including gene transcription, protein synthesis, cell cycle, apoptosis, cell differentiation, inflammation, and cytoskeletal rearrangements (Lewis *et al.* 1998, Widmann *et al.* 1999, Kyriakis & Avruch 2001, Edmunds & Mahadevan 2004, Raman *et al.* 2007, Whitmarsh 2007). MAPK signaling pathways can be triggered by a variety of stimuli including growth factors, cytokines, oxidative stress, environmental stress, and toxic chemical insults (Lewis *et al.* 1998, Martindale & Holbrook 2002, Matsuzawa & Ichijo 2005, McCubrey *et al.* 2006). There are three major groups of MAPKs: extracellular signal-regulated kinase (ERK)1/2, p38 ($\alpha/\beta/\gamma/\delta$), and c-Jun N-terminal kinase (JNK1/2/3) (Widmann *et al.* 1999, Kyriakis & Avruch 2001, Raman *et al.* 2007). These serine/threonine kinases are phosphorylated/activated by distinct

upstream dual specificity MAP kinases (MKKs or MEKs), which selectively target ERK (MKK1/2), JNK (MKK4/7), and p38 (MKK3/6) and phosphorylate both threonine and tyrosine in a regulatory T-X-Y motif present in all MAPKs (Widmann *et al.* 1999, Kyriakis & Avruch 2001, Raman *et al.* 2007). Once activated, MAPKs can be translocated to the nucleus and regulate gene expression at transcriptional, translational, and posttranslational levels. Presently it is believed that growth and differentiation factors preferentially activate ERK signaling pathway, whereas the JNK and p38 pathways are more responsive to environmental and physiochemical stresses.

It is well documented that StAR protein (presumably in cooperation with PBRT/TSPO) plays a major role in steroid hormone synthesis by enhancing the translocation of cholesterol substrate from the outer to inner mitochondrial membranes, where the cholesterol side-chain cleavage enzyme, P450_{scc} (CYP11A1), resides (Stocco & Clark 1996, Stocco 2000). The regulation of StAR protein is executed primarily at the transcription level (Manna *et al.* 2003, 2004, Hiroi *et al.* 2004, Clem & Clark 2006, Manna & Stocco 2007), but posttranscriptional (phosphorylation) modification of StAR also plays a significant role in its functional expression (Stocco & Clark 1996). Transcriptional regulations of StAR have been extensively studied, and a cluster of transcription factors is involved in the regulatory process (Manna *et al.* 2003, 2004, Hiroi *et al.* 2004, Clem & Clark 2006, Manna & Stocco 2007). One of these transcription factors is AP-1, a dimeric complex composed primarily of the Fos and Jun family of proteins (Chinenov & Kerppola 2001, Hess *et al.* 2004), which play a critical role in the regulation of StAR gene transcription (Manna *et al.* 2004). Since CRE2 site overlaps with AP-1 site in the proximal StAR promoter region, its activity is also subject to regulation via the crosstalk between CREB and Fos/Jun proteins (Manna *et al.* 2004, Manna & Stocco 2007). Interestingly, studies on the transcriptional regulation of another cholesterol transport protein, PBR/TSPO (which is believed to work in concert with StAR (Miller 2007, Papadopoulos *et al.* 2007)), have also identified an AP-1 binding site in its proximal promoter region (Giatzakis *et al.* 2007). Given the oxidant-sensitive nature of AP-1 (Dalton *et al.* 1999, Klaunig & Kamendulis 2004), involvement of stress-activated kinases, the MAPKs, in the regulation of AP-1 (Whitmarsh & Davis 1996, Pramanik *et al.* 2003, Tanos *et al.* 2005, Humar *et al.* 2007, Whitmarsh 2007) and the fact that functional expression of AP-1 is substantially reduced (Medicherla *et al.* 2001), while p38 MAPK is specifically activated in intact rat adrenal glands (Abidi *et al.* 2008) and Leydig cells (unpublished observations) during aging, it seems likely that oxidant stressors acting on p38 MAPK and AP-1 contribute to the age-related decline in StAR (and PBR/TSPO) expression and subsequently loss of steroidogenic function.

The present studies were initiated to further expand on the observations that p38 MAPK is specifically activated in intact rat adrenal glands (Abidi *et al.* 2008) and testicular Leydig cells

(unpublished observations) during aging and to critically evaluate whether excessive oxidative stress-induced inhibition of steroid synthesis is dependent upon activation of p38 MAPK. These studies were conducted using mouse hormone- and lipoprotein-sensitive adrenocortical cell line Y1-BS1 as an *in vitro* adrenal model system. Our data indicate that exposure of cells to oxidants leads to a significant reduction in hormone-stimulated steroid secretion, providing a direct demonstration that oxidative stress negatively impacts steroidogenesis. We also present data showing that both pharmacological and molecular inhibitors of p38 MAPK attenuate oxidant-dependent inhibition of steroid secretion, demonstrating that oxidative stress-mediated suppression of steroidogenesis is mediated through activation of p38 MAPK. These results define a novel mechanism for oxidative stress-mediated inhibition of steroid synthesis and identify p38 MAPK activation as a signaling pathway mediating oxidative stress-induced inhibition of steroidogenesis.

Materials and Methods

Reagents and antibodies

The following chemicals were purchased from Calbiochem (La Jolla, CA, USA): MEK/ERK inhibitors PD098059 and U0126, the p38 MAP kinase inhibitors SB203580, SB202190, and SB202474 (inactive analog), the JNK inhibitor SP600125, MnTMPyP (Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride), and MnTBAP (Mn(III)tetrakis(4-benzoic acid)porphyrin). Bt₂cAMP, fatty acid poor BSA, hydrogen peroxide (H₂O₂), *N*-acetylcysteine (NAC), xanthine and xanthine oxidase were supplied by Sigma-Aldrich. 4-Hydroxy-2-nonenal (HNE) was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA), and 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (DCFDA) was obtained from Molecular Probes/Invitrogen Corporation. All other reagents used were of analytical grade. Polyclonal antibodies against total ERK1/ERK2, JNK1/JNK2, and p38 MAPK α were purchased from Cell Signaling Technology (Beverly, MA, USA). Phospho-specific antibodies that recognize ERKs phosphorylated at Thr²⁰² and Tyr²⁰⁴, and p38 MAPK phosphorylated at Thr¹⁸⁰ and Tyr¹⁸² were also supplied by Cell Signaling Technology. Phospho-JNKs (Thr¹⁸³ and Tyr¹⁸⁵) antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-myc and anti-FLAG M2 monoclonal antibodies were purchased from Sigma-Aldrich.

Plasmids

Expression vectors of wild-type human FLAG-MKK3, constitutively active human FLAG-MKK3(Glu) (Ser¹⁸⁹ → Glu/Thr¹⁹³ → Glu), dominant-negative human FLAG MKK3(Ala) (Ser¹⁸⁹ → Ala/Thr¹⁹³ → Ala), wild-type human FLAG-MKK6, constitutively active human FLAG

MKK6(Glu) (Ser²⁰⁷ → Glu/Thr²²¹ → Glu), and dominant-negative human FLAG MKK6(K82A) (Lys⁸² → Ala), were generously provided by Dr Roger Davis, University of Massachusetts Medical School, Worcester, MA, USA (Raingeaud *et al.* 1996). Wild-type myc-tagged human MKK7, constitutively active myc-tagged human MKK7(MKK73E) (Ser²⁷¹ → Glu/Thr²⁷⁵ → Glu/Ser²⁷⁷ → Glu), dominant-negative mutant form of MKK7(MKK73A) (Ser²⁷¹ → Ala/Thr²⁷⁵ → Ala/Ser²⁷⁷ → Ala), and dominant-negative (kinase-dead) mutant form of MKK7(MKK7K149M) (Lys¹⁴⁹ → Met) were kindly provided by Dr Michael Kracht, Medical School, Hannover, Germany (Holtmann *et al.* 1999). The cloning of wild-type FLAG-tagged p38 α , p38 β , p38 γ , and p38 δ MAPK constructs have been described previously (Pramanik *et al.* 2003). Dominant-negative mutant forms of FLAG-tagged human p38 α (AF) (Thr¹⁸⁰ → Ala/Tyr¹⁸² → Phe), p38 β (AF) (Thr¹⁸⁸ → Ala/Tyr¹⁹⁰ → Phe), p38 γ (AF) (Thr¹⁸³ → Ala/Tyr¹⁸⁵ → Phe), and p38 δ (AF) (Thr¹⁸⁰ → Ala/Tyr¹⁸² → Phe) were created by substituting threonine and tyrosine with alanine and phenylalanine respectively in TGY motif (Pramanik *et al.* 2003).

Culture and treatment of Y1-BS1 mouse adrenocortical cells with oxidants, and/or antioxidants

The Y1-BS1 mouse adrenocortical cell line was initially obtained from Dr David William's laboratory (State University at Stony Brook, Stony Brook, NY, USA) in 1998. The cells were grown in F-10 medium supplemented with 12.5% horse serum, 2.5% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37 °C under 5% CO₂.

To induce oxidative stress, cells cultured in 60 mm dishes were treated with xanthine (0.25 mM) + xanthine oxidase (20 mU/ml) (to generate a flux of superoxide anion, O₂⁻, and H₂O₂), H₂O₂ (0.05, 0.1, 0.25, 0.5, or 1.0 mM) or a lipid peroxidation product, HNE (10, 20, 50, or 100 µM) for an appropriate time as specified under each figure and table. In other experiments, cells were co-incubated with xanthine (0.25 mM) + xanthine oxidase (20 mU/ml) ± the superoxide-dismutase (SOD)/catalase mimetic MnTMPyP (10 µM), H₂O₂ (0.1 mM) ± MnTMPyP (10 µM), or HNE (50 µM) ± antioxidant NAC (20 mM) for 1 h. Following treatment, cell samples were washed and were either used immediately for the measurement of steroid hormone secretion or stored frozen until analyzed for total and phosphorylated forms of ERK1/ERK2, p38 MAPK, and JNK1/JNK2. Cell viability was assessed by following the conversion of MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan crystals (Mosmann 1983), a reaction dependent upon mitochondrial respiratory chain activity that reflects mitochondrial redox state. After treatment with oxidants and washing, an MTT solution was added to the dishes at a final concentration of 500 µg/ml and the induction continued at 37 °C for 3 h. Following incubation, the medium was aspirated and accumulated formazan product was solubilized

with 1:1 solution of dimethyl sulfoxide/absolute ethanol. Cell viability was determined by the differences in absorbance at wavelength 570 nm minus 690 nm using a microplate reader.

Measurement of ROS

Oxidant-induced intracellular reactive oxygen species (ROS) generation and oxidative stress was monitored by measuring changes in fluorescence resulting from intracellular oxidation of 2',7'-dichlorofluorescein (DCFH) (Keller *et al.* 2004). The probe DCFDA enters the cell, is hydrolyzed by cellular esterases, where upon nonfluorescent DCFH is trapped inside the cell. Subsequent oxidation by ROS yields DCF. Y1-BS1 cells grown in 35 mm dishes were pre-loaded with DCFDA (10 μ M) for 60 min, washed and further incubated with buffer alone (control), xanthine (X, 0.25 mM) + xanthine oxidase (XO, 20 mU/ml), H_2O_2 (100 μ M), or HNE (50 μ M) in the presence of Bt_2cAMP (2.5 mM) for 1 h. At the end of incubation the media were removed and cells were immediately lysed and centrifuged. DCF fluorescence of the cell lysates was measured using a microplate reader with an excitation wavelength of 488 nm and emission wavelength of 515 nm. Results are expressed as percent change from medium-treated controls.

Transfection of Y1-BS1 adrenocortical cells

For transient transfection experiments, Y1-BS1 cells were sub-cultured at a density of 1.5×10^5 cells/dish in 60 mm tissue culture dishes the day prior to transfection. Each plate was transfected with 5 μ g vector alone or various MAPK constructs as noted under specific tables and figures using a FuGENE transfection reagent according to the manufacturer's instructions (Roche Molecular Diagnostics). After 48 h of transfection, cells were utilized for various measurements.

Measurement of steroid secretion by Y1-BS1 mouse adrenocortical cells

To examine hormone-stimulated and lipoprotein-supported steroid production, native Y1-BS1 cells, oxidant/antioxidant-treated Y1-BS1 cells, or Y1-BS1 cells transiently transfected with various MAPK constructs were incubated for 5 h with Bt_2cAMP (2.5 mM) $\pm$ hHDL₃ (500 μ g/ml), and following incubation the media were collected and stored frozen until analyzed. Steroids were extracted from the medium using methylene chloride and quantified by fluorescence in 65% sulfuric acid–35% ethanol using 20 α -dihydroprogesterone as a standard.

To evaluate the ability of MAPKs to modulate hormone-stimulated and lipoprotein-supported steroidogenesis, we utilized the specific MEK/ERK inhibitors PD098059 and U0126, the p38 MAP kinase inhibitors SB203580, SB202190, and SB202474 (inactive analog), and the JNK

inhibitor SP600125. Triplicate dishes of Y1-BS1 cells were pretreated with vehicle alone (control), PD098059 (30 μ M), U0126 (10 μ M), SB203580 (10 μ M), SB202190 (10 μ M), SB202474 (10 μ M), or SP600125 (10 μ M) for 1 h, and incubations continued for an additional 5 h following the addition of $Bt_2cAMP \pm$ hHDL₃ (500 μ g protein/ml). At the end of incubation, the media were collected, frozen, and stored frozen until analyzed for 20 α -dihydroprogesterone levels as described above. Cell viability after treatment was monitored using the MTT assay as described above.

Preparation of lipoproteins

Human low-density lipoprotein (hLDL) and human apoE-free high-density lipoproteins₃ (hHDL₃) were isolated and characterized as previously described (Reaven *et al.* 1990).

Western blot analysis of total and phosphorylated forms of ERKs, p38 MAPK, and JNKs

The Y1-BS1 adrenal cell samples were solubilized with lysis buffer (20 mM HEPES (pH 7.4), 1% Triton X-100 (vol/vol), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 20 mM β -glycerophosphate, 10 mM sodium pyrophosphate, 1 mM sodium vanadate, 10 nM okadaic acid, 1 mM dithiothreitol, 10 μ g/ml leupeptin, 10 μ g/ml pepstatin, 10 μ g/ml aprotinin, 0.5 mM 4-(2-aminoethyl)benzylsulfonyl fluoride, 10 μ M E-64, 50 μ M bestatin, 10 μ g/ml tosyl-L-phenylalanine chloromethyl ketone and 10 mg/ml N ^{α} -tosyl-L-lysine chloromethyl ketone) and incubated for 15 min at 4 °C. Subsequently, each cell lysate was sonicated briefly to disrupt chromatin (DNA), centrifuged at 15 000 *g* for 10 min at 4 °C and protein concentration of each clarified lysate was determined. All cell lysate samples were stored frozen until analyzed by western blotting (Kelley *et al.* 2004).

Samples containing an equal amount of protein (30–40 μ g) were fractionated by SDS-PAGE (10% polyacrylamide gel with 4% stacking gel) and transferred to Immobilon polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA, USA). After transfer, the membrane was washed in TBS containing 0.1% Tween 20 (TBS) and incubated in blocking buffer (TTBS containing 5% nonfat dry milk) for 90 min at room temperature, followed by overnight incubation at 4 °C with the primary antibody in blocking buffer (against total or phosphorylated forms of ERK1/ERK2, p38 MAPK, or JNK1/JNK2, or anti-FLAG monoclonal antibody). Subsequently, the membrane was washed in TTBS and incubated for 2 h with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG in blocking buffer. The immunoreactive bands were then visualized using a LumiGLO Chemiluminescent Detection System (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD, USA) followed by exposure to X-ray film (10–30 min) and quantified by Fluor-S-MultiImager scanning densitometry system (Bio-Rad).

Determination of p38 MAPK activity by western blotting

The activity of p38 MAPK was determined using a kit supplied by Cell Signaling Technology (catalog #9820). Briefly, cell lysates were incubated cold overnight with an immobilized primary phospho-p38 MAPK (Thr¹⁸⁰/Tyr¹⁸²) monoclonal primary antibody to selectively immunoprecipitate active p38 MAPK. Subsequently, immobilized immunoprecipitates were incubated in a buffer supplemented with ATP (200 µM) and ATF-2 fusion protein. The reactions were terminated by the addition of a 3× SDS sample buffer and subjected to SDS-PAGE followed by western blotting. The blots were developed with a polyclonal phospho-ATF-2 (Thr⁷¹) antibody and HRP-linked anti-rabbit IgG and if necessary, signals were quantified by the Fluor-S-MultiImager scanning densitometry system (Bio-Rad).

RNA isolation

Total RNA was isolated from young and old Leydig cells (pre-incubated with or without 10 ng/ml hCG) using TRIzol (Invitrogen Life Technologies) according to the manufacturer's protocol. The purity and concentration of RNA samples were determined by following absorbance (A₂₆₀/A₂₈₀) ratios. The integrity of the purified RNA samples was confirmed by 1.2% formaldehyde-agarose gel electrophoresis.

Measurement of mRNA levels by quantitative PCR (qPCR)

The specific primer sets used to detect the mRNA expression of p38 MAPKα, p38 MAPKβ, p38 MAPKγ and p38 MAPKδ were developed using Primer Express software (Applied Biosystems, Foster City, CA, USA) according to the recommended guidelines based on the sequences accessed through GenBank. Table 1 shows the primer sequences for qPCR assay used. First-strand cDNA was synthesized from 2 µg total RNA by incubating for 1 h at 50 °C with SuperScript II reverse transcriptase (Invitrogen Life Technologies) and random primers. Amplification of cDNAs was performed with an ABI Prism 7900 system according to the manufacturer's instructions. Each sample consisted of 1 µl cDNA, 4 mM MgCl₂, 0.2 mM deoxynucleoside triphosphate, 500 nM of each sense and antisense primer, 2 µl 10× PCR buffer, TaqMan polymerase, and SYBR Green master mix in a final volume of 20 µl. As an internal quantitative

control for gene expression, ribosomal protein 36B4 gene expression was also determined. Real-time PCR was performed with an ABI Prism. The relative mass of p38 MAPKα, p38 MAPKβ, p38 MAPKγ, p38 MAPKδ, or 36B4 mRNA was calculated by the comparative cycle of threshold detection method according to the manufacturer's instruction. Three independent sets of Taqman real-time PCR were performed using different RNA preparations; each run of Taqman real-time PCR was conducted in triplicate. The final data were normalized to 36B4 and the ratios of p38 MAPKα, p38 MAPKβ, p38 MAPKγ and p38 MAPKδ to 36B4 represented the normalized relative levels of each mRNA.

Analytical procedures

Protein was measured with a bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, IL, USA). The procedure of Markwell *et al.* (1978) was used to quantify the protein content of hHDL₃ and hLDL. Cholesterol content of hHDL₃ and hLDL was determined according to the procedure of Tercyak (1991).

Statistical analysis

All results are expressed as mean ± S.E.M. Data were analyzed using a nonpaired two-tailed Student's *t*-test using GraphPad Prism version 3.00 software for Windows (GraphPad Software, San Diego, CA, USA). Differences between groups was considered significant if *P* < 0.05.

Results

To investigate the molecular basis of the potential interaction between oxidative stress, p38 MAPK activation, and loss of steroidogenesis, we employed Y1-BS1 cells, a stable subclone of the Y1 mouse adrenocortical tumor cell line (Schimmer 1979). The Y1-BS1 cell line provides a useful *in vitro* model system for studying hormone-stimulated and lipoprotein-supported steroidogenesis (Faust *et al.* 1977, Rae *et al.* 1979, Temel *et al.* 1997). Unlike normal rodent adrenal cells that secrete corticosterone, Y1-BS1 cells chiefly secrete 20α-dihydroprogesterone and 11β-hydroxy-20α-dihydroprogesterone.

Table 1 Mouse-specific primer pairs used for quantitative PCR (qPCR)

	Forward primer	Reverse primer
Gene		
p38α	5'-CCCCAGAGATCATGCTGAAT-3'	5'-ACAACGTTCTTCCGGTCAAC-3'
p38β	5'-ATGTAGCGGTGAACGAGGAC-3'	5'-CCACATATCCGGTCATCTCC-3'
p38γ	5'-GAGACTTGAAGCCTGGCAAC-3'	5'-CCGGGTACCACATATCCTG-3'
p38δ	5'-CGCCACACAGACACTGAGAT-3'	5'-ATGATGCAACCAACAGACCA-3'
36B4	5'-CACTGGTCTAGGACCCGAGAAG-3'	5'-GGTGCTCTGGAGATTTCG-3'

Effect of various additives on steroid production by Y1-BS1 cells

To examine the effects of hormones and exogenous cholesterol on steroid production, Y1-BS1 cells were incubated without (basal) or with adrenocorticotrophin (ACTH) or its second messenger analog, $\text{Bt}_2\text{cAMP} \pm \text{LDL}$ or HDL for 5 h before harvesting aliquots of medium for quantification of the 20α -dihydroprogesterone content. As expected, both ACTH and Bt_2cAMP treatment increased steroid production by ~ 10 - to 11-fold (Fig. 1). Incubation of Y1-BS1 cells with either hLDL or hHDL also significantly increased the production of 20α -dihydroprogesterone. Steroid production further enhanced after treatment with ACTH or Bt_2cAMP + lipoprotein (hLDL or hHDL) (Fig. 1).

Oxidant-mediated inhibition of steroidogenesis

Next, we examined the inhibitory actions of enhanced oxidative stress on steroidogenesis. In the first set of experiments, we studied the effects of three oxidants, superoxide anion ($\text{O}_2^{\cdot -}$) (Englert & Shacter 2002), H_2O_2 (Nair *et al.* 2004), and HNE (Uchida *et al.* 1999) on steroid production in response to Bt_2cAMP or Bt_2cAMP + hHDL₃. HNE, an end product of the lipid peroxidation of membrane-associated polyunsaturated fatty acids, exhibits high reactivity towards various macromolecules (Uchida 2000, Esterbauer *et al.* 1991). The results in Fig. 2 show that exposure of Y1-BS1 cells to $\text{O}_2^{\cdot -}$ generated from xanthine oxidase (XO) and xanthine (X) significantly inhibited both Bt_2cAMP and Bt_2cAMP + hHDL₃-stimulated steroid synthesis. Likewise, treatment of cells with either H_2O_2 or HNE, an end product of lipid peroxidation (Uchida *et al.* 1999) also caused a reduction in 20α -dihydroprogesterone production in response to both Bt_2cAMP alone and Bt_2cAMP + hHDL₃

(Fig. 2). To ensure that the oxidants used to induce oxidative stress were indeed causing such an effect, simultaneous measurements of ROS production using DCFDA were performed during oxidant-induced oxidative stress. As shown in Fig. 3, the extent of DCF oxidation was significantly increased in cells treated with H_2O_2 or HNE. Relatively, very little fluorescence was noted in cells exposed to X/XO (superoxide anion, $\text{O}_2^{\cdot -}$); this is most likely due to the fact that $\text{O}_2^{\cdot -}$ reacts very poorly with the dye and the observed fluorescence represents only a fraction of the $\text{O}_2^{\cdot -}$ that reacted with NO to form peroxynitrite (ONOO^-) or have been dismutated to H_2O_2 by cellular superoxide dismutases (SODs) (Keller *et al.* 2002). To rule out the possibility that oxidant-induced inhibition of steroidogenesis was due to increased cell death, cell viability was measured under identical experimental conditions using a sensitive MTT assay (Mosmann 1983). None of these three oxidants, however, had any significant effect on the cell viability, which remained in the range of 85–90% (data not shown).

To examine whether oxidants directly mediate inhibition of steroidogenesis, we measured 20α -dihydroprogesterone production in the presence of ROS scavengers, manganese (III) tetrakis(1-methyl-4-pyridyl) porphyrin (MnTMPyP), and *N*-acetyl cysteine (NAC). MnTMPyP functions as a cell-permeable SOD/catalase mimetic (Pimental *et al.* 2001), while antioxidant NAC is the precursor of cellular glutathione (Jonassen *et al.* 1999) and is known to interfere with HNE action (Uchida *et al.* 2004). As shown in Fig. 2, pretreatment of Y1-BS1 cells with MnTMPyP attenuated the X/XO- or H_2O_2 -induced inhibition of steroid production. To address the possibility that MnTMPyP prevented the inhibition of steroidogenesis via a nonspecific effect, control experiments were conducted with light-inactivated, Mn(III)tetrakis (4-benzoic acid) porphyrin (MnTBAP). As expected,

Figure 1 ACTH- or Bt_2cAMP -stimulated and lipoprotein-supported steroid production by the Y1-BS1 mouse adrenocortical tumor cells. Results are mean $\pm$ S.E.M. of three independent experiments. Y1-BS1 mouse adrenocortical tumor cells were incubated in a serum-free medium in the absence or presence of ACTH (10 ng/ml), Bt_2cAMP (2.5 mM), hHDL₃ (500 μg protein/ml), hLDL (100 μg protein/ml), or both ACTH (10 ng/ml) or Bt_2cAMP (2.5 mM), and hHDL₃ (500 μg protein/ml) or hLDL (100 μg protein/ml) at 37 °C for 5 h. Following incubation, a suitable aliquot of the medium from each sample was collected and steroids were extracted from the medium using methylene chloride and quantified by fluorescence in 65% sulfuric acid–35% ethanol using 20α -dihydroprogesterone as standard.

Figure 2 Effect of the ROS scavengers MnTMPyP and NAC on oxidant-mediated inhibition of adrenocortical cell steroidogenesis. Results are mean $\pm$ S.E.M. of four independent experiments. (A) Y1-BS1 cells cultured in 60 mm dishes were treated with xanthine (0.25 mM) + xanthine oxidase (20 mU/ml) (to generate a flux of superoxide anion, $O_2^{\cdot-}$, and H_2O_2) $\pm$ the superoxide-dismutase (SOD)/catalase mimetic MnTMPyP (10 μ M), H_2O_2 (0.1 mM) $\pm$ MnTMPyP (10 μ M), or a lipid peroxidation product, HNE (50 μ M) $\pm$ antioxidant NAC (20 mM) for 1 h. Following treatment, dishes were rapidly washed and incubated with Bt_2cAMP (2.5 mM) $\pm$ hHDL₃ (500 μ g/ml) for 5 h. (B) Following incubation, media was collected and stored frozen until analyzed. Steroids were extracted from the medium using methylene chloride and quantified by fluorescence in 65% sulfuric acid–35% ethanol using 20 α -dihydroprogesterone as a standard. The basal amount of 20 α -dihydroprogesterone produced was 32.5 ± 4.6 ng/mg cell protein/5 h.

X/XO- or H_2O_2 -induced inhibition in 20 α -dihydroprogesterone production was unaffected in cells pretreated with light-inactivated MnTBAP (10 μ M; data not shown). The results of these experiments suggest that blockade of X/XO- or H_2O_2 -induced inhibition of steroidogenesis is not due to a nonspecific effect of MnTMPyP, but occurs in response to the scavenging of superoxide and H_2O_2 and suggest that these oxidants directly impair the steroidogenic function. In a similar manner, to investigate whether NAC could prevent an HNE-induced reduction in steroid formation, cells were co-incubated with NAC $\pm$ HNE. Addition of NAC to the incubation medium also effectively

Figure 3 Oxidant-induced reactive oxygen species (ROS) production and oxidative stress in Y1-BS1 cells. Triplicate dishes of cultured Y1-BS1 adrenal cells were pre-loaded with 10 mM 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (DCFDA) for 60 min. Subsequently, dishes were washed and incubated with *in situ* generated superoxide from xanthine (0.25 mM) + xanthine oxidase (20 mU/ml) (*in situ* generated superoxide), H_2O_2 (100 μ M) or HNE (50 μ M) for 1 h. At the end of incubation, the media were removed and cells were immediately lysed and centrifuged. DCF fluorescence of the cell lysates was measured using a microplate reader with excitation and emission wavelengths of 488 and 515 nm respectively.

blocked the HNE-induced suppression of steroidogenesis, further confirming a direct inhibitory action of HNE (Fig. 2). Since steroidogenic tissues are constantly exposed to ROS as byproducts of both oxidative phosphorylation and steroid biosynthetic pathways, we also incubated normal Y1-BS1 cells with these two antioxidants to determine whether such manipulations would also impact steroidogenesis in a positive manner. Upon incubation of Y1-BS1 cells with MnTMPyP or NAC, steroid synthesis in response to either Bt_2cAMP alone or in the presence of HDL₃ was significantly upregulated indicating inhibition of steroidogenesis by the endogenously produced ROS (Table 2). Overall, these results

Table 2 Effect of reactive oxygen species (ROS) scavengers on steroid hormone synthesis and secretion by the mouse Y1-BS1 mouse adrenocortical cells. Results are mean $\pm$ S.E.M. of four independent experiments. Y1-BS1 mouse adrenocortical tumor cells were incubated in a serum-free medium $\pm$ hHDL₃ (500 μ g protein/ml), $\pm$ Bt_2cAMP (2.5 mM), $\pm$ MnTMPyP (10 μ M), or $\pm$ NAC (20 mM) at 37 $^{\circ}$ C for 5 h. Following incubation, medium was collected separately from each dish and stored frozen until analyzed. Steroids were extracted from the medium using methylene chloride and quantified by fluorescence in 65% sulfuric acid–35% ethanol using 20 α -dihydroprogesterone as a standard. The cell lysates were analyzed for protein content

	20 α -dihydroprogesterone (ng/mg cell protein/5 h $\pm$ S.E.M.)		
	Control	MnTMPyP (10 mM)	NAC (20 mM)
Additions			
None	27.5 $\pm$ 4.8	26.0 $\pm$ 6.7	23.3 $\pm$ 4.3
hHDL ₃ (500 mg/ml)	56.3 $\pm$ 5.8	66.8 $\pm$ 9.4	68.3 $\pm$ 9.3
Bt_2cAMP (2.5 mM)	694 $\pm$ 51	1023 $\pm$ 105*	1094 $\pm$ 121 [†]
Bt_2cAMP + hHDL ₃	1673 $\pm$ 154	2663 $\pm$ 310 [‡]	2463 $\pm$ 276 [§]

* $P=0.0306$; [†] $P=0.0229$; [‡] $P=0.0416$; [§] $P=0.0467$.

suggest that steroidogenic pathway is highly sensitive to and negatively impacted by ROS possibly through enhanced lipid peroxidation and oxidative damage to membranes crucial for cholesterol to mitochondria or other events connected with the steroidogenesis.

Effects of superoxide- and HNE-induced oxidative stress on the activation state of MAPKs

Enhanced oxidative stress is known to activate multiple MAPK signaling cascades in many cellular systems (Finkel & Holbrook 2000, Martindale & Holbrook 2002, Matsuzawa & Ichijo 2005, McCubrey *et al.* 2006). To identify the MAPK signaling pathways activated in Y1-BS1 cells in response to superoxide, H_2O_2 , and HNE, we assessed the activation of ERK1/2, JNK1/2, and p38 MAPK using western blot analysis with antibodies that recognize either the phosphorylated or total content of each of the three MAPKs. Figure 4 shows total and phosphorylated forms of MAPKs assayed in cell extracts from Y1-BS1 cells pretreated with or without oxidants (X/XO or HNE). In control (vehicle-treated) cells, significant expression of the phosphorylated form of ERK1/2 and p38 MAPK was detected but very little of JNK1 (JNK-46) and JNK2 (JNK-54). The XO/X-generated superoxide treatment led to a marked increase in

Figure 4 Phosphorylation of ERK1/2, JNK1/2 and p38 MAPK by oxidants in cultured Y1-BS1 cells. Triplicate dishes of cultured Y1-BS1 adrenal cells were incubated with xanthine (0.25 mM) + xanthine oxidase (20 mU/ml) (to generate superoxide *in situ*), or HNE (50 μ M) for 1 h. Equal aliquots of solubilized samples (30–40 μ g protein) were subjected to SDS-PAGE and immunoblotted with antibodies against ERK1/2 phosphorylated at Thr²⁰²/Tyr²⁰⁴ (P-ERK1/2), against p38 MAPK phosphorylated at Thr¹⁸⁰/Tyr¹⁸² (P-p38 MAPK), or JNK1/2 phosphorylated at Thr¹⁸³/Tyr¹⁸⁵ (P-JNK1/2). The blots were stripped and re-probed using the antibodies that recognize total (phospho- plus non-phospho forms) ERK1/2, p38 α MAPK, and JNK1/2 proteins. The blots shown represent three independent experiments. Numerical results were obtained by densitometric scanning of the individual blots.

the amount of the phosphorylated form of p38 MAPK, but not of phosphorylated ERK1/2 and JNK1/2. Superoxide and HNE also increased the levels of phospho-p38 MAPK. Although, HNE treatment somewhat enhanced the phosphorylation of ERK1/2 and JNK1 (JNK-46) and JNK2 (JNK-54), the degree of their phosphorylation was several fold lower than that seen with p38 MAPK. Interestingly, the total amount (protein level) of p38 MAPK remained constant following treatment of cells with superoxide, or HNE for up to 1 h (Fig. 4), suggesting that oxidant effects on p38 MAPK are due to changes in the phosphorylation (activation) status of this kinase.

Kinetic dose-response studies were carried out to further establish the stimulatory actions of these three oxidants on p38 MAPK phosphorylation and the results are presented in Fig. 5A–C. The activation of p38 MAPK by XO/X generated superoxide was rapid (10 min) and sustained (Fig. 5A). Dose-response studies demonstrated that H_2O_2 significantly increased the phosphorylation of p38 MAPK (approximately fivefold) at 0.1 mM reaching a maximum around 0.25 mM (Fig. 5C). Likewise, the stimulatory action of HNE also showed concentration dependence with a maximal effect observed around 50 μ M (Fig. 5B).

Involvement of p38 MAPK during oxidant-mediated inhibition of steroidogenesis

Data shown in Figs 4 and 5 suggest possible involvement of the p38 MAPK signaling cascade in oxidant-induced loss of

Figure 5 Kinetics and dose-response effects of superoxide, H_2O_2 , and HNE on p38 MAPK activation. Y1-BS1 adrenal cells were treated with (A) xanthine (0.25 mM) + xanthine oxidase (20 mU/ml) for varying incubation time (10, 20, 30 or 60 min), (B) 0, 10, 20, 50, or 100 μ M HNE for 1 h and (C) 0, 0.1, 0.25, 0.5, or 1 mM H_2O_2 for 1 h. The cell lysates were analyzed by immunoblotting with antibodies against phosphorylated or total p38 MAPK (Fig. 4). Data shown are representative of two independent experiments.

adrenal steroid hormone production. We next investigated the potential relationship between oxidative stress-induced activation of p38 MAPK and the ability of excessive oxidative stress to inhibit hormone-stimulated and lipoprotein-supported steroid synthesis. One way to determine the involvement of p38 MAPK in mediating the inhibitory actions of oxidative stress on adrenal steroidogenesis is to examine the effects of two specific and cell-permeable chemical inhibitors of p38 MAPK, SB203580, and SB202190 (Lee *et al.* 1994, Cueda *et al.* 1995). Treatment of Y1-BS1 adrenal cells with a maximal effective dose of SB203580 (10 μ M) potentiated the stimulatory action effects of Bt₂cAMP and Bt₂cAMP + hHDL₃ (Fig. 6). The effect of SB202190 (10 μ M), a more potent inhibitor of p38 MAPK, was also examined in the face of Bt₂cAMP or Bt₂cAMP + hHDL₃-stimulated steroid production. In agreement with the effect of SB203580, SB202190 also increased the steroid production over that observed with Bt₂cAMP alone or Bt₂cAMP + hHDL₃ (Fig. 6). By contrast, SB202474, the biologically inactive inhibitor of p38 MAPK had no effect on steroidogenesis. To determine the potential involvement of ERK1/2 and JNK1/2 in the regulation of adrenal steroidogenesis, two MEK 1/2 inhibitors PD98059 (Alessi *et al.* 1995) and U0126 (Favata *et al.* 1998), and SP600125 a JNK1/2 inhibitor (Bennett *et al.* 2001) were utilized. (MEK1/2 are upstream kinases that activate ERKs). Treatments with either PD98059 (30 μ M), U0126 (10 μ M), inactive analog U0124 (10 μ M), or SP600125 were ineffective in upregulating Bt₂cAMP or Bt₂cAMP plus lipoprotein-stimulated steroid production (Fig. 6). None of the various inhibitors had any significant effect on the overall viability of the treated cells.

As shown above, treatment of Y1-BS1 cells with oxidants leads to increased stimulation of p38 MAPK activity, but at the same time inhibits hormone- and/or lipoprotein-supported steroid production (Figs 4 and 5). To investigate whether inhibition of the p38 MAPK cascade contributed to the protective effect conferred by ROS scavengers and p38 MAPK inhibitors in response to oxidant treatment, the levels of phospho-p38 MAPK were examined by western blot analysis. As described above, the level of phospho-p38 MAPK was enhanced in cell extracts prepared from superoxide (X/XO)- or HNE-treated Y1-BS1 cells. However, pretreatment with MnTMPyP and NAC completely suppressed the superoxide- and HNE-induced increase in the levels of phosphorylation of p38 MAPK respectively (data not shown). Likewise, use of p38 MAPK inhibitors completely prevented the superoxide- or HNE-induced enhanced phosphorylation of p38 MAPK. We, therefore, investigated the effect of p38 MAPK inhibition on superoxide- and HNE-inhibited steroidogenesis. In agreement with previous results, treatment with X/XO or HNE alone caused a significant reduction in Bt₂cAMP + hHDL₃-stimulated 20 α -dihydroprogesterone production (Fig. 7). Treatment of cells with either X/XO or HNE in the presence of SB203580 or SB202190 not only attenuated but restored steroidogenic function to almost control level (Fig. 7). Under identical experimental

Figure 6 Effect of MAPK signaling pathway inhibitors on (A) Bt₂cAMP and (B) Bt₂cAMP + hHDL₃-stimulated 20 α -dihydroprogesterone production. Y1-BS1 cells were incubated for 5 h in culture medium supplemented with Bt₂cAMP (2.5 mM) or Bt₂cAMP + hHDL₃ (500 μ g/ml) $\pm$ SB203580 (10 μ M), SB202190 (10 μ M), SB202474 (10 μ M), PD98059 (30 μ M), U0126 (10 μ M) or SP600125 (10 μ M). At the end of incubation, medium samples were quantified for the production of 20 α -dihydroprogesterone. In some cases, cell viability was determined using a sensitive MTT assay as described under the experimental section. The results are mean $\pm$ S.E.M. of four independent experiments. The amount of 20 α -dihydroprogesterone produced under basal condition was 26.5 ± 3.7 ng/mg cell protein/5 h. $P < 0.005$, Bt₂cAMP vs Bt₂cAMP + SB203580; $P < 0.02$, Bt₂cAMP + hHDL₃ versus Bt₂cAMP + hHDL₃ + SB203580; $P < 0.02$, Bt₂cAMP versus Bt₂cAMP + SB202190; $P < 0.01$, Bt₂cAMP + hHDL₃ versus Bt₂cAMP + hHDL₃ + SB202190.

conditions, both compounds also prevented oxidant-stimulated increases in p38 MAPK as indicated by inhibition of p38 MAPK-catalyzed threonine phosphorylation of ATF2 (data not shown).

Since SB203580 and SB202190 inhibit only p38 MAPK α / β isoforms, it raised the possibility that antisteroidogenic actions of excessive oxidative stress is primarily mediated by either p38 MAPK α , p38 MAPK β or both. To address this issue, qPCR was used to assess endogenous mRNA levels of p38 MAPK α , p38 MAPK β , p38 MAPK γ and p38 MAPK δ isoforms. The results presented in Fig. 8 indicate that Y1-BS1 cells predominantly

Figure 7 Effect of p38 MAPK inhibitors on superoxide or HNE inhibited steroid production. Results are mean $\pm$ s.e.m. of three independent experiments. Y1-BS1 cells cultured in 60 mm dishes were treated with xanthine (0.25 mM) + xanthine oxidase (20 mU/ml) (to generate a flux of superoxide anion, O_2^-) or HNE (50 μ M) $\pm$ SB203580 or SB202190 (10 μ M) for 1 h. Following treatment, dishes were rapidly washed and incubated with Bt_2 -cAMP (2.5 mM) $\pm$ hHDL₃ (500 μ g/ml) $\pm$ respective SB203580 or SB202190 (10 μ M) for 5 h. Following incubation, the incubation media was collected and stored frozen until analyzed. Steroids were extracted from the medium using methylene chloride and quantified by fluorescence in 65% sulfuric acid–35% ethanol using 20 α -dihydroprogesterone as a standard. The amount of 20 α -dihydroprogesterone produced under basal condition was 22.9 ± 4.0 ng/mg cell protein/5 h. (A) $P < 0.002$, Control versus X/XO; $P < 0.00$, control versus HNE; $P < 0.007$, control vs SB203580; $P < 0.01$, control versus X/XO + SB203580; $P < 0.01$, control vs HNE + SB203580. (B) $P < 0.0005$, Control versus X/XO; $P < 0.0008$, control vs HNE; $P < 0.003$, control vs SB202190; $P < 0.01$, control versus X/XO + SB202190; $P < 0.03$, control versus HNE + SB202190.

express p38 MAPK α isoform along with only low levels of p38 MAPK γ . By contrast, little or no expression of p38 MAPK β and p38 MAPK δ isoforms was noted. These results, thus, strongly support the possibility that p38 MAPK α is the predominant, if not the sole mediator of oxidative stress inhibition of steroid synthesis.

To further substantiate the above findings, we also employed various dominant-negative mutant forms of p38 MAPKs and consecutively active and dominant-negative MKK3/MKK6 constructs to genetically alter the function of p38 MAPK. MKK3 and MKK6 are upstream kinases that

Figure 8 p38 MAPK α , p38 MAPK β , p38MAPK γ and p38 MAPK δ mRNA levels in cultured Y1-BS1 cells. Total cellular RNA was extracted, cDNA synthesized, and aliquots of cDNA were used as template reactions containing either primers for p38 MAPK α , p38 MAPK β , p38MAPK γ , p38 MAPK δ or 36B4. Real-time PCR was performed with an ABI Prism 7900 System using SYBR Green master mix reagent and specific primers as listed in Table 1. Data are presented as ratio of p38 MAPK genes normalized to 36B4. Results are mean $\pm$ s.e.m. of three independent experiments performed in triplicates.

phosphorylate and activate p38 MAPK isoforms (Jiang *et al.* 1997, Enslen *et al.* 1998). Initially, Y1-BS1 cells were transiently transfected with FLAG- or c-myc-tagged dominant-negative(dn) dnMKK3, dnMKK6, dnMKK7, and dnMKK7, constitutively active (ca) caMKK3, caMKK6, or caMKK7 constructs and were subsequently evaluated for their steroidogenic potential. As a negative control, cells were transfected with an empty vector (pcDNA3.1). The immunoblot analysis of cell lysates prepared from transfected cells confirmed a robust expression of the respective MKK proteins (data not shown). Moreover, transfection of Y1-BS1 cells with a dn-MKK3 (Ala) plasmid construct increased steroid production by $\sim 50\%$ (Table 3). Expression of dnMKK6(K82A) also enhanced steroid synthesis in the range of 50–60%. Conversely, introduction of either caMKK3 (Glu) or caMkk6(Glu) constructs reduced the 20 α -dihydroprogesterone production to approximately half as compared with vector control (Table 3). By contrast, expression of either a constitutively active (caMKK7(73E)) or a dominant-negative form of MKK7 (dnMKK7(73A) or dnMKK7(7K149M)), an upstream activator of JNKs (Tournier *et al.* 1999), had no effect on the levels of steroid produced (Table 3). These results clearly establish that p38 MAPK acts as a negative modulator of steroidogenesis.

We next sought to define the contribution of individual p38 MAPK isoforms that may mediate the inhibition of steroidogenesis. Presently, there are four known isoforms of

Table 3 Overexpression of dominant-negative MKK3 and MKK6 upregulate while constitutively active MKK3 and MKK6 suppress steroidogenic response in Y1-BS1 mouse adrenocortical tumor cells. Results are expressed as mean $\pm$ s.e.m. of four independent experiments. Y1-BS1 cells were transiently transfected with an empty vector (control) or expression vectors for dnMKK3(Ala), caMKK3(Glu), dnMKK6(K82A), caMKK6(Glu), dnMKK7(73A), or caMKK7(73E) for 48 h. Bt_2cAMP (2.5 mM) + hHDL_3 ($500 \mu\text{g}$ protein/ml) were then added, and the incubation continued for another 5 h. At the end of incubation, the medium samples were collected and stored frozen until analyzed for steroid content. Steroids were extracted from the medium using methylene chloride and quantified by fluorescence in 65% sulfuric acid–35% ethanol using 20α -dihydroprogesterone as a standard. The cells were lysed and cell lysates were used for protein determination and immunoblotting to detect the expression of myc- or FLAG-tagged proteins

Expression constructs	20α -dihydroprogesterone (ng/mg cell protein/ 5 h $\pm$ s.e.m.)	P
Control (vector DNA alone)	8327 $\pm$ 1167	
dnMKK3(Ala)	12 210 $\pm$ 657	0.0275
caMKK3(Glu)	4889 $\pm$ 723	0.0463
dnMKK6(K82A)	13 760 $\pm$ 1167	0.0148
caMKK6(Glu)	4095 $\pm$ 502	0.0158
dnMKK7(73A)	9112 $\pm$ 1138	NS
dnMKK7(7K149M)	8891 $\pm$ 1037	NS
caMKK7(73E)	8022 $\pm$ 1285	NS

ca, catalytically active; dn, dominant negative.

p38 MAPK identified as p38 MAPK α , p38 MAPK β , p38 MAPK γ and p38 MAPK δ (Jiang *et al.* 1997, Enslen *et al.* 1998). To test their relative actions on steroidogenesis, we transfected Y1-BS1 cells with the dominant-negative (AF) constructs of each of these isoforms and subsequently measured steroid production. The dnp38 MAPK α (AF) and dnp38 MAPK β (AF) significantly increased steroidogenic function compared to vector transfected cells (Table 4). The other two dominant-negative mutants, dnp38 MAPK γ (AF) and dnp38 MAPK δ (AF) also slightly, but reproducibly, increased steroid production, although the data did not achieve statistical significance. From these detailed studies, we conclude that excessive oxidative stress-induced inhibition of steroidogenesis requires the activation of and is mediated by p38 MAPK.

Discussion

Recently we have shown that p38 MAPK is specifically activated in intact rat adrenals during aging and that this activation is linked to age-induced excessive oxidative stress and subsequent loss of steroidogenic response (Abidi *et al.* 2008). This study provides further evidence that oxidant-induced inhibition of adrenal steroidogenesis requires participation of p38 MAPK and that pharmacological and genetic inhibition of p38 MAPK activity prevents the

Table 4 Overexpression of dominant-negative p38 mitogen-activated protein kinase (MAPK) α and p38 MAPK β upregulate steroidogenic response in Y1-BS1 mouse adrenocortical tumor cells. Results are expressed as mean $\pm$ s.e.m. of four independent experiments. Y1-BS1 cells were transiently transfected with an empty vector (control) or expression vectors for dnp38 MAPK α , dnp38 MAPK β , dnp38 MAPK γ , or dnp38 MAPK δ for 48 h. Bt_2cAMP (2.5 mM) + hHDL_3 ($500 \mu\text{g}$ protein/ml) were then added and the incubation continued for another 5 h. At the end of incubation, the medium samples were collected and stored frozen until analyzed for steroid content. Steroids were extracted from the medium using methylene chloride and quantified by fluorescence in 65% sulfuric acid–35% ethanol using 20α -dihydroprogesterone as a standard. The cells were lysed and cell lysates used for protein determination and immunoblotting of FLAG-tagged p38 MAPK proteins

Expression constructs	20α -dihydroprogesterone (ng/mg cell protein/ 5 h $\pm$ s.e.m.)	P
Control (vector DNA alone)	9775 $\pm$ 883	
dnp38 MAPK α (AF)	14 580 $\pm$ 1276	0.0212
dnp38 MAPK β (AF)	15 380 $\pm$ 1709	0.0268
dnp38 MAPK γ (AF)	11 810 $\pm$ 812	NS
dnp38 MAPK δ (AF)	11 470 $\pm$ 1598	NS

dn, dominant negative.

oxidant-induced loss of steroid hormone production. This is the first demonstration of involvement of p38 MAPK in the negative regulation of steroidogenesis.

Initially our efforts were directed at determining the functional consequences of a potential interaction between oxidative stress and p38 MAPK using oxidant-treated mouse adrenocortical Y1-BS1 cells as an *in vitro* cell model system. Three different oxidants, namely superoxide, H_2O_2 , and HNE were selected on the basis that superoxide anion and H_2O_2 are the main oxidants generated as a byproduct of the oxidative phosphorylation and other cellular oxidative pathways (Azhar *et al.* 1995, Finkel & Holbrook 2000), while aldehydic product HNE is produced during lipid peroxidation of membrane polyunsaturated fatty acids (Esterbauer *et al.* 1991). Our results indicate that treatment of cells with any of the three types of oxidants caused a similar but significant reduction in steroidogenesis. Moreover, inhibitory effects of both superoxide anion and H_2O_2 were completely attenuated when cell incubations were conducted in the presence of MnTMPyP, a cell-permeable SOD/catalase mimetic (Pimental *et al.* 2001). Likewise, blockade of the HNE effect was observed when the cells were simultaneously exposed to the antioxidant NAC. These results suggest that superoxide anion, H_2O_2 , and HNE specifically inhibit steroid hormone production and that such oxidants are likely to be involved in the molecular mechanisms underlying the age-related decline in adrenal steroidogenesis. Furthermore, the fact that all three oxidants were equally effective in inhibiting steroid synthesis implies that they either act in concert or in a sequential manner to interfere with the steroidogenic process.

While relatively less reactive, $O_2^{\cdot-}$ and H_2O_2 can individually cause oxidative damage under certain conditions. However, they elicit more damaging effects when present together or in combination with other reactive species (Girotti 1998). The most likely possibility is the iron-mediated reduction of H_2O_2 by $O_2^{\cdot-}$ by a Haber–Weiss reaction [$O_2^{\cdot-} + Fe^{2+} \rightarrow [O_2^{\cdot-} + Fe^{2+} \rightarrow O_2 + Fe^{2+}]$ (a); $2O_2^{\cdot-} + 2H^+ \rightarrow O_2 + 2O_2^{\cdot-} + 2H^+ \rightarrow O_2 + H_2O_2$ (b); $H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + \cdot OH$ (c)], leading to formation of hydroxyl radical ($\cdot OH$), a highly reactive and strong oxidant (Kehrer 2000, Welch *et al.* 2002). Indeed, $\cdot OH$ is the well-accepted initiator of membrane lipid peroxidation and is responsible for the generation of lipid oxidation products including HNE. In this scenario, we believe that the combined actions of H_2O_2 and $O_2^{\cdot-}$ may facilitate the enhanced production of $\cdot OH$, thereby potentially increasing the extent of peroxidative damage of membrane lipids leading to the increased formation of HNE and consequently, inhibition of steroidogenesis.

Treatment of mouse adrenal Y1-BS1 cells with either $O_2^{\cdot-}$, H_2O_2 , or HNE also activated p38 MAPK in a time- and concentration-dependent manner. These results are in agreement with recent data from our laboratory showing increased stimulation of adrenal p38 MAPK in response to excessive oxidative stress that occurs during aging (Abidi *et al.* 2008). To elucidate the role of the p38 MAPK signaling cascade in oxidant-induced inhibition of steroid synthesis, we made use of both cell-permeable chemical inhibitors of p38 MAPK as well as genetic approaches involving either mutant cDNA constructs of p38 MAPKs or their upstream activators MKK3 and MKK6. Treatment of Y1-BS1 cells with specific inhibitors of p38 MAPK, SB203580, and SB202190 completely prevented oxidant-dependent inhibition of steroid production. Likewise, both inhibitors completely prevented oxidant-mediated increases in p38 MAPK activity as indicated by inhibition of ATF2 phosphorylation (a p38 MAPK substrate). By contrast, SB202474, a control compound that does not inhibit p38 MAPK activity had no effect on oxidant-mediated inhibition of steroidogenic response (Lee *et al.* 1994). Interestingly, the ability of two p38 MAPK inhibitors to enhance steroid production in untreated control cells further suggests that p38 MAPK functions as a negative modulator of adrenal steroidogenesis even under normal physiological conditions. To establish the specificity of p38 MAPK inhibitors, we also investigated the effects of PD98059 and U0126, structurally unrelated, specific inhibitors of the ERK activators, MEK1 and MEK2. Treatment of cells with either of the two inhibitors did not prevent oxidant-induced loss of steroidogenic function. Likewise, a specific JNK inhibitor, SP600125, had no significant effect on steroid synthesis following treatment of cells with either H_2O_2 or HNE.

The specificity of SB203580 and SB202190 noted above indicates that prevention of the oxidant-mediated inhibition of steroidogenesis resulted from the blockade of p38 MAPK activity. To further elucidate whether the p38 MAPK signaling cascades functions as a negative regulator of and directly interferes with adrenal steroidogenic function, we directly

examined the effects of various dominant-negative (dn) and constitutively active (ca) constructs of MKK3, MKK6, and p38 MAPK isoforms; MKK3 and MKK6 kinases are specific upstream activators of p38 MAPKs (Jiang *et al.* 1997, Enslen *et al.* 1998). Transient expression of a dominant-negative form of either MKK3(Ala) or MKK6(K82A) significantly increased the steroidogenic response, whereas expression of constitutively active forms of MKK3(Glu) and MKK6(Glu) reduced the steroidogenic response by $\sim 50\%$. A similar inhibitory effect was observed with the use of dominant-negative mutants of p38 MAPK isoforms (referred to here as dnp38 MAPK α (AF), dnp38 MAPK β (AF), dnp38 MAPK γ (AF), and dnp38 MAPK δ (AF)), although we noticed subtle differences in the relative contributions of the four isoforms. For instance, treatment with dnp38 MAPK α (AF) and dnp38 MAPK β (AF) increased stimulation of steroid production. On the other hand, although the use of dnp38 MAPK γ (AF) and dnp38 MAPK δ (AF) also enhanced steroidogenesis, the results were not statistically significant. The qPCR measurements further indicated that Y1-BS1 adrenal cells express extremely high levels of p38 MAPK α mRNA; approximately eightfold higher than second most expressed p38 MAPK γ mRNA. For p38 MAPK β and p38 MAPK δ , there was little or no mRNA expression. Taken together, these various observations suggest that activation of p38 MAPK by oxidative stress is a necessary event in the oxidant-mediated inhibition of steroid production. This is the first report implicating p38 MAPK signaling cascade as a negative modulator of adrenal steroidogenesis.

The exact role played by p38 MAPK in oxidative stress-dependent inhibition of steroidogenesis remains unclear at the present time. However, because multiple kinases and transcription factors have been identified as p38 MAPK substrates, this suggests that any of these processes could potentially contribute to the negative actions of p38 MAPK on steroidogenesis (Lewis *et al.* 1998, Widmann *et al.* 1999, Kyriakis & Avruch 2001, Raman *et al.* 2007, Whitmarsh 2007). One line of investigation supports a role for p38 MAPK in positively modulating cyclooxygenase-2 (COX-2), which is known to negatively impact steroid hormone synthesis (Nakamura & Sakamoto 2001, Wang *et al.* 2003, Frungieri *et al.* 2006). Several studies have demonstrated that expression of COX-2 is positively regulated by oxidants such as superoxide anion, H_2O_2 , and HNE (Nakamura & Sakamoto 2001, Kiritoshi *et al.* 2003, Kumgai *et al.* 2004, Yang *et al.* 2005, 2006). Furthermore, a number of studies have shown that engagement of p38 MAPK is necessary for oxidant-dependent induction of COX-2 (Guan *et al.* 1998, Lasa *et al.* 2000, Hendrickx *et al.* 2003, Zarrouki *et al.* 2007). When considering the possibility that COX-2 is a well-established negative modulator of steroid biosynthesis (Nakamura & Sakamoto 2001, Wang *et al.* 2003, Frungieri *et al.* 2006), there is a likely possibility that COX-2 may be involved in the p38 MAPK-mediated downregulation of steroidogenesis. Indeed, it has been recently demonstrated that COX-2 downregulates the gene expression of StAR (Wang *et al.* 2003) (the StAR protein, in concert with several other proteins, especially TSPO facilitate the rate-limiting transfer of

cholesterol to the mitochondrial inner membrane where the substrate cholesterol is converted to steroid precursor, pregnenolone by CYP11A1 (P450_{scc}; Liu *et al.* 2003, Manna & Stocco 2005)). Furthermore, our own unpublished preliminary data suggest that p38 MAPK functions as a suppressor of StAR promoter activity in adrenal and Leydig cell lines (S M Zaidi, unpublished observations). Recently, S M Zhao *et al.* (2006) have provided evidence for stabilization of 3.5 kb StAR mRNA transcript by arsenite/anisomycin- (non-specific stimulators of p38 MAPK) sensitive putative protein kinase(s) in an SB203580-independent manner. Use of p38 MAPK inhibitor, SB203580 also stabilized the 3.5 kb StAR mRNA transcript, which was completely blocked by an inhibitor of ERK pathway (MEK inhibitor PD98059). Interestingly, arsenite/anisomycin-mediated stabilization of 3.5 kb StAR mRNA transcript occurred in the absence of any detectable increases in StAR phosphorylation. Moreover, incubation of adrenal or Leydig cells with higher arsenite concentrations or longer incubation times resulted in inhibition of StAR expression. Thus, although physiological significance of these disjointed observations and their relevance to StAR function is not apparent, they are in line with our findings that p38 MAPK negatively impacts steroidogenesis.

In conclusion, our data provide direct evidence that activation of the p38 MAPK signaling pathway is functionally linked to the oxidative stress response and mediates its inhibitory effect on adrenal steroid production. This represents a novel cellular mechanism that allows negative modulation of steroidogenesis in response to excessive oxidative insult. Additional studies are underway in this laboratory to further delineate the mechanism by which oxidative stress-mediated activation of p38 MAPK signaling pathway negatively impacts steroidogenesis, particularly impact of oxidants/oxidative stress on the expression of other StARD proteins and potential interactions between StAR and PBR.

Declaration of Interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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