p38 MAPK regulates steroidogenesis through transcriptional repression of *STAR* gene

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Abstract

STAR/StarD1, part of a protein complex, mediates the transport of cholesterol from the outer to inner mitochondrial membrane, which is the rate-limiting step for steroidogenesis, and where steroid hormone synthesis begins. Herein, we examined the role of oxidant-sensitive p38 MAPKs in the regulation of *STAR* gene transcription, using model steroidogenic cell lines. Our data indicate that oxidant activation of p38 MAPK exhibits a negative regulatory role in the induction of functional expression of *STAR*, as evidenced by enhanced induction of *STAR* (mRNA/protein) expression and increased steroidogenesis during pharmacological inhibition of p38 MAPK or in cells with increased transient overexpression of a dominant-negative (dn) form of p38 MAPK α or p38 MAPK β . Studies with rat *Star*-promoter demonstrated that overexpression of p38 MAPK α -wt, - β , or - γ significantly reduced both basal and cAMP-sensitive promoter activity. In contrast, overexpression of p38 MAPK α -dn, - β , or - γ enhanced the *Star* promoter activity under basal conditions and in response to cAMP stimulation. Use of various constitutively active and dn constructs and designer knock-out cell lines demonstrated that MKK3 and MKK6, the upstream activators of p38 MAPKs, play a role in p38 MAPK α -mediated inhibition of *Star* promoter activity. In addition, our studies raised the possibility of CREB being a potential target of the p38 MAPK inhibitory effect on *Star* promoter activity. Collectively, these data provide novel mechanistic information about how oxidant-sensitive p38 MAPKs, particularly p38 MAPK α , contribute to the negative regulation of *Star* gene expression and inhibit steroidogenesis.

Key Words

- ▶ steroid hormones
- ▶ cAMP
- ▶ oxidative stress
- ▶ Y1 cells
- ▶ MLTC-1 cells
- ▶ CREB
- ▶ steroids

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Introduction

Extensive experimental evidence now indicates that aging in humans and animal models is associated with a general decline in steroid hormone production (Zaidi *et al.* 2012). Similarly, a number of *in vitro* studies have clearly shown that isolated adrenocortical cells and testicular Leydig cells (Zaidi *et al.* 2012) of older rats of several different strains synthesize and secrete less steroid hormone in response to tropic hormone or its second messenger, cAMP, than do cells from younger animals. These changes in steroid hormone production and secretion do not appear to be a function of reduced tropic hormone signaling or a defect in steroid hormone-synthesizing enzymes.

Previous work from this laboratory has shown that an adequate amount of cholesterol is not available to the adrenal (adrenocortical cells) and testis (Leydig cells) in aging rats (Poppellwell & Azhar 1987, Liao *et al.* 1993, Sun *et al.* 2008, Zaidi *et al.* 2012) for the initial and rate-limiting step in steroid biosynthesis, i.e. translocation of cholesterol from the outer mitochondrial membrane (OMM) to the P450 side-chain cleavage enzyme (P450_{scc}/Cyp11A1), which is localized in the matrix side of the inner mitochondrial membrane (IMM) and converts cholesterol to pregnenolone, the precursor of all steroid hormones (Stocco & Clark 1996, Stocco 2001, Hu *et al.* 2010, Miller & Bose 2011). This aging-induced attenuation of cholesterol transport to mitochondria is not due to a loss of cellular cholesterol stores, but results from the downregulation of STAR (Leers-Sucheta *et al.* 1999, Luo *et al.* 2001, Wang *et al.* 2005, Sun *et al.* 2008) and peripheral-type benzodiazepine receptor or translocator protein (TSPO; Culty *et al.* 2002, Sun *et al.* 2008). These components of the steroidogenic machinery (Stocco & Clark 1996, Stocco 2001) probably function in concert (Liu *et al.* 2006) to facilitate rate-limiting cholesterol transport from the OMM to the IMM (Hu *et al.* 2010, Miller & Bose 2011). Although various cellular and molecular mechanisms controlling this aging defect have not been definitely identified, excessive reactive oxygen species (ROS) production (Azhar *et al.* 1995, Cao *et al.* 2004, Abidi *et al.* 2008a,b, Zaidi *et al.* 2012) and ROS-induced oxidative damage to STAR have been suggested as a potential mechanism for impaired cholesterol transport to the IMM for steroid production during aging.

p38 MAPKs are members of the MAPK family that are activated by a variety of environmental stresses and inflammatory cytokines (Roux & Blenis 2004, Coulthard *et al.* 2009, Cuadrado & Nebreda 2010, Remy *et al.* 2010, Corrêa & Eales 2012). The four members of the p38 MAPK isoform family are p38 MAPK α (MAPK14), p38 MAPK β

(SAPK2b), p38 MAPK γ (SAPK3, ERK6, or MAPK14), and p38 MAPK δ (SAPK4 or MAPK13) (Han *et al.* 1994, Lee *et al.* 1994, Rouse *et al.* 1994, Coulthard *et al.* 2009, Cuadrado & Nebreda 2010, Remy *et al.* 2010). These isoforms differ in their organ/tissue distribution, regulation of kinase activation and subsequent phosphorylation of downstream substrates, kinases or transcription factors, and sensitivity to pharmacological inhibition by pyridinyl imidazole molecules such as SB203580 and SB202190 (Bain *et al.* 2007, Coulthard *et al.* 2009, Cuadrado & Nebreda 2010, Remy *et al.* 2010). Various forms of cellular stress, including oxidative stress, u.v. irradiation, hypoxia, and ischemia, as well as inflammatory cytokines, stimulate the activity of p38 MAPKs (Coulthard *et al.* 2009, Cuadrado & Nebreda 2010, Remy *et al.* 2010) by dual phosphorylation of Thr¹⁸⁰ and Tyr¹⁸² in a Thr–Gly–Tyr motif. This phosphorylation is mediated by upstream MAPKs (MAPKKs), MKK3 and MKK6 (Derijard *et al.* 1995, Raingeaud *et al.* 1995, 1996, Han *et al.* 1996), which are in turn activated by several different and overlapping sets of MAPKKKs or MEKKs (Coulthard *et al.* 2009, Cuadrado & Nebreda 2010, Remy *et al.* 2010).

Recently, we have shown that aging leads to increased activation of p38 MAPK α in rat adrenocortical cells (Abidi *et al.* 2008a). This effect appears to be specific for p38 MAPK α , as the expression of neither ERK1/2 nor JNK1/2 was altered during aging (Abidi *et al.* 2008a). We also showed that the use of p38 MAPK inhibitors (SB203580 and SB202190) and antioxidants (NAC and MnTMPyP) partially restored the steroidogenesis in adrenal cells from old animals, indicating that the p38 MAPK signaling cascade facilitates the inhibitory actions of excessive oxidative insult on adrenal steroid hormone production caused by aging (Abidi *et al.* 2008a). These results were further complemented by *in vitro* studies using a mouse adrenocortical tumor cell line, Y-1, showing that activated p38 MAPKs mediate oxidant (excessive oxidative stress)-induced inhibition of steroid production (Abidi *et al.* 2008b).

In the present study, we sought to determine molecular mechanisms by which the oxidant-sensitive p38 MAPK inhibits cAMP-induced steroidogenesis. Herein, we show that the inhibitory effect of p38 MAPK on cAMP-induced steroidogenesis in model steroidogenic cell lines is due to a decrease in StAR expression and its promoter activity. Furthermore, expression and functional activity of cAMP response-element binding protein (CREB), a mediator of tropic hormone/cAMP-stimulated STAR gene transcription, is also sensitive to the

inhibitory actions of p38 MAPK. The current study describes the molecular events responsible for the p38 MAPK-mediated negative regulation of STAR gene transcription and inhibition of steroidogenesis.

Materials and methods

Reagents and antibodies

N⁶, 2'-O-dibutyl cAMP (Bt₂cAMP), SB 203580, SB 202190, xanthine, xanthine oxidase (XO), microbial, fatty acid-free BSA, hydrogen peroxide, and monoclonal Anti-FLAG M2 antibody and mouse monoclonal anti-actin antibody were obtained from Sigma-Aldrich. 4-Hydroxy-2-nonenal (HNE) was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). The NEP-PER Nuclear Protein Extraction Kit, and Promega Dual-Luciferase Reporter Kit (E1980) were purchased from Thermo Fisher Scientific (Rockford, IL) and Promega Corporation respectively. Anti-phospho-CREB (Ser¹³³), anti-CREB, pan anti-p38 MAPK, and anti-phospho-p38 MAPK (Thr¹⁸⁰/Tyr¹⁸²) antibodies that detect endogenous levels of total and phosphorylated (activated) forms of p38 MAPK α , β , or γ protein, respectively, were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). IRDye 800CW goat anti-rabbit and IRDye 680LT goat anti-mouse secondary antibodies were purchased from LI-COR Corporate (Lincoln, NE, USA). SYBR Green Master Mix and Lipofectamine 2000 were supplied by Applied Biosystems and Invitrogen (Life Technologies) respectively.

Plasmids

The generation of Rat *Star* promoter-pGL3 constructs p-1862-luc, p-1413-luc, p-998-luc, p-545-luc, and p-342-luc has been described in an earlier publication (Sandhoff *et al.* 1998). The mouse *Star* promoter constructs, pGL3 (p-966-luc; Manna *et al.* 2002), were kindly supplied by Dr Douglas Stocco (Texas Tech University Health Sciences Center, Lubbock, TX, USA). The pcDNA3-Flag-tagged-p38 MAPK α -wt, p38 MAPK β -wt, p38 MAPK γ -wt, p38 MAPK δ -wt, p38 MAPK α /AF-dn (dominant negative) p38 MAPK β /AF-dn, p38 MAPK γ /AF-dn, and p38 MAPK δ /AF-dn constructs used here have been described previously (Pramanik *et al.* 2003). The dn forms (AF) of human p38 MAPK α , p38 MAPK β , p38 MAPK γ , and p38 MAPK δ cDNA constructs were created by substituting Thr¹⁸⁸ with Ala and Tyr¹⁹⁰ with Phe in the TGY dual phosphorylation site. Because AF mutant proteins cannot be phosphorylated, they act as dn proteins and interfere with the functions

of their respective p38 MAPKs. The pcDNA3-Flag-tagged MKK3-wt, MKK3 (AA)-dn, MKK3 (EE)-ca (constitutively active), MKK6-wt, MKK6 (AA)-dn, and MKK6 (EE)-ca expression constructs used here were generated as reported previously (Han *et al.* 1996, Raingeaud *et al.* 1996); ca MKK3 (EE)-ca and dn MKK3 (AA)-dn were constructed by replacing Ser¹⁸⁹ and Thr¹⁹³ with Glu and Ala residues respectively. Likewise, ca MKK6 (EE)-ca and dn MKK6 (AA)-dn were constructed by replacing Ser²⁰⁷ and Thr²¹¹ with Glu and Ala residues respectively. HA-MKK6-p38 MAPK α -ca, HA-MKK6-p38 MAPK γ -ca, HA-MKK6-p38 α /AGF-dn, and HA-MKK6-p38 γ /AGF-dn fusion constructs were kindly provided by Dr Guan Chen (University of Wisconsin, Madison, WI, USA). RSV-CREB plasmid was purchased from Addgene (Cambridge, MA, USA). CRE-Reporter (luc) and activator protein 1 (AP1) (TPA-response element (TRE)) Reporter (luc) kits were obtained from Qiagen-SA Biosciences (Valencia, CA, USA). Both CRE-Reporter (luc) and AP1 (TRE) Reporter (luc) are inducible reporter constructs that encode a firefly luciferase gene under the control of a basal promoter element (TATA box) joined to tandem six repeats (6 $\times$) of CRE and AP1/TRE transcriptional response elements respectively.

Cell culture

Mouse Y-1 adrenocortical tumor cells, mouse MLTC-1 testicular Leydig tumor cells, and human embryonic kidney-293 (HEK-293) cells were originally obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). MLTC-1 cells were maintained in RPMI 1640 medium with 10% fetal bovine serum (FBS), 100 μ g/ml streptomycin, 100 units/ml penicillin, and 1% L-glutamine in a humidified atmosphere of 5% CO₂ at 37 °C. Y-1 cells were maintained at 37 °C in a humidified 5% CO₂ atmosphere in Ham's F-10 medium supplemented with 2.5% FBS, 12.5% horse serum, 100 μ g/ml streptomycin, and 100 units/ml penicillin. MKK3/6 wt, MKK3^{-/-}, MKK6^{-/-}, and MKK3/6 double knock-out (DKO) mouse embryonic fibroblasts (MEFs) (Brancho *et al.* 2003) were maintained in DMEM containing 10% FBS, 100 μ g/ml streptomycin, and 100 units/ml penicillin at 37 °C with 5% CO₂.

Transfections and luciferase assays

MLTC-1 (or other cell types such as Y1, and MEFs, MKK3/6 wt, MKK3^{-/-}, MKK6^{-/-}, and MKK3/6^{-/-}) cells were seeded at a density of 1 $\times$ 10⁵ cells/well in a 12-well plate containing a cell-type-specific culture medium as

described earlier. After overnight incubation, cells were transiently transfected with a rat p-1862 Star-Luc (firefly luciferase) construct (1 µg) ± native p38 MAPKα (wt) (1 µg) or dn p38 MAPKα (1 µg) using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Life Technologies). A control reporter construct containing *Renilla* luciferase (*Rluc*, pRL-TK) (50 ng) was co-transfected for normalization of transfection efficiency. In some cases, MLTC-1 cells were transfected with rat p-1862 Star-Luc (firefly luciferase) construct (1 µg) + pRL-TK (50 ng) and ± p38 MAPKβ-wt (1 µg), ± p38 MAPKβ-dn, ± p38 MAPKγ-wt (1 µg), ± p38 MAPKγ-dn (1 µg), ± p38 MAPKδ-wt, ± p38 MAPKδ-dn, ± MKK6-p38 MAPKα-ca, ± MKK6-p38 MAPKα-dn, ± MKK6-p38 MAPKγ-ca, ± MKK6-p38 MAPKγ-dn, ± MKK3-wt, ± MKK3-dn (AA), ± MKK3-ca (EE), ± MKK6-wt, ± MKK6-dn (AA), or ± MKK6-ca (EE). In each case, total DNA concentration was kept constant to 3 µg with the addition of inert pcDNA3. After 48 h, cells were incubated with or without Bt₂cAMP for 5 h, washed, harvested using cell lysis buffer (Promega Corporation), and subsequently assayed for both firefly and *Renilla* luciferase activities using the Promega Dual-Luciferase Reporter Kit (E1980) and a SpectraMax L luminescence Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) or GloMax-20/20 Single-Tube Luminometer (Promega Corporation). Firefly luciferase activities were normalized to *Renilla* luciferase reporter activities and shown as -fold induction compared with control (empty vector). The results shown are the average of triplicate determinations with the error bars representing S.E.M. All the experiments were independently repeated at least two to three times.

In other assays, MLTC-1 cells were transfected with CRE-luc (firefly luciferase) + *Renilla* luciferase (*Rluc*, pRL-TK), ± CREB, or ± CREB + p38 MAPKα-wt or p38 MAPKα-dn, for 48 h. At the end of incubation, washed dishes were further incubated with or without Bt₂cAMP followed by measurement of firefly and *Renilla* luciferase activities. Likewise, MLTC-1 cells were transfected with AP1/TRE-luc (firefly luciferase) + *Renilla* luciferase (*Rluc*, pRL-TK) ± p38 MAPKα-wt or p38 MAPKα-dn and, after 48 h, dishes were further incubated with or without AP1/TRE activator TPA (10 nM) for 30 min. The cell lysates were then quantified for firefly and *Renilla* luciferase activities.

Transfection of MLTC-1 cells with p38 MAPK constructs and measurement of steroid (progesterone) secretion

MLTC-1 cells were seeded at a density of 1×10^5 cells/well in a 12-well plate containing RPMI 1640 medium

supplemented with 10% FBS, 100 µg/ml streptomycin, 100 units/ml penicillin, and 1% L-glutamine (under 5% CO₂ at 37 °C) for 24 h before transfection. The cells were transfected with native wt-p38 MAPKα (1 µg) and/or dn-p38 MAPKα (1 µg) or pcDNA3 (3 µg) using Lipofectamine 2000 as recommended by the manufacturer (Invitrogen, Life Technologies). In each case, the total amount of plasmid (3 µg) was kept the same with the addition of inert pcDNA3. Forty-eight hours after transfection, the medium was replaced with a fresh medium and the cells (triplicate wells) were treated with ± Bt₂cAMP (2.5 mM) and incubations continued for an additional 5 h. At the end of incubation, media were aspirated and stored frozen until analyzed for secreted progesterone content by RIA as described earlier (Reaven *et al.* 1995). The cells were washed once with PBS and then processed for the determination of *Star/StarD1* mRNA expression by quantitative RT-PCR as described below.

Quantitative real-time PCR

Total RNA from MLTC-1 and Y-1 cells was isolated by using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA concentration and purity were quantified using a NanoDrop Spectrophotometer (Thermo Scientific). Samples (2 µg) RNA were reverse transcribed to cDNA at 42 °C for 1 h using SuperScript II reverse transcriptase (Invitrogen) and oligo(dT) and random hexamers. Quantitative real-time PCR (qRT-PCR) was carried out using specific primers (Table 1) and amplified using SYBR Select Master Mix for CFX (Applied Biosystems) in an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The results are presented as relative mRNA expression levels calculated using the formula $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT = \Delta CT_{\text{target}} - \Delta CT_{\text{reference}}$ with 36B4 (60S acidic ribosomal protein P0) as the reference gene.

Western blotting analysis

The cells were washed with PBS and then harvested in RIPA buffer, containing a Complete Protease Inhibitor Cocktail and PhosSTOP phosphatase inhibitor cocktail (both from Roche Diagnostics) directly to plates and scraping into tubes. The cells were briefly sonicated, and centrifuged for 5 min. The protein concentrations of the lysates were measured using the Pierce BCA Protein Assay Reagent Kit (Thermo Fischer Scientific, Rockford, IL, USA). Proteins from cell lysates (20 µg) were resolved on a precast 4–20% gradient SDS-PAGE and electro-transferred onto

Table 1 Oligonucleotide sequences used in quantitative real-time PCR (qRT-PCR) analysis

Gene	Forward	Reverse
m- <i>Star/StarD1</i>	5'-GGATTAAGGCACCAAGCTGT-3'	5'-CCAGTTGAGAACCAAGCAGA-3'
m- <i>StarD2</i>	5'-TGTGCTGAACCTCAGAAACC-3'	5'-ATAAAGTCCACTTGGCTGGTC-3'
m- <i>StarD3</i>	5'-CCTGCTCTTCATCTCCCTTC-3'	5'-CTCCTGCTCCAAGTTCTTCC-3'
m- <i>StarD4</i>	5'-TCCTGTGGTGTGAGTGTTGA-3'	5'-TGGCTTGGACTGTCTTTGAG-3'
m- <i>StarD5</i>	5'-GGGAATCTGTACCGAGGAGA-3'	5'-AACTGGCTTTATGCAATCCC-3'
m- <i>StarD6</i>	5'-CACAGTTCGACTCCTCTGT-3'	5'-CTCTGCAGGAACAACTCCA-3'
m- <i>StarD7</i>	5'-ATGTTCCGGCGGTATAGTGTG-3'	5'-GGATAACCATTTGGGATTCG-3'
m- <i>StarD10</i>	5'-CTGGAACCTGACCTACAGCA-3'	5'-CCATCCGACACTTGATCTTG-3'
m-p38 MAPK α	5'-CCCCAGAGATCATGCTGAAT-3'	5'-ACAACGTTCTTCCGGTCAAC-3'
m-p38 MAPK β	5'-ATGTAGCGGTGAACGAGGA-3'	5'-CCACATATCCGGTCATCTCC-3'
m-p38 MAPK γ	5'-GAGACTTGAAGCCTGGCAAC-3'	5'-CCGGGTTACCACATATCCTG-3'
m-p38 MAPK δ	5'-CGCCACACAGACACTGAGA-3'	5'-ATGATGCAACCAACAGACC-3'
m-Cyclophilin	5'-TGGAGAGCACCAAGACAGACA-3'	5'-TGCCGGAGTCGACAATGAT-3'
m-36B4	5'-CCTGAAGTGCTCGACATCAC-3'	5'-CGATCTGCAGACACACTG-3'

a Hybond nitrocellulose membrane (Amersham, GE Healthcare, Piscataway, NJ, USA). The membranes were blocked, incubated with anti-p38 MAPK, anti-phospho-p38 MAPK, anti-CREB, anti-phospho-CREB, and mouse anti- β -actin antibodies, washed, and incubated with IRDye 800CW goat anti-rabbit and IRDye 680LT goat anti-mouse secondary antibodies. The membrane blots were then imaged using an ODYSSEY Infrared Imaging System (LI-COR Biosciences). Quantification of bands was made with the analysis software provided with the imaging system.

Statistical analysis

Data are presented as mean $\pm$ S.E.M. Statistical analyses were performed using ANOVA followed by the Bonferroni's post test using GraphPad Prism Software, Prism 4 (GraphPad Software, Inc., San Diego, CA, USA). A *P* value of <0.05 was considered statistically significant. All experiments were performed independently at least three times.

Results

Effect of Bt₂cAMP stimulation on mRNA levels of *Star* and *Star*-related lipid transfer proteins in MLTC-1 and Y-1 cells

The human and mouse genomes each contain 15 genes encoding *Star*-related lipid transfer (START) domain-containing proteins (STARD; Soccio & Breslow 2003). STAR (STARD1), the prototype of the STARD family of proteins, plays an essential role in moving cholesterol substrate to the IMM of steroidogenic cells of the adrenal gland and gonads, where the first committed step in steroidogenesis occurs (Stocco 2001, Miller & Bose 2011).

Herein, we assessed the mRNA levels of *Star* (*StarD1*) (subsequently referred to as *Star*) and other *StarD* family members under basal conditions and in response to cAMP stimulation in two steroidogenic cell lines, MLTC-1 and Y-1. Total RNA samples were analyzed for *Star*, *StarD2*, 3, 4, 5, 6, 7, and 10 mRNA expression by qRT-PCR. The results shown in Fig. 1 indicate that *Star* mRNA is most abundantly expressed in both MLTC-1 and Y-1 cells and that Bt₂cAMP (a cell-permeable analog of cAMP) stimulation further increased its mRNA levels by four- to fivefold in both cell types. Relatively low mRNA expression of *StarD4* and *StarD7* was also detected in MLTC-1 cells, and Bt₂cAMP (2.5 mM) treatment of these cells enhanced *StarD4* expression by four- to fivefold (Fig. 1). In Y-1 cells, low, but significant, mRNA expression was noted for *StarD4*, *StarD5*, and *StarD7*, although their levels were not affected by Bt₂cAMP stimulation. The higher levels of mRNA expression of STAR protein compared with other STARD proteins in MLTC-1 and Y-1 cells are consistent with its critical role in the regulation of steroidogenesis.

Expression of endogenous mRNA levels of p38 MAPK isoforms in MLTC-1 and Y-1 cells

Using qRT-PCR, we next determined the relative mRNA expression levels of each of the four p38 MAPK isoforms in MLTC-1 and Y-1 cells (Fig. 2). p38 MAPK α expression was most abundant in both cell types; only low levels of p38 MAPK β were detected in Y-1 cells, while its mRNA levels were roughly 40% of the levels of p38 MAPK α in MLTC-1 cells (Fig. 2). Low levels of p38 MAPK γ mRNA were also detected in Y-1 cells as well as in MLTC-1 cells. The expression of p38 MAPK δ , however, was barely detectable in MLTC-1, and Y-1 cells showed only low expression

Figure 1

qRT-PCR analysis of *StarD1/Star*, *StarD2*, *StarD3*, *StarD4*, *StarD5*, *StarD6*, *StarD7*, and *StarD10* mRNA levels in mouse Y-1 adrenal cells and mouse Leydig tumor cells, MLTC-1, treated with or without Bt₂cAMP (2.5 mM) for 6 h. Groups of RNA samples from Y-1 or MLTC-1 cells were analyzed by qRT-PCR as described in the 'Materials and methods' section. The levels of expression of *StarD1/Star*, *StarD2*, *StarD3*, *StarD4*, *StarD5*, *StarD6*, *StarD7*, and *StarD10* mRNAs are shown. Expression of cyclophilin was used for normalization. (A) Y-1 cells and (B) MLTC-1 cells. Each value represents mean ± S.E.M. of four separate determinations. ****P* < 0.0001 basal (control) vs Bt₂cAMP.

(Fig. 2). These results led us to conclude that p38 MAPK α is the predominant form in both cell types, but significant expression of p38 MAPK β and p38 MAPK γ was also detected in MLTC-1 and Y-1 cells respectively. Thus, most of the follow-up studies focused on the p38 MAPK α isoform.

Effects of oxidants and Bt₂cAMP on *Star* mRNA expression in steroidogenic cell lines

To examine the effect of oxidants on *Star* mRNA expression, MLTC-1 and Y-1 cells were pre-exposed to xanthine/XO (X/XO; superoxide anion), hydrogen peroxide, lipid peroxidation product, HNE, or vehicle

(control) for 1 h, the dishes washed and treated or not treated a cell membrane-permeable cAMP analog, Bt₂cAMP, for 5 h. Total RNA was isolated and *Star* mRNA measured using qRT-PCR. When cells were treated for 5 h, the results demonstrated that *Star* mRNA levels, as expected, increased threefold to fourfold following treatment with 2.5 mM Bt₂cAMP compared with nontreatment control (Fig. 3). Treatment of cells with a superoxide-generating system (X/XO), hydrogen peroxide, or a lipid peroxidation product, HNE, under basal conditions caused a significant reduction in *Star* mRNA levels in MLTC-1 and Y-1 cells, ranging from 30 to 80% (Fig. 3). Likewise, pre-exposure of cells to these three oxidants caused a variable, but significant, reduction in Bt₂cAMP-stimulated *Star* mRNA levels (Fig. 3). These results indicate that exposure of steroidogenic cells to different oxidants results in downregulation of STAR gene expression.

Figure 2

QRT-PCR analyses of p38 MAPK isoforms, p38 MAPK α , p38 MAPK β , p38 MAPK γ , and p38 MAPK δ mRNA levels in cultured Y-1 and MLTC-1 cells. Groups of RNA samples from Y-1 or MLTC-1 cells were analyzed by qRT-PCR as described in the 'Materials and methods' section. The levels of expression of p38 MAPK α , p38 MAPK β , p38 MAPK γ , and p38 MAPK δ mRNAs are shown. Expression of 36B4 was used for normalization. (A) Y-1 cells and (B) MLTC-1 cells. Each value represents mean ± S.E.M. of four separate determinations. **P* < 0.05 p38 α vs p38 β (MLTC-1) and ****P* < 0.001 p38 α vs p38 β (Y-1), p38 γ or p38 δ .

Figure 3

Exposure of Y-1 cells (A) and MLTC-1 cells (B) to xanthine/xanthine oxidase (X/XO) generated superoxide anion ($O_2^{\cdot-}$) or hydrogen peroxide (H_2O_2) attenuates mRNA expression of *Star/StarD1* both under basal conditions and in response to cAMP stimulation. Cultured Y-1 and MLTC-1 cells were exposed to ± 0.1 mM xanthine and 10 mU/ml XO or ± 50 μ M hydrogen peroxide (H_2O_2) for 1 h. At the end of incubation, the dishes were washed and subsequently incubated with or without Bt₂cAMP (2.5 mM) for 5 h, followed by isolation of total RNA and the quantification of the *Star/StarD1* mRNA levels by qRT-PCR. Each value represents mean $\pm$ S.E.M. of four separate determinations. *** $P < 0.0001$ basal (control) vs Bt₂cAMP.

Oxidant activation of p38 MAPK negatively affects cAMP transcriptional regulation of *Star*

The results described above indicate that oxidant exposure of steroidogenic cells results in downregulation of *Star* mRNA levels. As the p38 MAPK pathway has been implicated in mediating oxidative-stress-induced inhibition of steroid hormone synthesis (Abidi *et al.* 2008a,b), we investigated the role of p38 MAPK signaling in oxidant modulation of cAMP-mediated transcriptional regulation of STAR protein. MLTC-1 cells were transiently transfected with a construct containing the promoter region (–2862) of rat *Star*, a control reporter construct containing *Renilla* luciferase for normalization of transfection efficiency and with or without an expression plasmid

encoding FLAG-p38 MAPK α , and, after 48 h, cells were exposed to vehicle (control), superoxide anion ($O_2^{\cdot-}$), X/XO, the generator of $O_2^{\cdot-}$, H_2O_2 , or HNE (a lipid peroxidation product) for an additional 60 min. After extensive washing, cells were treated with $\pm$ Bt₂cAMP (2.5 mM) for 3 h, harvested and cell lysates analyzed for firefly and *Renilla* luciferase activities or subjected to western blotting analysis for the measurement of total and phosphorylated (activated) forms of p38 MAPK α . As shown in Fig. 4A, short-term exposure of transfected cells to optimal doses of superoxide anion, H_2O_2 , or HNE resulted in p38 MAPK α activation, as indicated by an increased level of phosphorylation at residues Thr¹⁸⁰ and

Figure 4

Evaluation of the effects of p38 MAPK α (wt) overexpression and the effects of X/XO generated $O_2^{\cdot-}$, H_2O_2 , and 4-HNE on basal and cAMP-stimulated rat *Star* promoter (luciferase) activity in MLTC-1 cells. MLTC-1 cells were transiently transfected with a rat p-1862 *Star-Luc* (firefly luciferase) construct (1 μ g) + *Renilla* luciferase (*Rluc*, pRL-TK) (50 ng) and $\pm$ native p38 MAPK α (wt) (1 μ g) using Lipofectamine 2000. After 48 h, dishes were exposed to $\pm$ xanthine (0.1 mM)/XO (10 mU/ml), ± 50 μ M H_2O_2 , or ± 50 μ M 4-hydroxynonenol (4-HNE) for 1 h, washed, incubated with or without Bt₂cAMP (2.5 mM) for 5 h, cells were lysed using cell lysis buffer (Promega Corporation) and subsequently assayed for both firefly and *Renilla* luciferase activities using a Promega Dual-Luciferase Reporter Kit (E1980). In some cases, cell lysates were subjected to SDS-PAGE followed by western blotting analysis and the quantification of the total and the phosphorylated p38 MAPK α bands. (A) Western blots showing the bands stained for either total p38 MAPK α or its phosphorylated form. (B) Rat *Star* promoter luciferase activity. ** $P < 0.01$ basal (control) vs Bt₂cAMP and *** $P < 0.0001$ basal (control) vs Bt₂cAMP.

Tyr¹⁸² (approximately 10–30% (phospho-p38 MAPK:total p38 MAPK ratios: control = 1.00; superoxide anion = 1.08; H₂O₂ = 1.34; and HNE = 1.24)). Basal levels of *Star* promoter luciferase activity were significantly decreased when cells were co-transfected with a p38 MAPK α construct (Fig. 4B). Basal promoter activity was further decreased when p38 MAPK α -transfected cells were exposed to oxidants: superoxide anion (O₂⁻), H₂O₂, or 4-HNE (Fig. 4B). Likewise, cAMP-stimulated promoter activity was also decreased with the introduction of p38 MAPK α . However, no additional decreases in cAMP-stimulated promoter activity were noted following oxidant exposure of p38 MAPK α -transfected cells (Fig. 4B). These p38 MAPK α overexpression studies indicate that inhibitory actions of oxidants on *Star* promoter activity are mediated by p38 MAPK α .

Effects of overexpression of native (wt) and dn p38 MAPK isoforms on *Star* promoter activity

The above findings indicate that p38 MAPK α exerts a negative effect on both basal and cAMP-stimulated *Star* promoter activity. To establish p38 MAPK isoform specificity, we next examined the relative efficacy of native (wt) p38 MAPK α , p38 MAPK β , p38 MAPK γ , and p38 MAPK δ and their respective dn forms on *Star* promoter activity. MLTC-1 cells were transfected with STAR-reporter luciferase plasmid alone or together with plasmids encoding native or dn FLAG-p38 MAPK isoforms, and luciferase activities were quantified. As before, expression of wt p38 MAPK α -wt reduced the basal promoter activity by approximately 50%, whereas expression of dn p38 MAPK α -dn enhanced the basal promoter activity by about twofold (Fig. 5A). Qualitatively similar results were

obtained when Y-1 or HEK-293 cells were substituted for MLTC-1 or rat *Star* promoter (p-1862-rat StAR) reporter construct was replaced with a mouse *Star* promoter reporter construct (p960-mouse *Star*; Manna *et al.* (2002); data not shown). Results presented in Fig. 5B and C show that overexpression of p38 MAPK β -wt or p38 MAPK γ -wt also significantly suppressed the transfected rat *Star* promoter reporter activity both under basal conditions and in response to cAMP stimulation. On the other hand, expression of p38 MAPK β -dn, similar to p38 MAPK α -dn, enhanced basal as well as cAMP-stimulated promoter activity, but the use of p38 MAPK γ -dn showed no such stimulatory effect. In contrast, expression of p38 MAPK δ -wt showed no inhibitory effect on promoter activity when measured under basal conditions, but significantly decreased cAMP-stimulated promoter activity (Fig. 5D). On the other hand, transient overexpression of p38 MAPK δ -dn variably but significantly enhanced cAMP-stimulated promoter activity (Fig. 5D). These results led us to conclude that p38 MAPK α and to a lesser extent p38 MAPK β , p38 MAPK γ , and p38 MAPK δ function as negative regulators of *Star* gene transcription.

To further establish the inhibitory action of p38 MAPK α on *Star* promoter activity, p38 MAPK inhibitor experiments were carried out. To this end, MLTC-1 cells were either transfected with *Star* promoter alone or *Star* promoter plus p38 MAPK α -wt construct and then treated with selective inhibitors of p38 MAPK, SB203580 and SB202190 (Bain *et al.* 2007). Luciferase measurement results indicated that SB203580 or SB202190 treatment resulted in a small increase in basal promoter activity in MLTC-1 cells transfected with *Star* promoter alone (Table 2). Co-transfection of *Star* promoter with p38

Figure 5

Effects of overexpression of wt and dn p38 MAPK α , p38 MAPK β , p38 MAPK γ , and p38 MAPK δ constructs on *Star* promoter activity in MLTC-1 cells. The cells were transiently transfected with p-1862 *Star*-Luc (firefly luciferase) construct plus *Renilla* luciferase (*Rluc*, pRL-TK), and empty vector, p38 MAPK α -wt, p38 MAPK α -dn, p38 MAPK β -wt, p38 MAPK β -dn, p38 MAPK γ -wt, p38 MAPK γ -dn, p38 MAPK δ -wt, or p38 MAPK δ -dn. Forty-eight hours after transfection, the dishes were washed and subsequently incubated with or without Bt₂cAMP (2.5 mM) for 5 h and cells lysed using

cell lysis buffer (Promega Corporation) and subsequently assayed for both firefly and *Renilla* luciferase activities. (A) p38 MAPK α , (B) p38 MAPK β , (C) p38 MAPK γ , and (D) p38 MAPK δ . Each value represents mean $\pm$ S.E.M. of four separate determinations. wt (native); dn, dominant negative. **P* < 0.01 basal (control) vs Bt₂cAMP; ***P* < 0.01 basal (control) vs Bt₂cAMP; ****P* < 0.0001 basal (control) vs Bt₂cAMP; †*P* < 0.01 Bt₂cAMP p38 δ -dn vs Bt₂cAMP p38 α -wt; and †††*P* < 0.0001 Bt₂cAMP control vs Bt₂cAMP p38 α -wt.

Table 2 Effects of p38 MAPK inhibitors and overexpression of native/wt and dominant negative (dn) forms of p38 MAPK α either separately or in combination on *Star* promoter (luciferase) activity in MLTC-1 cells. Mouse Leydig tumor cells MLTC-1 were transiently transfected with rat p-1862 rStar-luc (firefly luciferase) construct (1 μ g) $\pm$ plasmid-encoding native (wt) FLAG p38 MAPK α (1 μ g). A control reporter construct containing *Renilla* luciferase (*Rluc*, pRL-TK; 50 ng) was co-transfected for normalization of transfection efficiency. After 48 h, cells were incubated with or without Bt₂cAMP (2.5 mM) for 4 h and then with or without SB203580 (10 μ M) or SB202190 (10 μ M) for an additional hour, harvested and analyzed for firefly and *Renilla* luciferase activities. Firefly luciferase activities were normalized to *Renilla* luciferase reporter activities and shown as -fold induction compared with control (empty vector). Each value represents mean $\pm$ S.E.M. of four separate determinations

Additions	Relative p-1862 rStar-luc activity (U) $\pm$ S.E.M.
SB203580	6.56 $\pm$ 0.873*
SB202190	7.76 $\pm$ 0.850*
p38 MAPK α	2.96 $\pm$ 0.807
SB203580 + p38 MAPK α	7.18 $\pm$ 0.917 [†]
SB202190 + p38 MAPK α	9.42 $\pm$ 1.953 [†]

* $P < 0.01$ p38 MAPK α vs SB203580 or SB202190 and [†] $P < 0.01$ p38 MAPK α vs SB203580 + p38 MAPK α or SB202190 + p38 MAPK α .

MAPK α resulted in an efficient reduction in *Star* promoter activity under basal conditions. This reduction in basal promoter activity was completely restored following treatment of transfected cells with either SB203580 or SB202190 (Table 2). Qualitatively similar results were obtained when luciferase measurements were carried out using extracts from cAMP-treated cells (data not shown).

The results presented in Fig. 6 demonstrate that transient overexpression of p38 MAPK α -wt in MLTC-1 cells significantly decreased the endogenous mRNA levels of STAR protein both under basal conditions and in response to cAMP stimulation. In contrast, transient overexpression of a p38 MAPK α -dn significantly upregulated both basal as well as cAMP-stimulated *Star* mRNA levels. These results further confirmed that p38 MAPK α negatively regulates the gene transcription of STAR protein.

MKK3 and MKK6 contribute to the p38 MAPK-mediated suppression of *Star* promoter activity

Two closely related dual-specificity upstream protein kinases, MKK3 and MKK6, phosphorylate and activate p38 MAPK at the activation site Thr-Gly-Tyr (Han *et al.* 1996, Enslen *et al.* 1998, Wysk *et al.* 1999, Coulthard *et al.*

2009, Cuadrado & Nebreda 2010, Remy *et al.* 2010). We determined whether MKK3 and MKK6 play a role in p38 MAPK-mediated suppression of *Star* promoter activity. MLTC-1 cells (which express predominantly p38 MAPK α but also significant amount of p38 MAPK β , Fig. 2) were co-transfected with a rat *Star* firefly luciferase reporter construct as described earlier (rat p-1862 STAR-Luc) plus a reporter construct containing *Renilla* luciferase (*Rluc*, pRL-TK) with or without a plasmid encoding a ca form of MKK3/MMK6, FLAG-MKK3-ca (MKK3 (EE))/FLAG-MKK6-ca (MKK6 (EE)), or a dn MKK3/MKK6, FLAG-MKK3-dn (MKK (AA))/FLAG-MKK6-dn (MKK (AA)). After 48 h, the cells were treated with or without Bt₂cAMP (2.5 mM) for 5 h, harvested, and cell extracts analyzed for dual luciferase activities. As shown in Fig. 7A, overexpression of MKK3-ca significantly reduced both basal and cAMP-stimulated promoter activity. In contrast, transfections with an MKK3-dn construct upregulated both the basal as well as cAMP-stimulated reporter activity. Likewise, overexpression of MKK6-ca led to a significant inhibition of basal and cAMP-stimulated *Star* promoter activity (Fig. 7A). Similar to the effect of the dn form of MKK3, overexpression of MKK6-dn also enhanced promoter activity both under basal conditions and in response to cAMP stimulation. These results imply that both MKK3 and MKK6 are equally effective and participate in p38 MAPK-mediated repression of *Star* promoter activity.

Figure 6

Effects of transient overexpression of wt and dominant-negative p38 MAPK α on basal and cAMP-stimulated endogenous mRNA levels of *Star* in MLTC-1 cells. MLTC-1 cells were transiently transfected with empty vector, p38 MAPK α -wt, or p38 MAPK α -dn and, 48 h after transfection, the dishes were washed and subsequently incubated with or without Bt₂cAMP (2.5 mM) for 5 h. Following incubation, the dishes were subjected to total RNA isolation and the RNA samples quantified for *Star* (*StarD1*) mRNA levels by qRT-PCR. Each value represents mean $\pm$ S.E.M. of four separate determinations. * $P < 0.01$ Bt₂cAMP p38 α -dn vs basal (control) Bt₂cAMP; *** $P < 0.01$ Bt₂cAMP p38 α -wt vs basal (control) Bt₂cAMP; and ††† $P < 0.001$ Bt₂cAMP p38 α -dn vs Bt₂cAMP p38 α -wt.

Figure 7

Regulation of *Star* promoter activity in MLTC-1 cells in response to transient overexpression of MKK3, MKK6, or MKK6-p38 MAPK α/γ constructs and mouse embryonic fibroblasts (MEFs) lacking MKK3, MKK6, or both. (A) MLTC-1 cells were cotransfected with p-1862 *Star-Luc* (firefly luciferase) construct (1 μ g) plus *Renilla* luciferase (*Rluc*, pRL-TK) (50 ng), and empty vector, p38 MAPK α -wt, p38 MAPK α -dn, MKK3-ca, MKK3-dn, MKK6-ca, or MKK6-dn. At the end of 48 h, the dishes were treated with or without Bt₂cAMP (2.5 mM) for an additional 5 h and subsequently cell lysates assayed for firefly and *Renilla* luciferase activities. (B) MLTC-1 cells were transiently transfected with empty vector, p38 MAPK α -wt, p38 MAPK α -dn, MKK6-p38 MAPK α -ca, MKK6-p38 MAPK α -dn, MKK6-p38 MAPK γ -ca, or MKK6-p38 MAPK γ -dn and, after 48 h, further treated with or without Bt₂cAMP for 5 h. Cell lysates were assayed for firefly and *Renilla* luciferase activities as described above. (C) Wt (MKK3/6) MEFs and MEFs deficient in MKK3^{-/-}, MKK6^{-/-}, or both (MKK3^{-/-}/MKK6^{-/-}) were co-transfected with p-1862 *Star-Luc* (firefly luciferase) and *Renilla* luciferase (*Rluc*, pRL-TK) constructs for 48 h, stimulated with or without Bt₂cAMP for an additional 5 h, followed by determination of firefly and *Renilla* luciferase activities.

Next we undertook the approach of enzyme-substrate fusion (Robinson *et al.* 1998, Zheng *et al.* 1999, Qi *et al.* 2007) to further examine the MKK-p38 MAPK regulation of *Star* promoter activity. This approach is based on the fact that p38 MAPK becomes ca when linked to its upstream activator such as MKK6 (Qi *et al.* 2007). Herein, we used constructs in which p38 MAPK α -ca, p38 MAPK γ -ca, p38 α /AGF-dn, or p38 γ /AGF-dn (representative p38 MAPKs) were fused in-frame with the activator MKK6 (MKK6 activates all isoforms of p38 MAPK) through a decapeptide linker as reported previously by Qi *et al.* (2007). MLTC-1 cells were transiently transfected with *Star* promoter with or without expression vector encoding HA-MKK6-p38 MAPK α -ca, HA-MKK6-p38 MAPK γ -ca, HA-MKK6-p38 α /AGF-dn, or HA-MKK6-p38 γ /AGF-dn, and after 48 h cell lysates were analyzed for firefly and *Renilla* luciferase activities. As a positive control, some dishes were also transfected with p38 MAPK α (wt) or p38 MAPK α -dn plasmid DNA. Results presented in Fig. 7B demonstrate that transfection of cells with either MKK6-p38 MAPK α -ca or MKK6-p38 MAPK γ -ca suppressed the expression of *Star* promoter activity, whereas use of MKK6-p38 MAPK α /AGF-dn or MKK6-p38 MAPK γ /AGF-dn enhanced the promoter

(D) MKK3/6 and MKK3^{-/-}/MKK6^{-/-} DKO MEFs were co-transfected with p-1862 *Star-Luc* (firefly luciferase) and *Renilla* luciferase (*Rluc*, pRL-TK) constructs. In a third set MKK3^{-/-}/MKK6^{-/-} DKO MEFs were co-transfected with MKK3-ca + MKK6-ca plus p-1862 *Star-Luc* (firefly luciferase) and *Renilla* luciferase (*Rluc*, pRL-TK) constructs for 48 h followed by treatment with $\pm$ Bt₂cAMP. Cell lysates were assayed for firefly and *Renilla* luciferase activities, as described earlier. Each value represents mean $\pm$ S.E.M. of four separate determinations. ca, constitutively active; dn, dominant negative. All values presented (A) show statistically significant differences between basal vs Bt₂cAMP and basal (control) or basal Bt₂cAMP vs p38 α -wt, MKK3-ca, or MKK6-ca. The values presented in (B) show statistically significant differences between basal vs Bt₂cAMP and basal (control) or basal Bt₂cAMP vs p38 α -wt, MKK6-p38 α -ca, or MKK6-p38 γ -ca. (C) Basal (control) or basal Bt₂cAMP WT3/6 show statistically significant differences from the corresponding Bt₂cAMP MKK3^{-/-}, MKK6^{-/-}, or MKK3/6^{-/-} groups. (D) *** $P < 0.001$ wt MKK3/6 Bt₂cAMP vs MKK3/6^{-/-} Bt₂cAMP; and †† $P < 0.01$ MKK3/6^{-/-} Bt₂cAMP vs MKK3/6 plus MKK3-ca + MKK6-ca Bt₂cAMP.

activity. As expected, transfection of cells with p38 MAPK α -wt or p38 MAPK α -dn decreased and increased the promoter activity respectively. Thus, p38 MAPKs, including p38 MAPK α and their upstream activators MKK3 and MKK6, work in concert to modulate *Star* promoter activity.

To more directly define the roles that MKK3 and MKK6 play in p38 negative regulation of the *Star* promoter activity mediated by MAPKs, we assessed the expression of *Star* promoter activity in MEFs with targeted disruption of *Mkk3* (*Map2k3*), *Mkk6* (*Map2k6*) or both *Mkk3* and *Mkk6* genes. MKK3/6 (wt), MKK3^{-/-}, MKK6^{-/-}, and MKK3/6^{-/-} (DKO) MEFs (Branchio *et al.* 2003) were transfected with a rat *Star* reporter construct (48 h), treated with or without Bt₂cAMP (2.5 mM, 5 h), and, subsequently, cell lysates analyzed for luciferase activities. As shown in Fig. 7C, expression of *Star* promoter activity was substantially higher (both basal and cAMP stimulated) in single MKK3^{-/-} or MKK6^{-/-} MEFs as compared with wt MEFs that express both MKK3 and MKK6. Interestingly, use of MKK3/6^{-/-} DKO MEFs produced no additive effect, indicating that MKK3 and MKK6 play complementary roles and activate similar p38 MAPKs, such as p38 MAPK α that mediate negative regulation of *Star* promoter activity.

Finally, the results presented in Fig. 7D demonstrate that simultaneous overexpression of MKK3-ca and MKK6-ca in MKK3/6^{-/-} DKO MEFs significantly decreased both basal and cAMP-stimulated *Star* promoter luciferase activity as compared with cells transfected with the control DNA.

cAMP-stimulated *Star* mRNA and progesterone levels in relation to p38 MAPK

The above studies provided strong evidence that p38 MAPKs, particularly p38 MAPK α , function as negative regulators of *Star* promoter activity. We next evaluated the inhibitory actions of p38 MAPK α on steady-state *Star* mRNA levels and its relevance to steroidogenesis. Expression of p38 MAPK α -wt significantly decreased both basal and cAMP-induced *Star* mRNA levels. In contrast, when MLTC-1 cells were transfected with p38 MAPK α -dn, basal and cAMP-stimulated levels were significantly increased compared with levels for the control cells and the cells transfected with p38 MAPK α -wt (data not shown).

We also measured progesterone synthesis and secretion in the culture medium from cells transfected with either p38 MAPK α -wt or p38 MAPK α -dn and subsequently stimulated without or with Bt₂cAMP. As shown in Table 3, progesterone accumulation in each case paralleled changes in *Star* mRNA expression. The non-stimulated MLTC-1 cells transiently expressing p38 MAPK α (wt) decreased progesterone production by approximately 75–80%, whereas expression of p38

MAPK α -dn increased medium progesterone levels by approximately twofold. Likewise, cAMP-stimulated progesterone secretion by p38 MAPK α -wt-expressing cells was reduced by approximately 50% when compared with that seen in control cells. On the other hand, expression of p38 MAPK α -dn resulted in approximately a twofold increase in cAMP-stimulated progesterone production as compared with control cells (Table 3).

Effect of p38 MAPK on CREB-mediated regulation of *Star* promoter activity

Expression of *Star* is mainly regulated at the transcriptional level through a cAMP-protein kinase A (PKA)-dependent mechanism (Manna *et al.* 2009). Herein, we examined the effect of p38 MAPK on CREB-mediated regulation of *Star* promoter activity. As the CREB-dependent transcriptional response is primarily dependent on CREB phosphorylation (Shaywitz & Greenberg 1999, Johannessen *et al.* 2004, Altarejos & Montminy 2011), we initially examined the effects of p38 MAPK α -wt and p38 MAPK α -dn on the cAMP/PKA-stimulated phosphorylation (Ser¹³³) of CREB protein in MLTC-1 cells. The results presented in Fig. 8 demonstrate the effects of p38 MAPK α -wt and p38 MAPK α -dn constructs on the levels of the cAMP/PKA-stimulated phosphorylated form of CREB (p-CREB). Transfection of cells with p38 MAPK α -wt increased cAMP-stimulated phospho-CREB levels, and the use of p38 MAPK α -dn resulted in much greater stimulation of its phospho-levels.

The results presented in Fig. 8 also demonstrate that phosphorylation of ATF-1, which is 65% identical to CREB in its primary amino acid sequence (Shaywitz & Greenberg 1999), was also increased in response to the overexpression of p38 MAPK α -wt or p38 MAPK α -dn construct. The potential effect of p38 MAPK α on CREB was further studied in MLTC-1 cells using a CRE-reporter (luciferase) construct. The data presented in Fig. 9A show that transient transfection of MLTC-1 cells with CRE, CREB, and p38 MAPK α -wt resulted in a significant reduction in basal CRE-luciferase activity as compared with cells transfected with CRE alone or CRE+CREB. In contrast, transient expression of CRE, CREB, and p38 MAPK α -dn in these cells resulted in an approximately 50% increase in basal CRE-luciferase activity compared with results obtained with CRE or CRE+CREB construct. Results presented in Fig. 9B demonstrate that co-transfection of the cells with p38 MAPK α -wt decreased the Bt₂cAMP-induced luciferase activity. Expression of p38 MAPK α -dn construct, however, showed no effect on CRE-luciferase

Table 3 Effects of overexpression of native/wt and dominant-negative (dn) forms of p38 MAPK α on steroid secretion by MLTC-1 cells. Mouse Leydig tumor cells, MLTC, were transiently transfected with empty vector, plasmid-encoding native (wt) FLAG p38 MAPK α (1 μ g), or dn Flag p38 MAPK α (1 μ g). After 48 h, cells were treated with or without Bt₂cAMP (2.5 mM) for 5 h and subsequently culture medium samples collected and analyzed for progesterone secretion by RIA. Each value represents mean $\pm$ S.E.M. of four separate determinations

Additions	ng Progesterone secreted/10 ⁵ cells/5 h $\pm$ S.E.M.
Basal	8.53 $\pm$ 0.45*
Bt ₂ cAMP (2.5 mM)	34.83 $\pm$ 3.01 [†]
p38 MAPK α -wt	2.43 $\pm$ 0.41
p38 MAPK α -wt + Bt ₂ cAMP (2.5 mM)	17.20 $\pm$ 0.33 [†]
p38 MAPK α -dn	17.84 $\pm$ 0.95 [†]
p38 MAPK α -dn + Bt ₂ cAMP (2.5 mM)	72.33 $\pm$ 1.84 [§]

* $P < 0.05$ basal (control) vs p38 MAPK α -wt; [†] $P < 0.001$ Bt₂cAMP vs MAPK α -wt; [‡] $P < 0.01$ MAPK α -wt vs p38 MAPK α -wt + Bt₂cAMP or p38 MAPK α -dn; and [§] $P < 0.001$ MAPK α -wt vs p38 MAPK α -dn + Bt₂cAMP.

Figure 8

Effects of overexpression of p38 MAPK α -wt and p38 MAPK α -dn on the levels of total (t) and phospho-forms of CREB in response to treatment of cells with or without Bt₂cAMP. Cells were transfected with either an empty vector, p38 MAPK α -wt, or p38 MAPK α -dn, and, after 48 h, treated with or without Bt₂cAMP. The cell lysates were subjected to SDS-PAGE followed by

western blotting analysis. The blots were developed using anti-CREB (total) or anti-phospho-CREB (Ser¹³³). (A) Western blot of p-CREB, t-CREB and p-ATF. (B) Quantification of ratios of p-CREB:t-CREB. (C) Quantification of ratios of p-ATF:t-ATF. t, total; p, phosphorylated.

activity in response to cAMP stimulation (Fig. 9B). We also evaluated the effects of overexpression of p38 MAPK α -wt and p38 MAPK α -dn on AP1 (TRE) luciferase activity in MLTC-1 cells. As illustrated in Fig. 9C and D, transient expression of p38 MAPK α -wt or p38 MAPK α -dn, however, showed no effect on AP1 luciferase activity either under basal conditions or in response to TPA stimulation. These results led us to conclude that p38 MAPK α inhibits cAMP-induced Star promoter activity primarily by interfering

with the PKA-mediated CREB phosphorylation and its functional expression.

Discussion

STAR protein plays a pivotal role in the tropic peptide hormone-regulated acute phase of steroidogenesis, mediating the rate-limiting translocation of cholesterol from the OMM to the IMM, where side-chain cleavage enzyme

Figure 9

p38 MAPK α -mediated downregulation of CRE (A and B), but not AP1/TRE (C and D), reporter activity in MLTC-1 cells. MLTC-1 cells were transfected with Cre-Luc (firefly luciferase) + Renilla luciferase (Rluc, pRL-TK), with or without CREB, or CREB + p38 MAPK α -wt or p38 MAPK α -dn for 48 h. At the end of the incubation, washed dishes were further incubated with or without Bt₂cAMP followed by measurement of firefly and Renilla luciferase activities. Likewise, cells were transfected with AP1/TRE-luc (firefly

luciferase) + Renilla luciferase (Rluc, pRL-TK), with or without p38 MAPK α -wt or p38 MAPK α -dn, and, after 48 h, dishes were further incubated with or without AP1/TRE activator TPA (10 nM) for 30 min. The cell lysates were then quantified for firefly and Renilla luciferase activities. Each value represents mean ± S.E.M. of four separate determinations. ** P < 0.01 CRE + CREB p38 α -dn vs CRE + CREB p38 α -wt; *** P < 0.001 basal (control) vs Bt₂cAMP; ** P < 0.01 basal (control) vs TPA; and *** P < 0.001 basal (control) vs TPA.

(P450scc; Cyp11A) carries out the first committed step in steroidogenesis, i.e. conversion of cholesterol to pregnenolone, the precursor of all steroid hormones (Stocco & Clark 1996, Stocco 2001, Hu *et al.* 2010, Miller & Bose 2011). We and others previously reported that expression of STAR protein is downregulated in both the adrenal gland and testicular Leydig cells of aging rats, and, as a result, adequate amounts of cholesterol are not transported to mitochondria, leading to defective steroidogenesis during aging (Leers-Sucheta *et al.* 1999, Luo *et al.* 2005, Wang *et al.* 2005, Sun *et al.* 2008). These changes have been linked to excessive oxidative stress and increased ROS production (Azhar *et al.* 1995, Chen *et al.* 2001, 2008, Cao *et al.* 2004, Abidi *et al.* 2008a,b, Zaidi *et al.* 2012). The observed increased expression of the active (phospho) form of oxidant-sensitive p38 MAPK α during aging led to the suggestion that this kinase mediates the anti-steroidogenic actions of excessive oxidative stress during aging (Abidi *et al.* 2008a,b). The current study examined the molecular mechanisms by which oxidant-sensitive p38 MAPK negatively affects *Star* gene transcription. Our initial qRT-PCR data indicate that *Star* (*StarD1*) is most abundantly expressed in both Y-1 and MLTC-1 cells and that Bt₂cAMP stimulation further increased its mRNA levels by fourfold to fivefold in both cell types. The expression of *StarD2* and *StarD3*, which belong to the same subgroup as *StarD1* as well as a second subgroup of STARD proteins, STARD4, STARD5, and STARD6, was very low in Y-1 and MLTC-1 cells and their expression was not affected by cAMP stimulation of either cell type. These results are at variance with the results reported for another mouse Leydig tumor cell line, MA-10, where stimulation of cells with a cAMP analog was reported to stimulate mRNA expression of *StarD4*, in addition to a robust induction of *Star/StarD1* (Soccio *et al.* 2005). Similar to our data, however, the mRNA levels of *StarD3* and *StarD5* were induced by cAMP stimulation; the levels of *StarD2*, which is a phosphatidylcholine transfer protein (PCTP/*StarD2*) and *StarD6*, whose expression is limited to germ cells of testis (Soccio *et al.* 2002, Gomes *et al.* 2006, Bose *et al.* 2008), were not determined in this model Leydig cell line (Soccio *et al.* 2005). Interestingly, it has been shown that *StarD6* exhibits similar activity to that of *Star/StarD1* in inducing steroidogenesis in a cell-free mitochondrial system (Bose *et al.* 2008). Our qRT-PCR measurements also indicated that p38 MAPK α is the predominant endogenous isoform in both cell lines, although significant expression of p38 MAPK β was also noted in MLTC-1 cells. In contrast, only low levels of p38 MAPK γ and p38 MAPK δ were detected in both cell types. These results are

in agreement with an earlier report from this laboratory (Abidi *et al.* 2008b), but are at variance with another report showing that p38 MAPK δ is expressed at very high levels in human steroidogenic tissues (Wang *et al.* 1997). Whether the low expression of p38 MAPK δ noted here in mouse Y-1 adrenocortical tumor cells and mouse MLTC-1 Leydig tumor cells represents a species difference or tumor effect needs to be explored. As p38 MAPK α isoform (mRNA) is highly expressed in both Y-1 and MLTC-1 cells, most of the studies aimed at defining the effects of p38 MAPK on *Star/StarD1* gene transcription were carried out with p38 MAPK α . However, we acknowledge that mRNA levels of the four p38 MAPKs in theory may not reflect the actual protein levels. Technical difficulties associated with currently available isoform-specific p38 MAPK antibodies, as well as lack of availability of p38 MAPK δ antibody, prevented us from measuring the protein levels of individual p38 MAPK isoforms.

In accordance with a previous observation that ROS-mediated excessive oxidative stress inhibits steroidogenesis (Margolin *et al.* 1990, Gatzuli *et al.* 1991, Endo *et al.* 1993, Stocco *et al.* 1993, Kodaman *et al.* 1994, Musicki *et al.* 1994, Carlson *et al.* 1995, Diemer *et al.* 2003, Abidi *et al.* 2008b), our data demonstrate that treatment of Y-1 and MLTC-1 cells with oxidants, superoxide anion (O₂⁻), H₂O₂, or a lipid peroxidation product, 4-HNE results in a significant reduction in the levels of *Star* mRNA both under basal conditions and in response to cAMP stimulation. In addition, we provide evidence that exposure of MLTC-1 cells transiently overexpressing p38 MAPK α to the above mentioned three oxidants resulted in increased phosphorylation (activation) of p38 MAPK α , along with decreased *Star* promoter activity. The specificity studies demonstrated that p38 MAPK α , p38 MAPK β , and p38 MAPK γ were highly effective at suppressing *Star* promoter activity both under basal conditions and in response to cAMP stimulation, although p38 MAPK α was relatively more effective than p38 MAPK β or p38 MAPK γ . In contrast, expression of dn forms of p38 MAPK α , p38 MAPK β , or p38 MAPK γ increased the expression of basal as well as cAMP-stimulated promoter activity. Likewise p38 MAPK α/β inhibitors, SB203580 or SB202190, prevented p38 MAPK α -mediated suppression of promoter activity, further confirming that p38 MAPK functions as a negative regulator of *Star* gene transcription. Although overexpression of p38 MAPK δ -wt construct exhibited no significant effect on basal *Star* promoter activity, transient overexpression of p38 MAPK δ -dn, however, significantly increased the cAMP-stimulated promoter activity over the values seen with the use of p38 MAPK δ -wt construct.

Finally, use of various constructs of upstream activators of p38 MAPKs, MKK3 and MKK6 (Derijard *et al.* 1995, Raingeaud *et al.* 1995, 1996, Han *et al.* 1996), MKK6–p38-MAPK α , and MKK6–p38-MAPK γ fusion constructs (Robinson *et al.* 1998, Zheng *et al.* 1999, Qi *et al.* 2007) and MEFs with targeted disruption of *Mkk3*, *Mkk6*, or both *Mkk3* and *Mkk6* genes (Brancho *et al.* 2003, Kang *et al.* 2006) further established that p38 MAPKs, particularly oxidant-sensitive p38 MAPK α , are involved in the negative regulation of *Star* gene transcription.

Extensive evidence now indicates that the expression of *Star* is mainly regulated at the transcriptional level through a cAMP-PKA-dependent mechanism (Manna *et al.* 2009). Transcriptional activation of target genes by the cAMP-PKA signaling pathway is mainly achieved via cAMP-responsive element (CRE)-binding protein (CREB) transcription factor. cAMP-activated PKA catalyzes the phosphorylation of CREB at serine residue 133 (Altarejos & Montminy 2011). Phosphorylation of CREB at Ser¹³³ is required for CREB-induced gene transcription and promotes recruitment of the transcriptional co-activator CREB-binding protein (CBP) and its paralogue p300 (Mayr & Montminy 2001). Although *Star* promoter lacks a consensus CRE (TGACGTCA), three CRE-like sites, CRE1, CRE2, and CRE3, have been characterized in the proximal region of the *Star* promoter (Manna *et al.* 2002), of which the CRE2 site overlaps with an AP1/TRE (5-TGA^G/CTCA-3)-binding site (Manna & Stocco 2007) that can bind both CRE (CREB and its family member CRE modulator (CREM), and ATF1 and also ATF2) and AP1/TRE (Fos/Jun)-binding proteins (Shaywitz & Greenberg 1999, Shaulian & Karin 2002). Our findings demonstrate that transient overexpression of p38 MAPK α -wt increased phospho-CREB (p-Ser¹³³) levels, and the use of p38 MAPK α -dn resulted in a greater increase in phospho-CREB (p-Ser¹³³) levels. Interestingly, neither p38 MAPK α -wt nor p38 MAPK α -dn increased the phosphorylation status of ATF-1, which is 65% identical to CREB in its primary amino acid sequence (Shaywitz & Greenberg 1999). At present, we do not know the precise mechanism(s) by which p38 MAPK α and p38 MAPK α -dn upregulate the cAMP-PKA-mediated Ser¹³³ phosphorylation of CREB. As CREB is also a substrate for the p38 MAPK-regulated MSK kinase (Hauge & Frödin 2006), it is possible that both PKA and MSK simultaneously but variably catalyze CREB phosphorylation and in this process lead to increased CREB phosphorylation. The data obtained with the dn form of the p38 MAPK α raised another possibility that other kinases (e.g. PKC, calmodulin kinases, and glycogen synthase kinase; Impey & Goodman 2001, Johannessen

et al. 2004, Johannessen & Moens 2007) that also phosphorylate CREB may cooperate with p38 MAPK α /MSK and interfere with the ability of PKA to catalyze Ser¹³³ phosphorylation. These various possibilities are likely to decrease CREB activity and, consequently, *Star* gene transcription and its promoter activity.

The potential effect of p38 MAPK α on CREB function was studied in MLTC-1 cells using a CRE-reporter (luciferase) approach. Various measurements led to the conclusion that p38 MAPK α , by interfering with PKA-mediated Ser¹³³ phosphorylation of CREB, inhibits its function. On the other hand, both p38 MAPK α -wt and p38 MAPK α -dn proteins showed no significant effect on AP1 reporter activity. We used AP1 (TRE) as a control, because the CRE and AP1 (TRE) consensus sequences differ by only one nucleotide (CRE, TGACGTCA and AP1/TRE, TGACTCA) (Hai & Curran 1991, Shaulian & Karin 2002) and both recognition sequences can bind ATF/CREB and the Fos/Jun family of proteins, albeit with a variable affinity (Hai & Curran 1991).

In summary, our data indicate that oxidant/ROS exposure of steroidogenic cells leads to simultaneous inhibition and activation of *Star* mRNA and phospho-p38 MAPK protein levels respectively. In addition, results regarding *Star* promoter activity reported in this study indicate that p38 MAPK α and to some extent p38 MAPK β and p38 MAPK γ inhibit *Star* gene transcription and that upstream kinases, MKK3 and MKK6, play a significant role in this process. Finally, our data provide evidence that p38 MAPK α negatively regulates *Star* gene transcription by potentially interfering with cAMP-PKA-mediated phosphorylation and the functional expression of the CREB transcription factor.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

S K Z, W-J S, F B K, and S A conceived and designed the experiments; S K Z performed the experiments; W-J S, F B K, and S A interpreted the results; S K Z, S B, W-J S, F B K, and S A drafted and edited the manuscript; S B and A B prepared the figures; M P M, J H, and R J D provided critical reagents; and S K Z, W-J S, S B, A B, M P M, J H, R J D, F B K, and S A approved the final version of the manuscript.

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