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Evidence that age-related changes in p38 MAP kinase contribute to the decreased steroid production by the adrenocortical cells from old rats

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Summary

The current studies were initiated to investigate whether excessive oxidative stress exerts its antisteroidogenic action through modulation of oxidant-sensitive mitogen-activated protein kinase (MAPK) signaling pathways. Western blot analysis indicated that aging caused increased phosphorylation and activation of rat adrenal p38 MAPK, but not the ERK1/2 or JNK1/2. Lipid peroxidation measurements (an index of cellular oxidative stress) indicated that adrenal membranes from young animals contained only minimal levels of endogenous thiobarbituric acid-reactive substances (TBARS), and exposure of membranes to enzymatic and non-enzymatic pro-oxidants enhanced TBARS formation approximately 12- and 20-fold, respectively. The adrenal membranes from old animals showed much more susceptibility to lipid peroxidation and exhibited roughly 4- to 6-fold higher TBARS formation than young controls both under basal conditions and in response to pro-oxidants. Qualitatively similar results were obtained when lipid peroxide formation was measured using a sensitive FOXRS (ferrous oxidation-xylenol orange-reactive substances) technique. We next tested whether aging-induced excessive oxidative insult alters steroidogenesis through modulation of MAPK signaling pathway. Treatment of adrenocortical cells from old rats with specific p38 MAPK inhibitors restored Bt2cAMP-stimulated steroidogenesis ~60–70% of the value seen in cells of young animals. Likewise, pretreatment of cells with reactive oxygen species (ROS) scavengers MnTMPyP and *N*-acetyl cysteine also partially rescued age-induced loss of steroid production. In contrast, simultaneous treatment of cells with ROS scavengers and p38 MAPK inhibitor did not produce any additional effect suggesting that both types of inhibitors exert their stimulatory action through inhibition of p38 MAPK activation. Collectively, these results indicate that p38 MAPK functions as a signaling effector in oxidative stress-induced inhibition of steroidogenesis during aging.

Key words: antioxidants, corticosterone, MnTMPyP, NAC, oxidative stress, protein phosphorylation and cell signaling, steroidogenesis.

Introduction

Aging in both humans (Lamberts *et al.*, 1997; Harper *et al.*, 1999; Shifren & Schiff, 2000; Burger *et al.*, 2002; Arlt, 2004; Dharia & Parker, 2004; Harman, 2005; Kaufman & Vermeulen, 2005; Veldhuis *et al.*, 2005) and experimental animals (Popplewell *et al.*, 1986; Belloni *et al.*, 1992; Liao *et al.*, 1993; Zirkin & Chen, 2000; Cao *et al.*, 2004; Wang & Stocco, 2005 and references therein) is associated with a significant decline in the synthesis and secretion of steroid hormones. Work over the past several years from this laboratory has led to the realization that inefficient mobilization of stored cholesteryl esters and transport of free cholesterol from the putative 'cholesterol pool' to mitochondrial CYP11A1 (P450scc) sites for enzymatic conversion of cholesterol to pregnenolone and other steroid hormones are the two critical events responsible for age-related loss of steroidogenic response (Popplewell & Azhar, 1987; Liao *et al.*, 1993). Advancing age is also associated with reduced levels of the two cholesterol-binding proteins, steroidogenic acute regulatory (StAR) protein and peripheral-type benzodiazepine receptor (PBR) (Leers-Sucheta *et al.*, 1999; Luo *et al.*, 2001; Culty *et al.*, 2002). These proteins in concert with several other proteins have been implicated in the intramitochondrial transfer of cholesterol, the rate-limiting and regulated step in steroid biosynthesis (Liu *et al.*, 2003; Manna & Stocco, 2005).

Additional studies from this laboratory further demonstrated that aging also leads to excessive oxidative stress as a result of increased reactive oxygen species (ROS) production, inactivation of the enzymatic antioxidant system, and reduction in the levels of low-molecular weight non-enzymatic antioxidants in adrenal and testicular tissues (Azhar *et al.*, 1995; Cao *et al.*, 2004). Furthermore, we reported a relationship between age and inactivation of the adrenal oxidant-sensitive transcription

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factors, activator protein-1 (AP-1), and nuclear factor kappaB (NF-κB) (Medicherla *et al*., 2001, 2002). These observations were interpreted to suggest that excessive ROS generation and macromolecular damage, especially lipid peroxidation-mediated oxidative damage of cellular membranes involved in intracellular cholesterol transport and steroidogenesis, may impair their function leading to decreased steroidogenesis. The potential risk for damage to macromolecules from excessive oxidative insult is especially high for steroidogenic tissues, which use molecular oxygen not only for energy (ATP) production, but also for steroid biosynthesis, and thus exhibit high rates of oxidative metabolism and generation of ROS (Azhar *et al*., 1995; Hanukoglu, 2006). Indeed, it has been shown that free radicals inhibit steroidogenesis by interfering with cellular events connected with cholesterol transport to mitochondria and/or catalytic function of P450 enzymes (Kodaman *et al*., 1994; Musicki *et al*., 1994; Diemer *et al*., 2003; Abidi *et al*., 2004). Although the exact mechanism by which excessive oxidative stress contributes to the age-related loss of steroidogenic response is not well understood, accumulating evidence in other systems now indicates that persistent low-level, long-term oxidative stress especially during aging can trigger activation of mitogen-activated MAP kinase (MAPK) pathways leading to altered gene expression that may potentially affect cellular metabolic processes and biological responses (Finkel & Holbrook, 2000; Martindale & Holbrook, 2002; Matsuzawa & Ichijo, 2005; McCubrey *et al*., 2006). Given this, there is a likely possibility that chronic oxidative stress-induced modulation of MAPK cascade(s) may be an important event in the age-related decline in steroidogenesis. However, until now the identity of such intracellular signaling cascade(s) by which excessive oxidative stress may negatively impact steroidogenesis during aging has not been evaluated.

In mammalian cells, several different subfamilies of MAPK have been identified (Kyriakis & Avruch, 2001; Pearson *et al*., 2001). Among these, three best characterized MAPKs are the extracellular signaling-regulated kinases (ERKs) p44 MAPK (ERK 1) and p42 MAPK (ERK2); c-Jun NH_2-terminal kinases (JNKs), also referred to as stress-activated protein kinases (SAPKs) that include JNK2 (p54 SAPK, SAPKα/β) and JNK1 (p45 SAPK, SAPKγ); and p38 MAPKs (α, β, γ, and δ). Each of these MAPKs is phosphorylated and catalytically activated by a signaling cascade comprising a specific upstream MAPK kinase (MKKs), which dually phosphorylates MAPKs on threonine (T) and tyrosine (Y) residue separated by an intervening amino acid characteristic for each MAPK subfamily (i.e. TEY for ERKs, TPY for JNKs, and TGY for p38 MAPKs). MKK is activated by MKK kinase (MKKK) through the phosphorylation of specific T and Y residues in MKKs. Whereas ERKs are typically stimulated by growth factors and mitogenic stimuli, p38 MAPK and JNK are strongly activated by various cellular stresses including ROS (Kyriakis & Avruch, 2001; Pearson *et al*., 2001; Martindale & Holbrook, 2002; Matsuzawa & Ichijo, 2005; McCubrey *et al*., 2006). The phosphorylated and fully activated MAPKs are subsequently translocated to the cell nucleus where they phosphorylate and activate multiple transcription factors, ultimately resulting in the altered transcription of specific genes (Edmunds & Mahadevan, 2004).

In an attempt to assess whether MAPKs facilitate oxidative stress-dependent loss of steroidogenesis during aging, we have investigated effects of aging, antioxidants and MAPK inhibitors on steroidogenesis in adrenal and adrenal cells from old rats. Our data indicate that expression of p38 MAPK is significantly increased in response to aging. This effect appears to be specific for p38 MAPK as the expression of neither ERKs nor JNKs was altered during aging. We also present data showing that p38 inhibitors and antioxidants partially restore the steroidogenesis in cells from old animals and that the observed rescue of steroidogenesis is likely to stem from effects of the inhibitors on p38 MAPKs. From these studies we conclude that p38 MAPK signaling pathway facilitates the inhibitory actions of excessive oxidative insult on adrenal steroid hormone production caused by aging.

Results

Aging and corticosterone production by isolated rat adrenocortical cells

Initially, experiments were conducted to confirm the previous observations that aging is associated with a decline in hormone-stimulated corticosterone production by freshly isolated rat adrenocortical cells. Figure 1 shows corticosterone production by adrenocortical cells from the young mature (5 months old) and old (24–27 months old) rats and incubated for 5 h in the absence (basal) or presence of a maximally effective dose of Bt_2cAMP (2.5 mM) (the second messenger of ACTH action). The addition of a maximally stimulating dose of Bt_2cAMP to cells from control animals (adequate circulating levels of cholesterol)

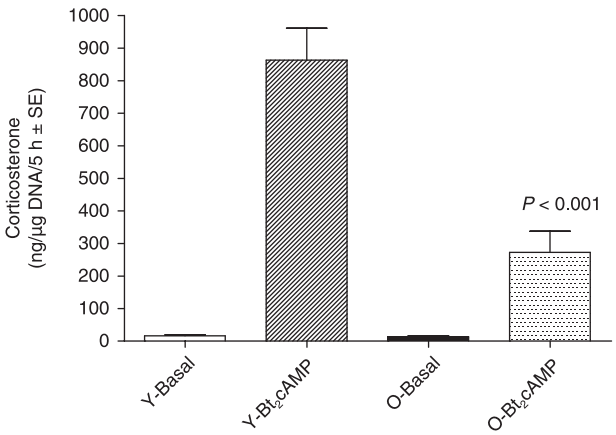

Fig. 1 Basal and cAMP-stimulated corticosterone production by isolated adrenocortical from young and old rats. Adrenocortical cell suspensions were prepared from young (5 months old) and old (4–27 months old) animals, and suitable aliquots (~5×10^5 cells) were incubated in the presence and absence of Bt_2cAMP (2.5 mM) for 5 h at 37 °C. The amount of corticosterone produced was measured by RIA as described under 'Materials and Methods'. Results are mean ± standard error of four separate experiments.

Conditions	Young	Old
TBARS		
(nmol MDA equivalent formed mg^{-1} protein h^{-1} ± SE)		
Basal	0.109 ± 0.026	0.463 ± 0.089*
Enzymatic (NADPH + $FeSO_4$ + ADP)	1.330 ± 0.283	7.847 ± 1.268†
Non-enzymatic ($FeSO_4$ + ascorbic acid)	2.171 ± 0.456	9.600 ± 1.120‡
FOXRS		
(nmol lipid peroxides formed mg^{-1} protein h^{-1} ± SE)		
Basal	3.112 ± 0.756	13.010 ± 1.867§
Enzymatic (NADPH + $FeSO_4$ + ADP)	34.390 ± 8.800	183.000 ± 36.320¶
Non-enzymatic ($FeSO_4$ + ascorbic acid)	ND	ND

Table 1 Effect of aging on TBARS and FOXRS formation in particulate fractions of adrenal glands from young mature (5 months old) and old rats (24–27 months old) with or without prior exposure to enzymatic or non-enzymatic pro-oxidants

*P = 0.0072; †P = 0.0024; ‡P = 0.0009; §P = 0.0027; ¶P = 0.0073.
Results are mean ± standard error (SE) of four separate experiments.
The incubation mixture in a final volume of 1.0–3.0 mL contained total adrenal membrane fraction (equivalent to 5 mg tissue mL^{-1}) from young (5 months old) and old (24–27 months old) rats and 1 mM NADPH + 50 μM $FeSO_4$ + 5 mM ADP (enzymatic) or 5 μM $FeSO_4$ + 500 μM sodium ascorbate (non-enzymatic). Following incubation at 37 °C for 60 min, 1 mL aliquots were analyzed for the formation of TBARS and FOXRS as described in Materials and Methods. ND, not determined; TBARS, thiobarbituric acid-reactive substances; FOXRS, ferrous oxidation-xylenol orange-reactive substances.

resulted in a general increase in hormone production over basal levels, but showed a significant reduction (~70%) in old vs. young rats. In contrast, the corticosterone response to 20α-hydroxycholesterol (20α-hydroxycholesterol, a freely diffusible form of cholesterol) was unaffected by aging, further indicating that the observed decline in steroidogenesis was related to an inability of aging cells to effectively transport steroid substrate, cholesterol to mitochondria and not due to alterations in second messenger signaling or the enzymes involved in corticosterone production (data not shown).

Effect of age on basal (endogenous) and pro-oxidant induced membrane lipidperoxidation

TBARS (thiobarbituric acid-reactive substances) and FOXRS (ferrous oxidation-xylenol orange-reactive substances) assays were employed to measure age-induced oxidative damage (i.e. peroxidative damage or lipid peroxidation) in the isolated adrenal cell membranes. These measurements were made both under basal conditions and in response to enzymatic (Fe^{2+}/ADP/NADPH) or non-enzymatic (Fe^{2+}/ascorbate) lipid-peroxidation initiators. Although we realize that malondialdehyde-thiobarbituric acid (MDA-TBA) (TBARS) technique as employed here has limited specificity and sensitivity (Bird & Draper, 1984; Lykkesfeldt, 2007), we used this technique to simply quantify for the oxidative damage that may be occurring in aging adrenals. In the past, we have used both a direct colorimetric method and a highly specific but technically challenging HPLC method to quantify for the formation of MDA-TBA products (TBARS) in the adrenal homogenates (Azhar *et al*., 1995). Interestingly, both methods gave qualitatively similar results (Azhar *et al*., 1995). The FOXRS method also has limitation in that it not only measures lipid hydroperoxides but also measures protein hydroperoxides (Gay & Gebicki, 2003). Again, we used this technique to get an independent estimate of age-related oxidative damage to adrenal membranes. Moreover, measurement of protein and lipid hydroperoxides by the modified ferric-xylenol orange method of Gay & Gebicki (2003) indicated that in adrenal homogenates from old animals, lipid hydroperoxides represent > 90% of the total hydroperoxides formed (data not shown).

The results presented in Table 1 indicate that adrenal membranes from young animals contained only minimal levels of endogenous TBARS, and that exposure of membranes to enzymatic and non-enzymatic pro-oxidants enhanced TBARS formation by approximately 12- and 20-fold, respectively. The adrenal membranes from old animals showed much more susceptibility to oxidative damage and exhibited roughly 4- to 6-fold higher TBARS formation than young controls both under basal conditions and in response to pro-oxidants. Qualitatively similar results were obtained when lipid peroxide formation was measured using a sensitive FOXRS technique (Table 1).

Activation of MAPK signaling pathways in response to aging

We next examined the effect of aging on the activation of ERKs, JNKs and p38 MAPK, the three well-characterized members of the MAP kinase family (Kyriakis & Avruch, 2001; Pearson *et al*., 2001) that are known to be activated by oxidative stress (Martindale & Holbrook, 2002; Matsuzawa & Ichijo, 2005; McCubrey *et al*., 2006). As phosphorylation of residues Thr^{202} and Tyr^{204} in ERK1/2, residues Thr^{180} and Tyr^{182} in p38 MAPKs, and residues Thr^{183} and Tyr^{185} in JNKs results in enzymatic activation, we employed Western blot analysis and phospho-specific antibodies to monitor phosphorylation of these sites as an index of kinase activation (Kelley *et al*., 2004). Significant levels of endogenous phosphorylation of all three MAPKs were observed in whole adrenal homogenates from both young and old rats (Fig. 2). Furthermore, we observed that aging significantly increased the phosphorylation of p38 MAPK (~3-fold), but not

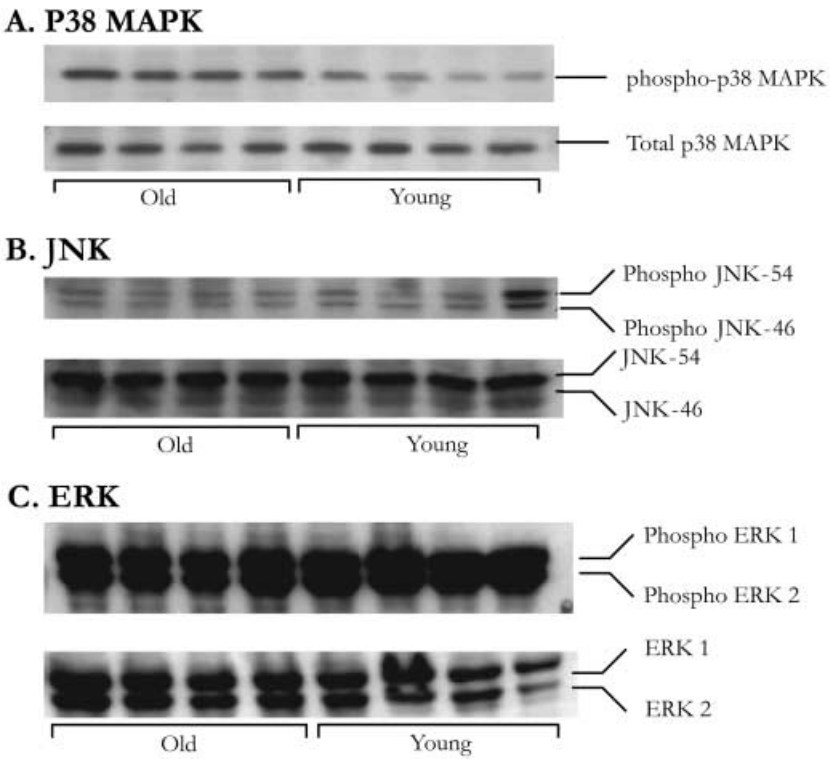

Fig. 2 Age-dependent changes in the activation of rat adrenal ERKs, JNKs, and p38 MAPK. Whole adrenal extracts were prepared from young (5 months old) and old (24–27 months old) rats as described under 'Experimental Procedures'. Equal aliquots of solubilized samples (30–40 μg protein) were subjected to SDS-PAGE and immunoblotted with antibodies against ERK1/2 phosphorylated at Thr^{202}/Tyr^{204} (P-ERK1/2), against p38 MAPK phosphorylated at Thr^{180}/Tyr^{182} (P-p38 MAPK), or JNK1/2 phosphorylated at Thr^{183}/Tyr^{185} (P-JNK1/2). The blots were stripped and re-probed using the antibodies that recognize total (phospho- plus nonphospho-forms) ERK1/2, p38α MAPK, and JNK1/2 proteins. The blots shown represent four independent experiments. Numerical results were obtained by densitometric scanning of the individual bands.

of ERK1/2 or JNK1/2 in adrenal samples (Figs 2 and 3). As the phospho-p38 MAPK antibody used in our study does not differentiate among the type of p38 MAPK isoforms expressed in rat adrenals, specific age-related changes in the activation and expression of individual isoforms could not be assessed.

Involvement of p38 MAPK during oxidant-mediated inhibition of steroidogenesis

Data shown in Figs 2 and 3 suggest possible involvement of the p38 MAPK signaling cascade in age-related and oxidative stress-mediated loss of adrenal steroid hormone production. Here we sought to further examine the potential relationship between oxidative stress-induced activation of p38 MAPK and the ability of excessive oxidative stress to inhibit hormone-stimulated 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 separate and combined effects of cell-permeable chemical inhibitors of p38 MAPK (Lee *et al*., 1994; Cueda *et al*., 1995; Guan *et al*., 1997; Page *et al*., 2001; Natale *et al*., 2004) and non-enzymatic antioxidants (Pimentel *et al*., 2001; Uchida *et al*., 2004).

Treatment of adrenal cells from old animals with 5.0 and 10 μM concentrations of SB202190, SC68376 or SB220025 (structurally divergent inhibitors of p38 MAPK α/β) resulted in a significant increased production of corticosterone as compared to vehicle control (Fig. 4B). In each case, however, the addition of a higher concentration of inhibitor (10 μM) produced slightly better stimulation relative to lower concentration.

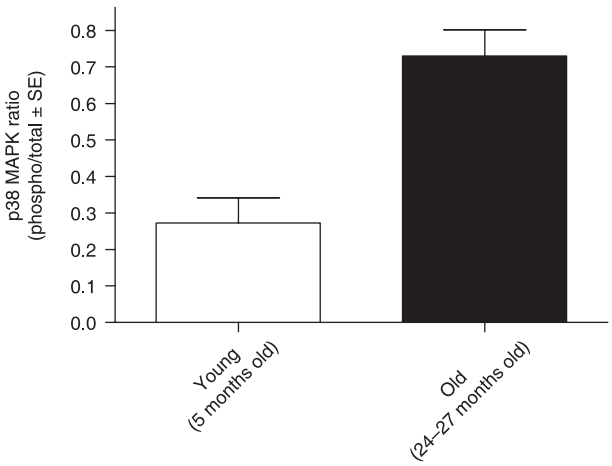

Fig. 3 Activation of p38α MAPK in response to advancing age. Individual bands representing phospho- and total-forms of p38α MAPK from Fig. 2 were analyzed by densitometric scanning. After scanning, data from four separate experiments (Fig. 2) were expressed as a ratio of phospho-p38α MAPK/total p38α MAPK and results are shown as mean ± standard error (SE).

In contrast, the biologically inactive inhibitor of p38 MAPK, SB202474 at 10 μM had no effect on steroidogenesis. To determine the potential contribution of ERK1/2 and JNK1/2 in the regulation of adrenal steroidogenesis during aging, two MEK 1/2 inhibitors PD98059 (Alessi *et al*., 1995) and U0126 (Favata *et al*., 1998) and a JNK1/2 inhibitor, SP600125 (Bennett *et al*., 2001), were utilized. (MEK1/2 is upstream kinase that activates ERKs).

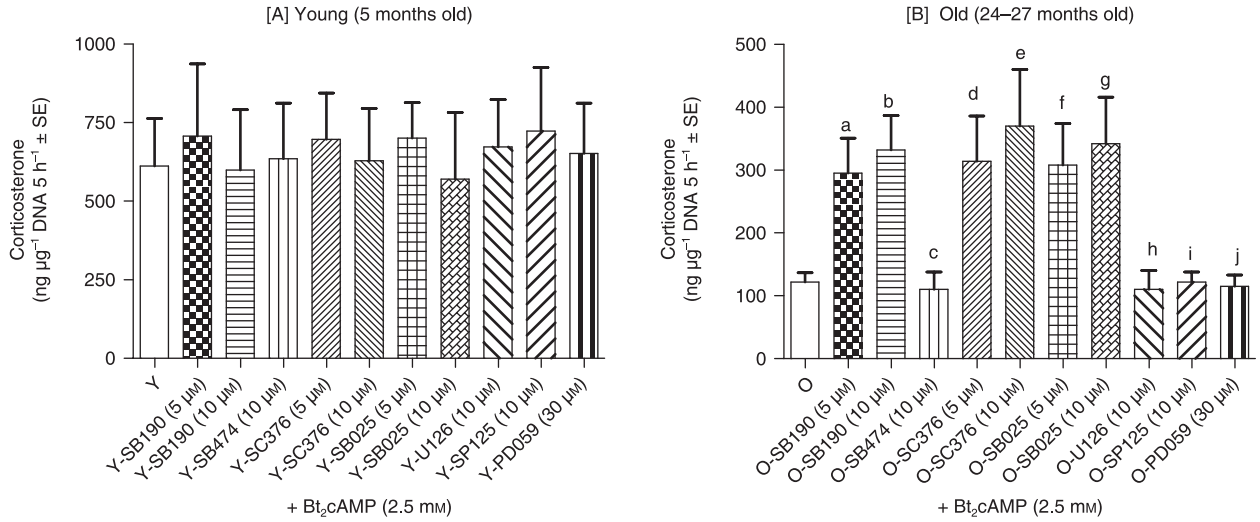

Fig. 4 Effect of MAPK signaling pathway inhibitors and ROS inhibitors on Bt_2cAMP-stimulated corticosterone production in isolated adrenal cells from young and old rats. Freshly isolated adrenal cells from young mature (5 months old) or old (24–27 months old) animals were incubated for 5 h in culture medium supplemented with ± Bt_2cAMP (2.5 mM) ± SB202190 (SB190; 5.0 or 10 μM), ± SC68376 (SC376; 5.0 or 10 μM), ± SB220025 (SB025; 5.0 or 10 μM), ± SB202474 (SB474; 10 μM), ± PD98059 (PD059; 30 μM), ± U0126 (10 μM), or ± SP600125 (SP125; 10 μM). At the end of incubation, medium samples were quantified for the production of corticosterone by RIA. In some cases, cell viability was determined using a sensitive MTT assay as described under Experimental Section. The results are mean ± standard error (SE) of four separate experiments. [a]$P = 0.0246$ vs. O-Bt_2cAMP; [b]$P = 0.0099$ vs. O-Bt_2cAMP; [c]P = NS vs. O-Bt_2cAMP; [d]$P = 0.0403$ vs. O-Bt_2cAMP; [e]$P = 0.0034$ vs. O-Bt_2cAMP; [f]$P = 0.0033$ vs. O-Bt_2cAMP; [g]$P = 0.0267$ vs. O-Bt_2cAMP; [h]P = NS vs. O-Bt_2cAMP; [i]P = NS vs. O-Bt_2cAMP; [j]P = NS vs. O-Bt_2cAMP.

Treatments with either PD98059 (30 μM), U0126 (10 μM), inactive analog U0124 (10 μM) or SP600125 were ineffective in up-regulating either basal or Bt_2cAMP-stimulated steroid production in adrenal cells from old rats (Fig. 4B). Finally, none of the various MAPK inhibitors tried had any significant effect on the overall viability of the treated cells. In contrast, treatment of adrenal cells from young mature (5 months old) rats with p38 MAPK inhibitors had no significant effect on steroid secretion (Fig. 4A). These latter studies provide additional support to the notion that selective activation of p38 MAPK mediates the age-related decline in adrenal steroidogenesis.

We next investigated the potential interaction between excessive oxidative stress, p38 MAPK activation and age-related decline in adrenal steroidogenesis by evaluating the separate and combined actions of non-enzymatic antioxidants and p38 MAPK inhibitors. We reasoned that since excessive oxidative stress mediates the activation of adrenal p38 MAPK during aging that, in turn, leads to inhibition of corticosterone production, the use of ROS scavengers, such as manganese (III) tetrakis(1-methyl-4-pyridyl) porphyrin (MnTMPyP) and *N*-acetyl cysteine (NAC) should rescue the adrenals from age-induced loss of corticosteroid protection. We further predicted that under this scenario, simultaneous addition of antioxidant and p38 MAPK inhibitor should not produce an additive effect. MnTMPyP functions as a cell-permeable superoxide-dismutase/catalase mimetic (Pimentel *et al.*, 2001), while NAC, an intermediary (along with glutamic acid and glycine) in the conversion of cysteine to glutathione (Wu *et al.*, 2004) is known to interfere with 2-hydoxy-nonenal action (Uchida *et al.*, 2004).

As shown in Table 2, pretreatment of adrenal cells from old rats with MnTMPyP partially restored the aging-induced decline in 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, corticosterone production in old adrenal cells was unaffected by treatment of cells pretreated with light-inactivated MnTBAP (10 μM; data not shown). The results of these experiments suggest that reversal of steroidogenesis is not due to a nonspecific effect of MnTMPyP, but occurs because of the scavenging of superoxide and H_2O_2 and provides additional support to the notion that excessive oxidative stress impairs steroidogenic function. Likewise, addition of NAC to the incubation medium also partially restored the steroidogenic response in adrenal cells from old animals.

To investigate whether inhibition of the p38 MAPK cascade contributed to the stimulatory effect conferred by ROS scavengers and p38 MAPK inhibitors (Fig. 3) in response to aging, we evaluated the combined actions of ROS and p38 MAPK inhibitors on steroid production in old cells. As before, pretreatment with SB202190 (10 μM), a highly potent inhibitor of p38 MAPK, restored steroidogenesis in old adrenal cells to ~60–70% of the level seen in adrenal cells from young animals (Table 2). Likewise, use of MnTMPyP and NAC partially restored steroid production in old adrenal cells (Table 2). In contrast, treatment of cells with MnTMPyP + SB202190 or NAC + SB202190 did not produce any additive or synergistic effect suggesting that both types of inhibitors exert their stimulatory action through inhibition of p38 MAPK activation.

Table 2 Effect of non-enzymatic antioxidant, NAC and MnTMPyP and/or p38 MAPK inhibitor (SB202190) on corticosterone production by primary adrenocortical cells isolated from old rats

Additions	Corticosterone (ng μg^{-1} DNA 5 h^{-1} ± SE)
Basal	7.3 ± 1.4
Bt_2cAMP (2.5 mM)	131 ± 16
NAC (10 mM)	9.8 ± 1.4
MnTMPyP (10 μM)	11.5 ± 2.5
SB202190 (5 μM)	6.6 ± 0.96
Bt_2cAMP + SB190	292 ± 31
Bt_2cAMP + NAC	240 ± 27*
Bt_2cAMP + MnTMPyP	208 ± 18†
Bt_2cAMP + NAC + SB190	320 ± 29‡
Bt_2cAMP + MnTMPyP + SB190	305 ± 27§

*P = 0.0130 vs. O-Bt_2cAMP; †P = 0.0170 vs. O-Bt_2cAMP; ‡P = 0.0012 vs. O-Bt_2cAMP; §P = 0.0029 vs. O-Bt_2cAMP.
Results are mean ± standard error (SE) of four separate experiments. The incubation medium in a final volume 0.5 mL contained DME ± Bt_2cAMP (2.5 mM), ± NAC (10 mM), ± MnTMPyP (10 μM) or ± SB202190 (**SB190**; 5 μM) and a suitable aliquot of adrenal cells isolated from individual old rats. Cell samples were preincubated with medium alone (basal), NAC or MnTMPyP for 1 h, and then ± Bt_2cAMP was added and incubations were continued for an additional 5 h at 37 °C. At the end of incubation each medium sample was analyzed for corticosterone content by the radioimmunoassay. The data shown above were derived using adrenal cells isolated from old rats. Under identical experimental conditions, the basal and Bt_2cAMP-stimulated corticosterone production rates by adrenal cells from young animals were in the range of 9.5 ± 1.7 and 565 ± 88 ng μg^{-1} DNA 5 h^{-1} (P = 0.0028 vs. O-Bt_2cMP), respectively.

Discussion

In this report, we describe two key findings as follows. First, oxidative stress-mediated inhibition of adrenal steroidogenesis during aging is accompanied by a selective activation of p38 MAPK. Second, the inhibitory actions of enhanced oxidative stress on adrenal steroidogenesis, such as that which occurs during aging, are mediated by the p38 MAPK pathway. These findings strongly suggest that p38 MAPK activity is a negative determinant of adrenal steroidogenesis and that it plays a critical role in age-related loss of steroidogenic function.

Our initial efforts were directed to find an association between age-induced excessive oxidative stress and alterations in the expression of MAPK signaling cascades. As reported earlier and further confirmed here, aging decreased the corticosterone production in isolated adrenocortical cells (Popplewell *et al*., 1986). Aging-induced adrenal lipid peroxidation was also significantly increased both under basal condition and following exposure to either enzymatic or non-enzymatic pro-oxidants. The latter observations were confirmed using a generally utilized measure of lipid peroxide decomposition product, malondialdehyde (Ohkawa *et al*., 1979) as well as a sensitive assay utilizing direct detection of lipid peroxides with ferric-xylenol orange complex (Hermes-Lima *et al*., 1995). Under identical conditions, we examined phosphorylation-dependent activation of ERK1/2, JNK1/2 and p38 MAPK in order to clarify the signal transduction pathway responsive to oxidative stress conditions during aging. Activation of each of these MAPKs is mediated by a specific dual phosphorylation of the threonine (T) and tyrosine (T) residues of ERK, JNK and p38 MAPK (i.e. TEY for ERKs, TPY for JNKs, and TGY for p38 MAPKs). The results showed significant levels of endogenous phosphorylation of all three MAPKs in whole adrenal extracts from both young and old rats (Fig. 2). Moreover, we observed that phosphorylation-mediated activation of p38 MAPK, but not of ERK1/2 or JNK1/2, significantly increased in adrenal glands from old animals. These data are consistent with the recent findings reported in other systems, where increased activation of p38 MAPK has been linked to various aging-induced alterations in metabolic parameters (Haq *et al*., 2002; Davis *et al*., 2005; Kang *et al*., 2005; Hsieh & Papaconstantinou, 2006; Ito *et al*., 2006). In the present study, we also observed an association between oxidative stress and p38 MAPK activation, raising the possibility that aging-induced enhanced oxidative stress leads to activation of p38 MAPK, which, in turn, attenuates the steroidogenic response seen during aging.

The functional consequences of a potential relationship between oxidative stress and p38 MAPK were further investigated using both cell-permeable chemical inhibitors of p38 MAPK as well as potent ROS scavengers. Treatment of adrenal cells from old rats with specific but structurally divergent inhibitors of p38 MAPK, SB202190, SB220025 or SC68376 significantly (60–70%) restored the age-related decline in steroid production. In 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). To further 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 up-regulate the steroidogenic function in old adrenal cells. Likewise, a specific JNK inhibitor, SP600125, had no significant effect on steroid synthesis in an adrenal cell model exposed to a set of oxidants under *in vitro* conditions. Additional studies demonstrated that the use of MnTMPyP, a cell-permeable superoxide-dismutase/catalase mimetic (Pimentel *et al*., 2001), partially restored the steroidogenic response in old adrenal cells. Likewise, blockade of the inhibitory actions of lipid peroxidation products (e.g. HNE) with antioxidant NAC also improved the corticosterone production in adrenal cells derived from old animals. Simultaneous addition of p38 MAPK inhibitor and ROS inhibitors, however, did not yield any additional stimulatory effect. These results suggest that the chosen antioxidant partially nullifies the inhibitory action of aging on steroid hormone production and, thus, enhanced oxidative stress is central to the molecular mechanisms underlying the age-related decline in adrenal steroidogenesis. Furthermore, the fact that both antioxidants and p38 MAPK inhibitors can, to some extent, restore steroidogenesis in old cells, and that the simultaneous presence of two types of inhibitors yielded no additional effect implies 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.

Precisely how p38 MAPK interferes with the steroidogenesis is not clear. However, given that p38 MAPK is involved in a wide spectrum of cellular processes and that multiple kinases and transcription factors have been identified as p38 MAPK substrates suggests that any of these processes could potentially contribute to the negative actions of p38 MAPK on steriodogenesis (McDermott & O'Neill, 2002; Pramanik *et al*., 2003). In this context, there is evidence that oxidants such as superoxide anion, H_2O_2 and HNE can cause induction of cyclooxygenase-2 (COX-2) (Nakamura & Sakamoto, 2001; Kiritoshi *et al*., 2003; Kumagai *et al*., 2004; Yang *et al*., 2005, 2006). Furthermore, oxidant-dependent activation of p38 MAPK is known to directly regulate the expression of cycolooxygenase-2 (Guan *et al*., 1998; Lasa *et al*., 2000; Hendrickx *et al*., 2003). Other studies have also demonstrated that, when activated, COX-2 inhibits steroidogenesis in several steroidogenic systems (Nakamura & Sakamoto, 2001; Wang *et al*., 2003; Frungieri *et al*., 2006). From this we speculate that COX-2 may be involved in the negative regulation of steroidogenesis by p38 MAPK. This possibility is consistent with findings reported for steroidogenic cells, where cyclooxygenase-2 is shown to down-regulate steroidogenic acute regulatory (StAR) gene expression (Wang *et al*., 2003). [The StAR protein in concert with several other proteins facilitates the rate-limiting transfer of cholesterol to the mitochondrial inner membrane where the substrate cholesterol is converted to steroid precursor, pregnenolone by CYP11A1 (P450scc) (Liu *et al*., 2003; Manna & Stocco, 2005).] Our own unpublished preliminary data suggest that p38 MAPK functions as a suppressor of StAR promoter activity in adrenal cells. Additional studies are underway in this laboratory to further evaluate the functional interactions between p38 MAPK and COX-2 in an effort to delineate the exact mechanism by which oxidative stress-mediated activation of p38 MAPK negatively impacts steroidogenesis.

In conclusion, our data provide direct evidence that oxidative stress is involved in the selective inhibition of steroidogenesis both *in vivo* in response to aging and *in vitro* after treatment with oxidants. Furthermore, we provide 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 during aging-induced oxidative stress.

Experimental procedures

Animals

All experimental animal protocols were approved by the Institutional Animal Care and Use Committee of the Department of Veterans Affairs Palo Alto Health Care System. Sprague-Dawley male rats were used for all studies. The animals had free access to rodent chow and water. Young rats were obtained at 2 months of age and were used 3 months later as young mature (Y, 5 months old) rats. Old rats were obtained at 11–12 months of age and were maintained in our facility until 24–27 months of age (O, 24–27 months old). The 5-month-old rat is a young mature animal whose weight is comparable to that of the aged rats; the 24-month-old rat shows all the age-related changes we are attempting to define. These two groups are standard age groups commonly used in aging studies (Cheng *et al*., 1998; Culty *et al*., 2002; Ivell *et al*., 2003). Animals were monitored on a quarterly basis for various viruses and infectious agents as described previously (Reaven *et al*., 1988). All animals were checked for gross pathology before use. Those with visible kidney, pituitary, adrenal, or testicular tumors, or other apparent defects, were not used (~6% of 24- to 27-month-old rats).

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 SB202190 and SB202474 (inactive analog), the JNK inhibitor SP600125, MnTMPyP [Mn(III)tetrakis1-methyl-4-pyridylporphyrin pentachloride] and MnTBAP [Mn(III)tetrakis(4-benzoic acid)porphyrin], Bt_2cAMP; fatty acid-poor bovine serum albumin; hydrogen peroxide (H_2O_2); and *N*-acetyl cysteine (NAC) were supplied by Sigma-Aldrich (St. Louis, MO, USA). p38 MAPK inhibitors, SC68376 and SB220025, were purchased from Calbiochem (EMD Chemicals Inc., San Diego, CA, USA). All other reagents used were of analytical grade.

Polyclonal antibodies against total ERK1/ERK2, JNK1/JNK2, and p38 MAPKα were purchased from Cell Signaling Technology (Beverly, MA, USA). Phospho-specific antibodies that recognize ERKs phosphorylated at Thr^{202} and Tyr^{204}, and p38 MAPK phosphorylated at Thr^{180} and Tyr^{182} were also supplied by Cell Signaling Technology. Phospho-JNKs (Thr^{183} and Tyr^{185}) antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Isolation of primary rat adrenocortical cells and measurement of corticosterone secretion

Rat adrenocortical cells from young mature (5 months old) and old (24–27 months old) rats were prepared by a procedure described previously (Azhar *et al*., 1991). In brief, rats were killed by cervical dislocation, and the adrenal glands aseptically removed, decapsulated, and dissected free of fat. A group of two adrenals from each rat was finely minced with scissors, and tissue fragments were suspended in sterilized Medium 199 containing 40 mg mL^{-1} of bovine serum albumin, 3.7 mg mL^{-1} of collagenase, and 5 μg mL^{-1} DNAse and incubated with shaking for 1 h in an atmosphere of 95% O_2 to 5% CO_2. The tissue suspension was then dissociated by repeated pipetting with a tuberculin syringe, the resulting suspension filtered

through a nylon mesh, washed by centrifugation, and the final pellet re-suspended in DME:F12 (1:1). These cell preparations were used immediately for the measurement of steroid production and secretion.

To assay steroidogenesis, triplicate samples of cells were incubated for 5 h without (basal) or with Bt_2cAMP (2.5 mM), and subsequently samples of incubation medium were collected, frozen, and stored frozen until analyzed for corticosterone production by the radioimmunoassay technique (Reaven *et al*., 1988).

To evaluate the effects of MAPKs and/or ROS inhibition to modulate Bt_2cAMP-stimulated steroidogenesis in adrenal cells from old rats, we utilized specific MEK/ERK inhibitors (PD098059 and U0126), the p38 MAP kinase inhibitors (SB203580, SB202190 and SB202474 [inactive analog]), and the JNK inhibitor (SP600125) either separately or in a specific combination. Triplicate dishes of old-adrenal cells were pretreated with vehicle alone (control), PD098059 (30 μM), U0126 (10 μM), SB203580 (10 μM), SB202190 (10 μM), SB202474 (10 μM) or SP60012 (10 μM) for 1 h, and incubations continued for an additional 5 h following the addition of Bt_2cAMP ± hHDL3 (500 μg protein mL^{-1}). At the end of incubation, the media were collected, frozen, and stored frozen until analyzed for 20α-dihydroprogesterone levels as described above. Cell viability after treatment was monitored using the MTT assay as described above.

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 on mitochondrial respiratory chain activity and reflecting the mitochondrial redox state. After treatment with antioxidants and/or p38 MAPK inhibitors, MTT solution was added to the dishes at a final concentration of 500 μg mL^{-1} and the induction continued at 37 °C for 3 h. Following incubation, the medium was aspirated and the accumulated formazan product was solubilized with a 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.

Determination of lipid peroxidation by measurement of lipid hydroperoxides

We employed TBARS and FOXRS assay procedures for measurement of lipid peroxides in untreated adrenal homogenates or homogenates subjected to enzymatic or non-enzymatic pro-oxidants. Freshly excised adrenals (two per sample) were homogenized in buffer (0.15 M KCl, 5 mM Tris maleate, pH 7.4, and 1 mM EDTA), and subsequently centrifuged at 800 × *g* for 15 min to sediment unbroken cells and nuclei. A total membrane fraction was obtained by centrifugation of the supernatant at 105 000 × *g* for 60 min and was stored in liquid nitrogen until assayed for *in vitro* lipid peroxidation.

To determine TBARS and FOXRS formation, aliquots of adrenal membrane fractions were incubated with buffer alone (endogenous), or in the presence of pro-oxidants, 1 mM NADPH + 50 μM $FeSO_4$ (enzymatic), or 5 μM $FeSO_4$ + 500 μM sodium ascorbate (non-enzymatic) in a final volume of 1.0–3.0 mL as described previously (Azhar *et al*., 1995; Abidi *et al*., 2004). Following incubation at 37 °C for 60 min, 1.0 mL aliquots were analyzed for the formation of TBARS and FOXRS. (Note: FOXRS measurements were conducted either under basal conditions or in the presence of enzymatic pro-oxidants; no measurements were carried using non-enzymatic pro-oxidants as the presence of ascorbate interferes with the color formation.)

To quantify TBARS formation, 0.8 mL aliquot of incubation mixture was mixed with 3.2 mL of a stock TBA reagent to achieve a final concentration of 0.3% TBA, 7.5% of acetic acid buffered to pH 3.5, and 0.405% of sodium dodecyl sulfate (Ohkawa *et al*., 1979). The tubes were covered with glass-marbles, heated at 95 °C for 60 min, cooled and extracted with 1.0 mL distilled water + 5.0 mL of a mixture of *n*-butanol and pyridine (15;1, v/v). The absorbance of the upper organic layer was simultaneously determined at 510, 532 and 560 nm and the concentration of TBARS (MDA equivalent) was calculated as follows: $MDA\text{-}A_{532} = 1.22[(A_{532}) - (0.56)(A_{510}) + (0.44)(A_{560})]$ (Pyles *et al*., 1993). For FOXRS measurement, a 0.1 mL aliquot of incubation mixture +0.4 mL of distilled water was mixed with 0.5 mL of 2× xylenol orange (XO) reagent containing 0.5 mM $FeSO_4$, 50 mM H_2SO_4 and 0.2 mM XO (Hermes-Lima *et al*., 1995). Following incubation of the tubes in the dark for 60 min at room temperature, the absorbance of each tube was measured at 580 nm. Levels of lipid hydroperoxides are expressed as cumene hydroperoxide equivalents.

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

Adrenals (two per sample, ~50 mg) were homogenized using a Potter-Elvehjem homogenizer (Thomas Scientific, Swedesboro, NJ, USA) in three volumes of lysis buffer A, and incubated for 30 min at 4 °C on an orbital shaker for complete lysis. The lysates were cleared by centrifugation at 15 000 × *g* for 10 min, and protein concentration of each solubilized lysate was determined. All cell/tissue lysate samples were stored frozen until analyzed by Western blotting (Kelley *et al*., 2004).

Samples containing an equal amount of protein (30–40 μg) were fractionated by SDS-PAGE (10% polyacrylamide gel with 4% stacking gel) and transferred to Immobilon® polyvinyllidene difluoride membrane (PVDF, Millipore Corp., Bedford, MA, USA). After transfer, the membrane was washed in Tris-buffered saline 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 primary antibody in blocking buffer (total or phosphorylated form 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 antirabbit or antimouse 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-Multilmager scanning densitometry system (Bio-Rad, Hercules, CA, USA).

Analytical procedures

Protein was measured with a bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, IL, USA).

Statistics

The results were analyzed by GraphPad Prism version 3.00 software for Windows (GraphPad Software, San Diego, CA, USA) on a Dell PC computer. Student's *t*-test was used to compare data between two groups. Multiple group comparisons were performed using one- or two-way ANOVA with Bonferroni post-test. All data are presented as mean ± standard error of mean. The value of $P < 0.05$ was considered significant.

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