The roles of p38 MAPK → COX2 and NF-κB → COX2 signal pathways in age-related testosterone reduction

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In our study, we explored changes in the redox status and inflammatory response in the testes of the SAMP8 model of varying ages (2, 4, 8, 10 months old) compared with control mice SAMR1 by the methods of immunohistochemical staining, Western blotting, RT-PCR and Luminex multi-analyte cytokine profiling. We found that as ROS and inflammation levels increased during aging, steroidogenic enzymes (StAR and P450scc) reduced and led to the decline of testosterone production eventually. The pathways of P38 MAPK → COX2 and NF-κB → COX2 were detected by using specific inhibitors of SB203580 and Bay 11-7082 in isolated Leydig cells. These results indicated that activation of both p38 MAPK → COX2 and NF-κB → COX2 signaling pathways are functionally linked to the oxidative stress response and chronic inflammation during aging, and mediate their inhibitory effects on testosterone production.

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Male aging is usually accompanied with decrease in serum testosterone concentration, which are associated with osteoporosis, metabolic syndrome, depression and sexual dysfunction. Although testosterone-replacement therapy can be used, some undesired side effects occur such as increased risk of cardiovascular diseases, prostate cancer, luteinizing hormone (LH) suppression and obesity. Therefore, it is necessary to thoroughly understand the mechanism by which steroidogenic function decreases with aging and further develop therapies for hypogonadism.

Testosterone is secreted primarily by testicular Leydig cells and then released into the circulation. The serum testosterone level is known to be affected by a variety of physiological and biochemical factors related with aging. Studies over the past several years showed that ROS level increasing with aging in Leydig cells, particularly lipid peroxidation-mediated damage to cell membrane, is related with intracellular cholesterol transport, which may weaken the function of Leydig cells leading to decreased steroidogenesis. It is also known that inflammatory events are involved in the decline of physiological functions of aging organs. Compared to the young, two- to four-fold increments in the levels of blood pro-inflammatory cytokines, such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6), are typical in the elderly. It has been shown that TNF-α and IL-1β have an inhibitory effect on the expression of steroidogenic enzymes in Leydig cells.

Although the mechanism by which excessive oxidative stress leads to age-related loss of steroidogenesis is unclear, evidence accumulated in other systems now indicates sustained low levels of oxidative stress during aging can activate the mitogen-activated protein kinase (MAPK) pathway, leading to changes in gene expression that may influence cellular biological reactions and metabolic processes. Recent reports showed that oxidative damage-associated p38 MAPK signaling pathway was activated in aged rat adrenal cells and Leydig cells. Interestingly, p38 MAPK activity blocked by pharmacological inhibitor in aged adrenal cells led to decrease in phosphorylation of p38 MAPK and increase in cellular steroid synthesis. This finding supports the possibility of the role of ROS and p38 MAPK in the reduction of steroid production in elderly steroidogenic cells.

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Cyclooxygenase-2 (COX2), an isofrom of cyclooxygenase, is a prostanooid enzyme that is responsible for synthesis of thromboxane and prostaglandins. According to the cell type, the expression of COX2 can be induced by cytokines, bacterial endotoxin lipopolysaccharide(LPS) and the tumor promoter phorbol myristate acetate (PMA)12. COX2 gene in aged Leydig cells is expressed at higher levels, and has been shown to inhibit the expression of steroidogenic acute regulatory protein (STAR)31. Further studies showed that COX2 could also be induced by oxidative stress, a process depending on p38 MAPK signaling14. There is no clear evidence that elevating oxidative products in aged Leydig cells may inhibit STAR expression and steroid production by p38 MAPK activation and higher levels of COX2. In our study, Leydig cells were used to verify the effect of p38 MAPK pathway on testosterone synthesis.

NF-κB is an important transcription factor involved in immune reaction, inflammatory diseases and development15,16. NF-κB can be activated and transferred from the cytoplasm to the nucleus where it is combined with the promoter regions of several genes, including Cox2, TNF-α and IL-1β17. Our study indicated that NF-κB expressed in the nucleus of testis increases with age, but it is not clear whether NF-κB has an effect on testosterone reduction in age-related Leydig cells of by regulating Cox2 expression.

In our study, we observed the age-associated changes in testosterone production, the expression of cholesterol side-chain cleavage enzyme (P450scs) and StAR, which are two key enzymes in testosterone synthesis, and investigated the age-related alterations in COX2, IL-1 and TNF-α in 2, 4, 8, and 10-month-old male mice. The results demonstrated that oxidative stress and chronic inflammation are involved in the decline of testosterone production both in vivo and in vitro with aging. Furthermore, we provide evidence that NF-κB → COX2 and p38 MAPK → COX2 signaling pathways are functionally linked to the oxidative stress response and chronic inflammation during aging and mediates their inhibitory effects on testosterone production. We concluded that both the p38 MAPK → COX2 and NF-κB → COX2 signal pathways played roles in the testosterone reduction with age change.

Material and Methods

Animals. Male senescence-accelerated mouse prone 8 (SAMP8, P8) and senescence-resistant inbred strain (SAMR1, R1) mice at 2, 4, 8 and 10 months of age were provided by Hebei Medical University (The Animal approval number is SCXK2014-0004, n = 6 mice per age group). All mice were kept under controlled environment (24–28°C, 65 ± 5% relative humidity, 12 hour light/12 hour dark cycle) and fed with food and water ad libitum. All animals were handled according to Beijing Laboratory Animal Center’s guidelines, and all programme were appraised and allowed by the Ethics Committee of Hebei Medical University.

Tissue collection and preparation. All mice were anesthetized with an intraperitoneal (i.p.) injection of chloral hydrate (400 mg/kg body weight). Testes were collected and prepared as my previous paper described3. Briefly, the fixed samples were submitted to immunohistochemical staining procedures. Each frozen sample was submitted to protein detection by Western blotting, RT-PCR and Luminex multi-analyte cytokine profiling.

Isolation of primary Leydig cells. Mice were killed by cervical dislocation, and the testes were aseptically removed and the adipose tissue was dissected. Testes were decapsulated, and then were dissociated with collagenase 1 (500 μg/mL) at 34°C, with low-speed shaking (90 cycles/min) for 5–10 min until the seminiferous tubules were isolated but retained their structural integrity. Two volumes of DME:F12 (1:1) was added to stop the digestion. Seminiferous tubules were filtered and removed by a 100-μm pore size nylon mesh, then the dissociated cells were collected from the filtrate, centrifuged (15 min, 1000 rpm) and then subjected to centrifugal washing (5 min, 1000 rpm). The final pellet that collected to the 1.0 μm pore size nylon mesh, then the dissociated cells were collected from the filtrate, centrifuged (15 min, 1500 rpm) and then subjected to centrifugal washing. Seminiferous tubules were isolated but retained their structural integrity. Two volumes of DME:F12 (1:1) containing 10% FBS, 100 U mL⁻¹ streptomycin and 100 U mL⁻¹ penicillin, followed by incubation at 37°C with 5% CO₂. After 2 h of culture, the medium was dispensed with fresh medium after three washes using PBS. Leydig cells were obtained using the differential adhesion speed method. The viability of Leydig cell preparations was detected by incubating with 0.4% Trypan blue for 5 minutes. The viability was 96.5–97%. The purity of cell preparations was evaluated by measuring the percentage of cells stained by immunohistochemical staining of 3β-hydroxysteroid dehydrogenase (3β-HSD)28. The >95% purity was achieved. These cell preparations were used to measure the secretion of steroid.

The production of testosterone was measured as previously described15,16. Briefly, Leydig cells from young (4 month age) and old (8–10 month age) mice were incubated in the absence (basal) or in the presence of the maximum dose of LH (100 ng/ml) for 2 h. Subsequently, media of incubation were collected and cryopreserved until testosterone production was analyzed by the radioimmunoassay (RIA) technique.

To assess the roles of p38 MAPKs and/or NF-κB inhibitor in regulating steroidogenesis induced by LH in Leydig cells of aged mice, we used the specific p38 MAP kinase inhibitor SB203580 (group SB) and the NF-κB inhibitor Bay 11–7082 either separately (Bay group) or in a specific combination (SB + Bay group). To assess the concentration and the incubation times of Bay as previously described17, old Leydig cells were treated with 0–10 μM of Bay for 12 h and incubated with 5 μM Bay for the different time periods (from 0 to 24 h). The concentration and the incubation times of Bay depended on cell viability and the inhibition on the NF-κB expression. The concentration and the incubation times of SB based on a previously published protocol10. Triplicate old Leydig cells were pretreated with vehicle alone (control), SB203580 (10 μM) for 1 h, 5 μM Bay 11-7082 for 12 h or SB203580 (10 μM) for 1 h, followed by 5 μM Bay 11-7082 for 12 h. The incubation continued for 2 hours after the addition of LH (100 ng/ml). The medium were collected, frozen and cryopreserved until testosterone levels were analyzed as described above.

LH and testosterone measurements. Serum testosterone and LH concentrations were evaluated as my last paper described3. Briefly, serum testosterone and LH concentrations from individual mice and aliquots
of supernatants from testicular homogenates were evaluated using RIA. The sensitivity and intra-assay and inter-assay coefficients of variation of RIA were 13 pg/tube, 8.9%, and 13.6%, respectively.

Cytokine measurements. xMAP technology (Austin, TX, USA) was used to measure cytokine levels as described previously. Briefly, bead sets were coated with capture antibodies for TNF-α and IL-1β. The two cytokines in the samples were recognized by differences in bead sets with fluoregenic emission detection using flow cytometric analysis (Niu and Ro, 2011).

Measurement of intracellular ROS. The level of ROS was analyzed by measuring the fluorescence of DCFH-DA as previously described. Briefly, the relative levels of fluorescence were measured with a flow cytometer (Becton-Dickinson, Franklin Lakes, NJ).

mRNA analysis by semi-quantitative reverse transcriptase or real-time PCR. Total RNA was extracted from treated cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was frozen at −70°C until use. Total RNA (2 µg) was reverse transcribed into cDNA by using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). The mRNA was analyzed by semi-quantitative reverse transcription as described previously. The primer sequences are listed in Supplement 1.

Quantification of mRNA was performed using real-time PCR according to the manufacturer’s instructions for SYBR Premix Ex Taq using a real-time thermal cycler (Bio-Rad, Hercules, CA, USA). Results were expressed as optimal density ratios to β-actin. Gene-specific primers used are listed in Supplement 1.

Western blotting. The protein of COX2, p-p38, p38, StAR, NF-κB was detected by Western blot as described previously. Briefly, the frozen testes and collected cells were homogenized in lysis buffer. NF-κB nuclear localization experiments referred to manufacturer’s protocol (Thermo Scientific, Pierce Protein Biology Products, Rockford, IL, USA). Each protein sample (50 µg) was separated by SDS-PAGE on different gels (10% gel for NF-κB, COX2, p-p38, p38 or 12% gel for StAR) and then transferred to nitrocellulose membranes. The membranes were incubated with antibodies against NF-κB (Santa Cruz, CA, USA, 1:1000), COX2 (Epitomics Burlingame, CA, USA, 1:1000), p38 (Abcam, Cambridge, MA, USA, 1:1000), p-p38 (Abcam, Cambridge, MA, USA, 1:1000) and StAR (Santa Cruz, CA, USA, 1:1000). Immunoreactive proteins were detected and quantified. To ensure even loading of the samples, the same membrane was probed with β-actin (Santa Cruz, CA, USA, 1:5000) or H3 (Santa Cruz, CA, USA, 1:1000) antibody.

Data analysis. All data were analyzed using SPSS 16, and results expressed as mean ± standard deviation (SD). For all statistical analyses, value of P < 0.05 was considered significant. Data were analyzed using two-way ANOVA with complete random design, and then multiple pair-wise comparison was performed using Student-Newman-Keuls post hoc tests.

Results

Age-dependent changes in LH and testosterone. Experiments were conducted to observe the age-related changes of LH concentration in P8 and R1 mice in different ages (Fig. 1A). A two-way ANOVA was conducted on serum LH levels for the mouse strains (P8, RI) by age (2, 4, 8, 10 months old). The results indicated that age and strain had a significant effect (P < 0.01) (Fig. 1A). The interaction of age and strain was significant (P < 0.01). A one-way ANOVA conducted on serum LH across P8 mice of 2, 4, and 8 months of age yielded increase with aging but no significant effect (Fig. 1A). The LH levels for 10-month-old was higher compared to younger groups.
with 2, 4-month-old in P8 mice (P < 0.01), whereas LH levels for 2, 4, 8 and 10-month-old had no significant difference in RI mice.

Experiments were conducted to observe the age-related changes of testosterone production in P8 and R1 mice (Fig. 1B). A two-way ANOVA was conducted on serum testosterone levels for the mouse strains (P8, RI) by age (2, 4, 8, 10 months old). The results showed that age had a significant impact (P < 0.01), and that the strain did not (P > 0.05) (Fig. 1B). Our results were basically consistent with the results of the previous research21. The interaction of strain and age was significant (P < 0.01). The testosterone value of the 8-month-old was significantly lower than that of the 4-month-old in P8 mice (P < 0.01), while the testosterone value of the 4 and 8-month-old RI mice had no significant difference. One-way ANOVA of plasma testosterone in 2, 4, 8 and 10-month-old P8 mice produced a significant effect, with testosterone values declining with age (Fig. 1B). The testosterone level of the 4-month-old increased compared with those at 8 or 10 months in P8 mice (P < 0.01) (Fig. 1B). Testosterone level of 8-month-old were reduced by ~44% compared to 4-month-old in P8 mice.

**Age-dependent expression changes in StAR and Cyp11a1 mRNA and protein in testis samples.** A two-way ANOVA was used on the expression of StAR and Cyp11a1 for strain (P8, RI) by age (2, 4, 8, 10 months old) (Fig. 2A–D). The analysis of the StAR expression showed that the interaction of strain and age was significant. However, the interaction of strain and age have no significant impact on Cyp11a1 expression. In P8 mice across age groups of 2, 4, 8, and 10 months, a significant effect was seen in the StAR and Cyp11a1 expression, which decreased with age (Fig. 2A–D). The expression level of StAR in 8-month-old decreased compared with
that of 4-month-old in P8 mice (P < 0.05), however there was no significant difference between the 4-month-old and 8-month-old in R1 mice. The expression of Cyp11a1 had no differences between 4-and 8-month-old in P8 mice. The levels of StAR were significantly higher at 2 and 4 months, and they were significantly lower at 8 and 10 months in P8 mice than that of R1 mice (P < 0.05). The levels of P450scc at the mRNA level was not consistent with that at the protein level. However, the levels of P450scc at 10 months of age declined compared with those of 2, 4 and 8-month-old mice in P8 and R1 mice. (P < 0.01). The levels of P450scc protein were significantly lower at 10 months in P8 mice compared with that of R1 mice.

The P8 mice is characterized by a rapid decline of testosterone at 8 months of age. In order to identify the reasons for this decrease, we detected the change in expression of StAR and Cyp11a1 in testes of P8 mice with age. The level of StAR of 8-month-old mice was significantly lower than those of 2- and 4-month-old mice in P8 mice (P < 0.01). This result was consistent with changes in testosterone.

Age-related alterations in pro-inflammatory cytokines in testis samples. A two-way ANOVA was used to test the values of IL-1β and TNF-α in the two strains (P8, R1) by age (2, 4, 8, 10 months old) (Fig. 3A,B). The result showed that the interaction of strain and age was not significant. In both strains of mice at 8 months old, increases in the levels of TNF-α and IL-1β were observed (P < 0.001) (Fig. 3A,B). Differences in the levels of IL-1β and TNF-α between the strains were detected at 4, 8 and 10 months (P < 0.001). Thus, pro-inflammatory cytokines TNF-α and IL-1β showed age-related elevation, and the cells were in a chronic inflammatory state.

Age-related increase in COX2 expression in testis samples. The levels of COX2 were tested using a two-way ANOVA in the two strains (P8, R1) by age (2, 4, 8, 10 months old) (Fig. 4A–F). The result showed that the interaction of age and strain had no differences. The protein level of COX2 in testes increased in an age-related manner in P8 and R1 mice. The level of COX2 increased at 8 and 10 months compared with that of 2 and 4 months-old mice in P8 mice. The level of COX2 in P8 became significantly higher at 8 months old than that of age-matching R1 mice (P < 0.05) (Fig. 4B).

A significant increment of COX2 protein level was observed in the testes of 8 months old P8 mice (Fig. 4B). Furthermore, we assessed the COX2 protein level by immunohistochemical staining, which revealed COX2 is located in the cytoplasm of interstitial cells, and the intensity of positive products elevated with aging in P8 mice (Fig. 4A–E). In P8 mice, the COX2 immunohistochemical staining was much more intense than that in R1 mice at 8 months old (Fig. 4D,F).

Effect of age on testosterone production from isolated Leydig cells. The young Leydig cells stimulated by the maximum dose of LH showed a significantly increase in testosterone production compared to basal levels. However, the level of testosterone in old Leydig cells stimulated by the maximum dose of LH was significantly lower than that of young Leydig cells (Fig. 5).

Effect of age on ROS production of isolated Leydig cells. Levels of ROS were significantly increased in old Leydig cells compared to the young Leydig cells (P < 0.05) (Fig. 6). The results indicate that aging significantly increases the intracellular ROS production, and excess ROS production may cause oxidative damage to old Leydig cells.

Effects of p38MAPK → COX2 and NF-κB → COX2 signaling pathways on testosterone reduction of aging Leydig cells. Inhibition of Bay 11-7082 on NF-κB translocation to nucleus and inhibition of SB203580 on p38 MAPK. We assessed the effect of Bay 11-7082 at different concentrations and different times on the inhibition of NF-κB activity using western blot and MTT assays, as well as the role of SB203580 in...
Figure 4. COX2 expression in P8 and R1 mice of varying ages. Testes sections were examined from the P8 groups at 2, 4, 8 and 10 months and the R1 group at 8 months of age. (A,C–F) COX2 protein expression by immunohistochemistry (×200, Bar:20 μm). The arrow show positive Leydig cells in the interstitial tissue. (B) COX2 protein expression by Western blot. The expression of COX2 are based on normalized ratios to β-actin. Values are present as means ± SD (n = 6). *P < 0.05 vs. 2-month-old P8; †P < 0.05 vs. 4-month-old P8; ‡P < 0.05 vs. 8-month-old P8; §P < 0.05 vs. 10-month-old P8; a P < 0.05 vs. 2-month-old R1; b P < 0.05 vs. 4-month-old R1; c P < 0.05 vs. 8-month-old R1; p2 P < 0.05 vs. 2-month-old P8; p4 P < 0.05 vs. 4-month-old P8; p8 P < 0.05 vs. 8-month-old P8; p10 P < 0.05 vs. 10-month-old P8.
inhibiting p38 MAPK. Old Leydig cells were incubated with Bay 11-7082 at a doses of 0–10 μM for 12 h. As shown in Fig. 7A, the expression of NF-κB decreased in a dose-dependent manner when treated with Bay 11-7082. The MTT assay showed that 5 μM Bay 11-7082 treatment resulted in a 25% reduction in cell viability, but dose of 10 μM led to a 60% decrease (Fig. 7B). Bay 11-7082 treatment of old Leydig cells at the 5 μM concentration seems to be more appropriate for this study. Therefore, Leydig cells were treated with 5 μM Bay 11-7082 for the specified time (Fig. 7C). The decrease in NF-κB protein was first detected at 9 h, reached the lowest level at 12 h, then progressively increased in the next 12 h. Old Leydig cells were incubated with SB203580 (10 μM) for 1 h based on a previously published protocol10.

**Effects of NF-κB and p38 MAPK inhibitor on testosterone production of young and old Leydig cells.** The roles of p38 MAPK and NF-κB activity in regulating testosterone production during aging were analyzed using SB203580 and Bay 11-7082. The values were analyzed by two-way ANOVA. The results showed that SB203580 and Bay 11-7082 didn’t influence the testosterone production of Leydig cells from young mice (4 months old) (Fig. 8).

NF-κB activity increases with age, and previous data have shown that p38 MAPK pathway is closely related to NF-κB22. Therefore, we explored the potential relationship between p38 MAPK, NF-κB and age-related decrease in testosterone production by assessing the separated and combined effects of p38 MAPK and NF-κB inhibitors. As shown in Fig. 8, testosterone production in old Leydig cells pretreated with the p38 MAPK inhibitor SB203580 restored to approximately 40% of the level seen in young Leydig cells (P < 0.05). Likewise, using Bay 11-7082 partially restored testosterone level in old Leydig cells (P < 0.05) (Fig. 8). There are interactions between these two factors (P < 0.05). Therefore, our results showed that SB203580 and Bay 11-7082 exerted their respective effects and also produced synergistic effects on testosterone production in old Leydig cells.

**Effects of SB203580 and/or Bay 11-7082 on the StAR and COX2 expression of aged Leydig cells.** The roles of SB203580 and Bay 11-7082 in the induction of COX2 and StAR gene expression were assessed by western blotting and real-time PCR. The values were analyzed by two-way ANOVA. Bay 11-7082 or SB203580 led to a significant decline in mRNA and protein level of COX2 (P < 0.05) (Fig. 9A,B), and a significant increase in protein and mRNA level of StAR (P < 0.05) (Fig. 9C,D). There are interactions between these two factors (P < 0.05).
Treatment of cells with SB203580 and Bay 11-7082 produced a synergistic effect on Cox2 and StAR gene expression. Therefore, both the NF-κB-COX2 and p38 MAPK-COX2 pathways played important roles in the reduction of testosterone production with aging.

**Discussion**

Senescence has been hypothesized to be the result of accumulated oxidative injury and chronic inflammatory damage. The SAMP mice strains provide a useful tool to explore the mechanism of age-associated diseases. In comparison with the SAMR strains, the SAMP strains display an earlier onset and more accelerated progression of age-related pathological phenotypes resembling human geriatric diseases\(^2^1\). SAMP8 were used in our study to investigate serum LH across P8 mice of 2, 4, and 8 months of age yielded age-related increases but no significant effect, and decline in testosterone production and its possible mechanism. Our results are consistent with those reported in the literature\(^2^1\).
in old Leydig cells. Our results delineated the mechanism by which oxidative stress-associated pathway of p38 MAPK signaling has a well-documented link to the NF-κB pathway. We found activation of p38 MAPK in 8-month-old P8 mice. It is known that the activation of p38 MAPK depends on ROS directly modulates COX2 expression. Thus, we speculate that COX2 involvement in inhibition of testosterone production may be mediated by p38 MAPK. This hypothesis is in coincidence with our results that p38 MAPK inhibitor SB203580 was shown to down-regulate COX2 gene expression and up-regulate StAR gene expression, along with the increase of testosterone production in old Leydig cells. Our experiments show evidence that the higher expression of COX2 and its negative regulation on testosterone production are mediated by pathway of p38 MAPK in old Leydig cells. Our results delineated the mechanism by which oxidative stress-associated pathway of p38 MAPK → COX2 inhibits testosterone production.

The potential relationship between p38 MAPK → COX2 and NF-κB → COX2 pathways were further explored. Simultaneous addition of SB203580 and Bay 11-7082 yielded an additive or synergistic effect on up-regulation of StAR expression and testosterone secretion in old Leydig cells. Perhaps both of the two signaling pathways, p38 MAPK → COX2 and NF-κB → COX2, play negative and synergistic regulatory roles in testosterone production with aging.
Summary and Conclusions

Summary diagram shows a model of old Leydig cells reported in this paper. Our results supply direct evidence that oxidative stress and chronic inflammation are implicated in the negative regulation of testosterone production both in vivo and in vitro in the process of aging. Furthermore, we provide evidence that activation of both p38 MAPK → COX2 and NF-κB → COX2 signaling pathways are functionally linked to the oxidative stress response and chronic inflammation during aging and mediates their inhibitory effects on testosterone production.

References

1. Papadopoulos, V. et al. Translocator protein-mediated pharmacology of cholesterol transport and steroidogenesis. Mol Cell Endocrinol. 408, 90–8 (2015).
2. Aghazadeh, Y., Zirkin, B. R. & Papadopoulos, V. Pharmacological regulation of the cholesterol transport machinery in steroidogenic cells of the testis. Vitam Horm. 98, 189–227 (2015).
3. Zhao, X. et al. Effects of moderate exercise over different phases on age-related physiological dysfunction in testes of SAMP8 mice. Experimental Gerontology. 48, 869–880 (2013).
4. Li, W. et al. Effects of apigenin on steroidogenesis and steroidogenic acute regulatory gene expression in mouse Leydig cells. J Nutr Biochem. 22(3), 212–8 (2011).
5. Abidi, P. et al. Evidence that age-related changes in p38 MAPK contribute to the decreased steroid production by the adrenocortical cells from old rats. Aging Cell 7(2), 168–78 (2008).
6. Hanukoglu, I. Antioxidant protective mechanisms against reactive oxygen species (ROS) generated by mitochondrial P450 systems in steroidogenic cells. Drug metabolism reviews. 38, 171–96 (2006).
7. Rosario, E. R. & Pike, C. J. Androgen regulation of beta-amyloid protein and the risk of Alzheimer's disease. Brain research reviews. 57, 444–53 (2008).
8. Matsuzawa, A. & Ichijo, H. Stress-responsive protein kinases in redox-regulated apoptosis signaling. Antioxidants & redox signaling 7, 472–81 (2005).
9. McCubrey, J. A., Lahair, M. M. & Franklin, R. A. Reactive oxygen species-induced activation of the MAP kinase signaling pathways. *Antioxidants & redox signaling*. 8, 1775–89 (2006).
10. Midzak, A. S. et al. Leydig cell aging and the mechanisms of reduced testosterone synthesis. *Molecular and Cellular Endocrinology*. 299(1), 23–31 (2009).
11. Sokanovic, S. J. et al. Age related changes of CAMP and MAPK signaling in Leydig cells of Wistar rats. *Exp Gerontol*. 58, 19–29 (2014).
12. Kang, Y. J., Mbonye, U. R., DeLong, C. J., Wada, M. & Smith, W. L. Regulation of intracellular cyclooxygenase levels by gene transcription and protein degradation. *Prog Lipid Res.* 46(2), 108–25 (2007).
13. Chen, H., Luo, L., Liu, J. & Zirkin, B. R. Cyclooxygenases in rat Leydig cells: effects of luteinizing hormone and aging. *Endocrinology*. 148(2), 735–42 (2007).
14. Yang, T. et al. Nitric oxide stimulates COX2 expression in cultured collecting ducts through MAP kinases and superoxide but not cGMP. *American journal of physiology Renal physiology*. 291, F891–5 (2006).
15. Bacher, S. & Schmitz, M. L. The NF-kappaB pathway as a potential target for autoimmune disease therapy. *Current pharmaceutical design*. 10, 2827–37 (2004).
16. Dolcet, X., Llobet, D., Pallares, J. & Matias-Guiu, X. NF-kB in development and progression of human cancer. *Virchows Archiv: an international journal of pathology*. 446, 475–82 (2005).
17. Min, K.-j. et al. An Ile-Box phosphorylation inhibitor induces heme oxygenase-1 (HO-1) expression through the activation of reactive oxygen species (ROS)-Nrf2-ARE signaling and ROS-PI3K/Akt signaling in an NF-kappaB-independent mechanism. *Cellular Signalling*. 23(9), 1505–13 (2011).
18. Klinefelter, G. R., Hall, P. F. & Ewing, L. L. Effect of luteinizing hormone deprivation in situ on steroidogenesis of rat Leydig cells purified by a multistep procedure. *Biology of reproduction*. 36, 769–83 (1987).
19. Reaven, E., Kostrna, M., Ramachandran, J. & Azhar, S. Structure and function changes in rat adrenal glands during aging. *The American journal of physiology*. 255, E903–11 (1988).
20. You, L.-hao et al. Mitochondrial ferritin suppresses MPTP-induced cell damage by regulating iron metabolism and attenuating oxidative stress. *Brain Research*. 1642, 33–42 (2016).
21. Flood, J. F. et al. Age-related decrease of plasma testosterone in SAMP8 mice: Replacement improves age-related impairment of learning and memory. *Physiology & Behavior*. 57(4), 69–73 (1995).
22. Min, K. J., Lee, J. T., Joe, E. H. & Kwon, T. K. An IkappaBalpha phosphorylation inhibitor induces heme oxygenase-1 (HO-1) expression through the activation of reactive oxygen species (ROS)-Nrf2-ARE signaling and ROS-PI3K/Akt signaling in an NF-kappaB-independent mechanism. *Cellular Signalling*. 23, 1505–13 (2011).
23. Wang, J. H. et al. Neuroendocrine immunomodulation network dysfunction in SAMP8 mice and PPr-hA/PPrSw/Ps1<sup>ΔE9</sup> mice: potential mechanism underlying cognitive impairment. *Oncotarget*. 7(17), 22988–23005 (2016).
24. Akbar, S. et al. Spontaneous occurrence of photoaging-like phenotypes in the dorsal skin of old SAM7 mice, an oxidative stress model. *Exp Dermatol*. 22(1), 62–4 (2013).
25. Orrenius, S., Gogvadze, V. & Zhivotovsky, B. Mitochondrial oxidative stress: implications for cell death. *Annual review of pharmacology and toxicology*. 47, 143–83 (2007).
26. Lee, J. S. & Surh, Y. J. Nrf2 as a novel molecular target for chemoprevention. *Cancer letters*. 234, 171–84 (2005).
27. Hoesel, B. & Schmid, J. A. The complexity of NF-kB signaling in invertebrates and cancer. *Exp Dermatol*. 22, 4265–74 (2000).
28. Hendrickx, N. et al. Up-regulation of cyclooxygenase-2 and apoptosis resistance by p38 MAPK in hypericin-mediated photodynamic therapy of human cancer cells. *The journal of biological chemistry*. 278, 52231–9 (2003).

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**Author Contributions**

Yu Zhao and Yine Qu performed isolation of primary Leydig cells from SAMP8 mouse and related experimental work. Chunfang Zhao, Dan Geng and Lixuan Wang prepared and performed tissue collection and related experimental work. Wei Chen and Li Li performed the information collection and analysis. Xiujun Zhao guided the research and offer critical discussion of experimental results. Yu Zhao revised and wrote the manuscript. Xuehui Liu, Yangyang Tian and Shiyang Chang contributed to revise paper. Pin Lv contributed to supplement the experiment. All authors have read and approved the final manuscript.

**Additional Information**

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