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Cyclooxygenase-2 Regulation of the Age-Related Decline in Testosterone Biosynthesis

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The age-related decline in testosterone biosynthesis in testicular Leydig cells has been well documented, but the mechanisms involved in the decline are not clear. Recent studies have described a cyclooxygenase-2 (COX2)-dependent tonic inhibition of Leydig cell steroidogenesis and expression of the steroidogenic acute regulatory protein (StAR). The present study was conducted to determine whether COX2 protein increases with age in rat Leydig cells and whether COX2 plays a role in the age-related decline in testosterone biosynthesis. Our results indicate that from 3 months of age to 30 months, COX2 protein in aged rat Leydig cells increased by 346% over that of young Leydig cells, StAR protein decreased to 33%, and blood testosterone concentration and testosterone biosynthesis in Leydig cells decreased to 41 and 33%, respectively. Further experiments demonstrated that overexpressing COX2 in MA-10 mouse Leydig cells inhibited StAR gene expression and steroidogenesis and that the inhibitory effects of COX2 could be reversed by blocking COX2 activity. Notably, incubation of aged Leydig cells with the COX2 inhibitor NS398 enhanced their testosterone biosynthesis. Blood testosterone concentrations in aged rats fed the COX2 inhibitor DFU, at doses of 5, 10, 15, and 20 mg/kg body weight per day were increased by 15, 23, 56, and 120%, respectively, over the levels in the rats receiving no DFU. The present study suggests a novel mechanism in male aging involving COX2 and a potential application of the mechanism to delay the age-related decline in testosterone biosynthesis. (*Endocrinology* 146: 4202–4208, 2005)

TESTOSTERONE IS PRINCIPALLY synthesized in testicular Leydig cells and then released into the blood. It is well known that blood testosterone concentration decreases during the course of male aging and results in declines in sexual function, muscle function, bone density, and other physiological parameters (1–3). It has also been reported that blood testosterone concentrations were lower in male patients suffering from Alzheimer's disease (4, 5). Supplementation with testosterone reduced neuronal β-amyloid peptide and hyperphosphorylation of τ-protein, two markers of Alzheimer's disease, suggesting that low blood testosterone is a possible risk factor for development of this disease (6–8). Whereas testosterone-associated physiological changes accompanying male aging are well described, the molecular mechanism(s) responsible for the decline of blood testosterone during the aging process remains unclear. Although multiple factors have been reported to be involved in the age-associated decrease in blood testosterone (1, 2, 9), it appears that the primary site for this decrease is testosterone biosynthesis in aging Leydig cells (1, 10). The rate-limiting step in testosterone biosynthesis is the transfer of the substrate cholesterol to the mitochondrial inner membrane, thereby initiating the steroidogenic process (11). We previously demonstrated that the steroidogenic acute regulatory (StAR) protein plays a critical function in this step by facilitating cholesterol transfer to the mitochondrial inner membrane (12–14). However, StAR protein also declines during Leydig cell aging, and the process of mitochondrial cholesterol transfer in aged Leydig cells is defective (15–17). Therefore, the mechanism regulating the age-related decline in StAR gene expression becomes an important consideration in elucidating the decline in testosterone biosynthesis during male aging.

StAR gene expression and testosterone biosynthesis are regulated by LH released from the pituitary gland. LH stimulation of Leydig cells induces cAMP formation, which activates protein kinase A (PKA). PKA in turn phosphorylates transcription factors that regulate StAR gene expression. LH and cAMP also stimulate arachidonic acid (AA) release (18–21), and AA metabolites transduce signals to the nucleus that are also involved in regulating StAR gene transcription (22). These two LH-activated signaling pathways act to coregulate StAR gene expression and steroidogenesis. Positive or negative changes in the signals in either pathway will dramatically affect StAR gene expression, which will subsequently affect steroid hormone biosynthesis in Leydig cells (22, 23). Regarding AA-mediated signaling, AA is primarily metabolized by the lipoxygenase, epoxygenase, or cyclooxygenase enzyme pathway. Whereas AA metabolites produced by lipoxygenase and epoxygenase activities may enhance steroid hormone biosynthesis (24), previous studies suggested that cyclooxygenase-2 (COX2), an isoform of cyclooxygenase, produces a tonic inhibition of StAR gene expression and

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Abbreviations: AA, Arachidonic acid; COX2, cyclooxygenase-2; db-cAMP, N6,2-*O*-dibutyryladenosine cAMP; hCG, human chorionic gonadotropin; 5-HETE, 5-hydroxyeicosatetraenoic acid; 5-HPETE, 5-hydroperoxyeicosatetraenoic acid; PG, prostaglandin; PKA, protein kinase A; StAR, steroidogenic acute regulatory protein; SV40, Simian virus 40.

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steroidogenesis in Leydig cells (23). It has been reported that COX2 expression is up-regulated during the aging process in various tissues (25, 26). Also, using cDNA microarray screening, it has previously been demonstrated that COX2 mRNA levels were increased in aged Leydig cells (27), but a cause-and-effect relationship between COX2 and aging was not apparent in that study. Our reasoning was that if COX2 protein increases in aging Leydig cells, it is possible that the tonic inhibition caused by COX2 activity plays an important role in the age-related decrease in testosterone production. The present study sought to determine whether COX2 protein increases during the course of normal aging in Leydig cells and, if so, to investigate the impact of this increase on StAR gene expression and testosterone biosynthesis.

Materials and Methods

Reagents

N6,2-*O*-dibutyryladenosine 3:5-cAMP (dbcAMP) was purchased from Sigma (St. Louis, MO). The selective COX2 inhibitor, NS398, AA metabolites, (±)5(6)-epoxy-8Z,11Z,14Z-eicosatrienoic acid, (±)8(9)-epoxy-5Z,11Z,14Z-eicosatrienoic acid, (±)11(12)-epoxy-5Z,8Z,14Z-eicosatrienoic acid, 5-hydroxyeicosatetraenoic acid (5-HETE), and 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and the enzyme immunoassay kit for prostaglandin (PG) E2 assay and antibody against COX2 were purchased from Cayman (Ann Arbor, MI). The selective COX2 inhibitor DFU was obtained from Merck Co. (Rahway, NJ). Rabbit antiserum generated against StAR protein was a generous gift from Miller and colleagues (28). Donkey antirabbit IgG antibody conjugated with horseradish peroxidase was purchased from Amersham (Arlington Heights, IL). Waymouth's MB/752 medium, DMEM, fetal bovine serum, and horse serum were purchased from Gibco-BRL Life Technologies (Gaithersburg, MD). The MSCV retroviral expression system was obtained from Clontech (Palo Alto, CA). The dual-luciferase reporter assay system was purchased from Promega (Madison, WI). Other common chemicals used in this study were obtained from either Sigma or Fisher Chemicals (Pittsburgh, PA).

Animal experiments

Forty 16-month-old male Brown Norway rats were purchased from the National Institute of Aging and housed individually in cages in a room maintained at 22 C with a 12-h light, 12-h dark cycle (0800–2000 h). The rats were fed with AIN-93M powder diet (Dyets, Bethlehem, PA) as a basal diet and divided into five groups of eight rats each. The selective COX2 inhibitor, DFU, was mixed in the basal diet every day for individual rats to give dosages of 0 (control), 5, 10, 15, or 20 mg DFU per kilogram body weight per day, respectively, based on daily food intake and body weight of each rat. The experiment was controlled, and the animals were closely monitored each day to avoid possible factors that could affect COX2 activity. After 30 d, the rats were killed in a chamber filled with carbon dioxide. Blood was collected by heart puncture. Testes were collected for Leydig cell isolation. The organs of each animal, including heart, stomach, intestines, kidney, adrenal, and testis were examined to ensure that the animals in the experiment were healthy. All procedures were approved by the Texas Tech University Health Sciences Center Animal Care and Use Committee.

Leydig cell isolation and cell culture

Rat Leydig cells were isolated from the testes using density gradient centrifugation as previously described. Based on our previous studies, when isolated with this procedure, 80% of the cells were positive for 3β-hydroxysteroid dehydrogenase and less than 1% of the contaminating cells were macrophages (29). The cells were cultured in 6-well plates with DMEM/F12 medium containing 0.1% BSA. After 2 h of culture at 32 C and 5% CO_2, the cells were cultured in DMEM/F12 medium without BSA for 4 h. Culture medium and Leydig cells were collected and stored at −80 C. The MA-10 mouse Leydig tumor cells were a generous gift from Dr. Mario Ascoli (Department of Pharmacology, University of Iowa, College of Medicine, Iowa City, IA) and were cultured in 6-well culture plates in Waymouth's MB/752 medium containing 15% horse serum as previously described (30). MA-10 cells were cultured at 37 C and 5% CO_2.

Virus preparation and infection

COX2 cDNA was excised from the plasmid PCR3.1/COX2 (23) using the restriction enzymes *BamH*I/*Xho*I and inserted into the retroviral vector pMSCV (Clontech) digested with *Bgl*II/*Xho*I to construct viral plasmid pMSCV/COX2, expressing COX2. PT67 packaging cells were transfected with pMSCV plasmid or pMSCV/COX2 plasmid using FuGENE6 transfection reagent (Roche, Indianapolis, IN). The stably transfected PT67 cells were selected with puromycin. The pMSCV and pMSCV/COX2 viruses were prepared following the manufacturer's instructions (Clontech). MA-10 mouse Leydig cells were infected with the control pMSCV virus or the pMSCV/COX2 virus following the instructions and the infected cells were used for further experiments.

Steroid hormone production

The infected MA-10 cells were cultured for 30 min in serum-free Waymouth's medium with or without 5 μM of the COX2 inhibitor NS398 and then stimulated with 0.5 mM dbcAMP for 6 h. Samples of the culture medium and cells were collected at the end of each experiment and stored at −80 C. For *in vitro* experiments with the COX2 inhibitor NS398, Leydig cells isolated from 24-month-old Brown Norway rats were cultured in DMEM/F12 medium with or without 20 μM NS398 for 30 min and then stimulated with 0.1 mM dbcAMP for 4 h. Samples of rat Leydig cells and culture medium were collected and stored at −80 C. Plasma samples from the rats were prepared from heparinized blood. Testosterone concentrations in blood plasma and the culture medium of rat Leydig cells were determined by RIA, using standards with added charcoal-stripped plasma or DMEM/F12 medium, respectively. Progesterone levels in the medium of MA-10 cell cultures were determined by RIA (31).

PGE2 assay

To confirm the inhibitory effect of the selective COX2 inhibitor, DFU, on COX2 activity, PGE2 concentrations in blood plasma were assayed using an enzyme immunoassay kit following the manufacturer's instructions (Cayman).

Transfection

MA-10 cells infected with pMSCV or pMSCV/COX2 virus were cultured in 6-well plates (0.5×10^6 cells/well) overnight. The cells in each well were transfected with 1.0 μg DNA of the StAR promoter/luciferase plasmid PGL2/StAR expressing firefly luciferase driven by the −966-bp sequence of the StAR promoter (32). Transfections also included 12.5 ng of the pRL-Simian virus 40 (SV40) vector DNA (a plasmid that constitutively expresses *Renilla* luciferase, a control reporter under the control of the SV40 promoter; Promega). Transfections were performed using FuGENE6 transfection reagent (Roche) following the manufacturer's instructions. After 48 h in culture, the cells were used for further experiments.

Luciferase assay

After experiments, the cells were washed three times with ice-cold PBS and lysed with passive lysis buffer (Promega). The supernatants were used for luciferase assays using a dual-luciferase reporter assay system following the manufacturer's instructions (Promega). The relative light units (expressed as the reading from the StAR promoter/luciferase divided by the reading from *Renilla* luciferase) were measured using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA).

Northern blot analysis

In experiments designed to determine StAR expression at the mRNA level, cells were washed three times with cold PBS and used for total RNA purification using TRIzol reagent following the manufacturer's

instructions (Gibco BRL, Grand Island, NY). The RNA was separated by electrophoresis in an agarose/formaldehyde gel (1%/6%) and blotted onto a Hybond-N^+ membrane (Amersham). StAR mRNA on the membrane was probed with ^{32}P-labeled mouse StAR cDNA and detected by autoradiography. The membrane was stripped with a buffer containing 15 mM NaCl, 15 mM sodium citrate, and 1% sodium dodecyl sulfate (pH7.0) for 30 min at 55 C. 18S rRNA on the membrane was probed to adjust for the RNA loading in each lane.

Western blot analysis

StAR protein and COX2 protein in the cells were detected by Western blot analysis as described previously (14). Western blots were performed at least three times and the results of one representative experiment are shown.

HPLC analysis of arachidonic acid metabolites

The methods previously described (33, 34) were modified for extraction and HPLC separation of AA metabolites. Briefly, MA-10 cells in 100 mm-culture plates were incubated in 5 ml Waymouth's medium containing 1.5% horse serum and 25 μCi/ml ^{3}H-AA for 4 h. The labeled cells were washed twice with 5 ml Waymouth's medium containing 0.1% fatty acid-free BSA. The cells were then incubated for 30 min in 5 ml of Waymouth's medium containing 20 μM of the selective COX2 inhibitor, NS398, and then stimulated with 0.05 mM dbcAMP for 6 h. Stimulation was terminated by transfer to −80 C and the addition of 1.0 ml cold methanol containing 5 μl of 37% HCl, 15 μg of cold AA, and a mixture of AA metabolites (3 μg each of (±)5(6)-epoxy-8Z,11Z,14Z-eicosatrienoic acid, (±)8(9)-epoxy-5Z,11Z,14Z-eicosatrienoic acid, (±)11(12)-epoxy-5Z,8Z,14Z-eicosatrienoic acid, 5-HETE, and 5-HPETE). The cells were collected and centrifuged at 8000 × *g* for 20 min. The resulting supernatants were loaded onto 3 ml/500 mg Discovery DSC-18 SPE tubes (Supelco, Bellefonte, PA) preconditioned with washes of 3 ml methanol followed by 3 ml water. The tubes were then eluted with 3 ml methanol, followed by 3 ml acetonitrile. The eluates from the tubes were dried under nitrogen and resuspended in 350 μl of 60% acetonitrile. The concentrated extracts were immediately analyzed by HPLC with a gradient component system with two 515 HPLC pumps (Waters, Milford, MA) on a Discovery C18 column (4.6 mm × 25 cm, 5 μm particle size; Supelco) using a stepped gradient of solvent A, containing water/acetonitrile/methanol/phosphoric acid (75:12.5:12.5:0.01) and solvent B, containing water/acetonitrile/methanol/phosphoric acid (5:63:32:0.01). The gradient steps used were as follows: 0 min, 100% A, 1.0 ml/min; 0–10 min, 44% A + 56% B, 0.98 ml/min; 10–89 min, 40% A +60% B, 0.96 ml/min; and 89–140 min, 100% B, 0.96 ml/min. The sample was analyzed spectrophotometrically at 206 and 235 nm using a Waters 2487 dual λ absorbance detector (Waters). The column eluate was collected directly into scintillation vials, and the radioactivities of the fractions were measured using a LS 6500 scintillation counter (Beckman, Fullerton, CA).

Statistical analysis

Each experiment was repeated at least three times. Statistical analysis of the data were performed with ANOVA and Fisher's protected least significant difference test using the StatView SE system (Abacus Concepts, Berkeley, CA). The data are shown as the means ± SE.

Results

Age-related increase in COX2 protein and concomitant decreases in StAR protein and testosterone production

To determine whether COX2 protein in Leydig cells increases in the aging process, Leydig cells were isolated from healthy Brown Norway rats aged 3, 20, and 30 months and analyzed for their COX2 and StAR protein content and their ability to synthesize testosterone. As shown in Figs. 1 and 2, the levels of COX2 protein in the Leydig cells significantly increased as the rats aged ($P < 0.01$). Concomitant with the increase in COX2 were the significant decreases in StAR protein ($P < 0.01$), blood testosterone concentrations ($P < 0.05$), and testosterone biosynthesis in isolated Leydig cells ($P < 0.05$). From 3 to 30 months of age, COX2 protein in the aged Leydig cells increased by 346% over that of the young Leydig cells, StAR protein decreased to 33%, and blood testosterone concentration and testosterone biosynthesis in the aged Leydig cells decreased to 41 and 33%, respectively.

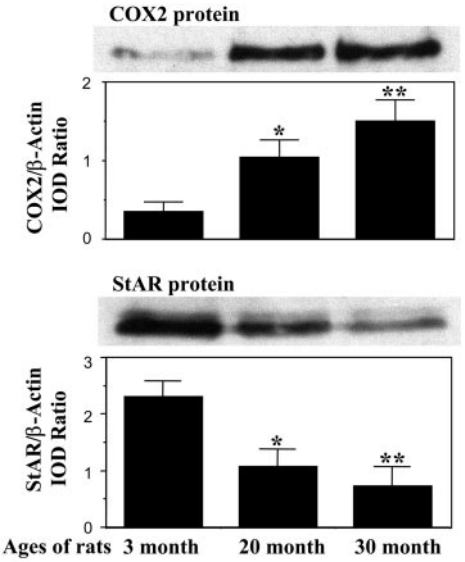

FIG. 1. Age-related increase in COX2 protein and its associated decrease in StAR protein in rat Leydig cells. Leydig cells were isolated from male Brown Norway rats aged 3, 20, and 30 months. COX2, StAR, and β-actin protein in the cells were detected by Western blot analysis. Bands specific for β-actin, COX2, and StAR were quantified using the BioImage Visage 2000 (B.I. Systems Corp., Ann Arbor, MI) and expressed as integrated OD (IOD). The amount of COX2 or StAR protein in the Leydig cells was expressed as the COX2 to β-actin or StAR to β-actin IOD ratio. *, Significantly different from that of 3-month-old rats ($P < 0.05$, n = 4). **, Highly significantly different from that of 3-month-old rats ($P < 0.01$, n = 4).

Increased COX2 expression resulted in decreased StAR gene expression and steroidogenesis

To directly demonstrate that increased COX2 expression in Leydig cells results in decreased StAR gene expression and steroidogenesis, MA-10 mouse Leydig cells were infected with pMSCV/COX2 virus to express COX2 and examined its effects. Infection with pMSCV/COX2 virus markedly increased COX2 expression and dramatically reduced db-cAMP-stimulated StAR protein level and progesterone synthesis (Fig. 3). Furthermore, StAR promoter activity and StAR transcript levels were also reduced in the cells overexpressing COX2 (Fig. 4). Importantly, the reduced StAR promoter activity, StAR mRNA levels, and StAR protein and steroid hormone production were reversed by inhibition of COX2 activity using 5 μM of the selective COX2 inhibitor, NS398.

Inhibition of COX2 activity increased testosterone biosynthesis in isolated aged Leydig cells and blood testosterone concentrations in aged rats

To confirm the above observations, Leydig cells were isolated from 24-month-old Brown Norway rats and treated

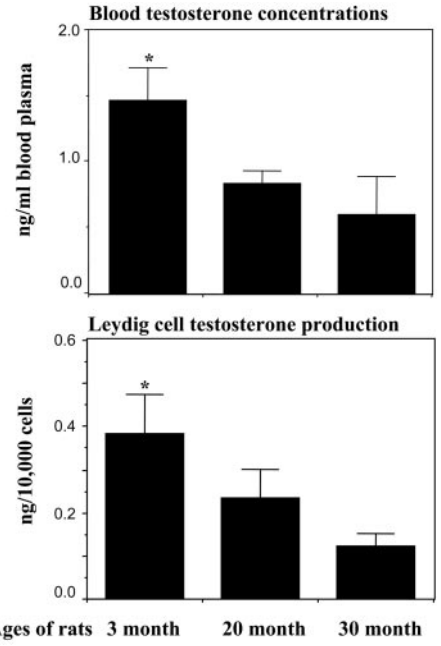

FIG. 2. Age-related decreases in blood testosterone levels and testosterone production in isolated rat Leydig cells. Blood samples were collected from male Brown Norway rats aged 3, 20, and 30 months. Blood plasma was prepared from the blood samples and stored at −80 C. Leydig cells were isolated from the testes and cultured with DMEM/F12 medium containing 0.1% BSA in 6-well plates. After 2 h of culture at 32 C and 5% CO_2, the cells were cultured in DMEM/F12 medium without BSA for 4 h. The culture medium was collected and stored at −80 C. Testosterone concentrations in the culture medium and blood plasma were determined by RIA. *, Significantly different from that of 30-month-old rats ($P < 0.05$, n = 6).

with the COX2 inhibitor NS398 to determine StAR protein levels and testosterone biosynthetic capacity. Figure 5 shows that inhibition of COX2 activity increased StAR protein expression and testosterone biosynthesis ($P < 0.05$) in the isolated aged Leydig cells. During 4 h of culture, testosterone production and StAR protein expression in COX2 inhibitor-treated cells increased by 76.4 and 110.5%, respectively, over those of the cells stimulated with 0.1 mM dbcAMP alone. To determine whether dietary supplementation with a COX2 inhibitor is able to increase blood testosterone concentrations, animal experiments were conducted with 40 16-month-old Brown Norway rats as described in *Materials and Methods*. During the course of the experiment, all of the animals were healthy. It was found that inhibition of COX2 activity with DFU, as assessed by measuring prostaglandin E2 levels, increased blood testosterone concentrations ($P < 0.05$) and StAR protein in Leydig cells in a dose-dependent manner. Blood testosterone concentrations in the rats fed 5, 10, 15, and 20 mg DFU per kilogram body weight per day were increased by 15, 23, 56, and 120%, respectively, over the levels in rats receiving no DFU (Fig. 6).

Inhibition of COX2 activity increased 5-lipoxygenase-generated AA metabolites

To investigate the mechanism for the COX2-dependent tonic inhibition of StAR gene expression and steroidogenesis in Leydig cells, HPLC was employed to determine which AA

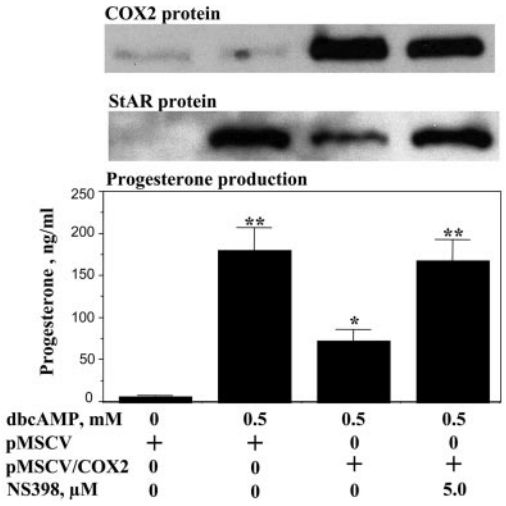

FIG. 3. Overexpression of COX2 inhibited StAR protein expression and steroid production in MA-10 mouse Leydig cells. The pMSCV and pMSCV/COX2 retroviruses were prepared and used to infect MA-10 cells. The infected cells were incubated with 5 μM of the selective COX2 inhibitor NS398 for 30 min and then stimulated with 0.5 mM dbcAMP for 6 h. COX2 protein and StAR protein in the cells were analyzed by Western blot. Progesterone production in the culture medium was analyzed by RIA (26). *, Significantly different from control ($P < 0.05$, n = 5). **, Highly significantly different from that of pMSCV/COX2-infected cells without NS398 ($P < 0.01$, n = 5);

metabolites in Leydig cells are affected by COX2 activity. The results in Fig. 7 show that inhibition of COX2 activity in MA-10 Leydig cells with 20 μM of the COX2 inhibitor NS398 significantly increased 5-HPETE ($P < 0.05$) and 5-HETE ($P <$

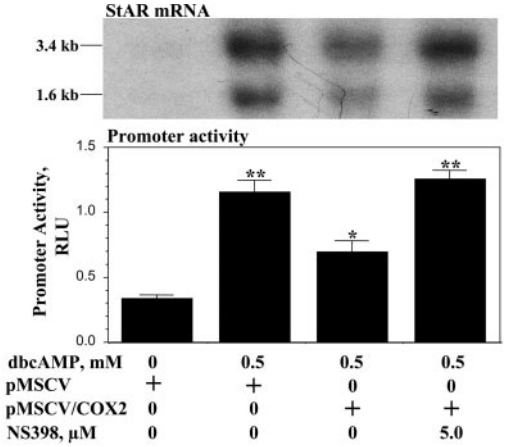

FIG. 4. Overexpression of COX2 inhibited StAR gene transcription in MA-10 mouse Leydig cells. MA-10 cells infected with pMSCV or pMSCV/COX2 virus were incubated with 5 μM NS398 for 30 min and then stimulated with 0.5 mM dbcAMP for 6 h. StAR mRNA levels in the cells were analyzed by Northern blot. For StAR promoter activity assay, the infected MA-10 cells in each well were transfected with StAR promoter/luciferase plasmid PGL2/StAR DNA (32). Transfections also included pRL-SV40 vector DNA (a plasmid that constitutively expresses *Renilla* luciferase, a control reporter under the control of the SV40 promoter). After 48 h in culture, the cells were treated as described above and then washed and lysed with passive lysis buffer. The supernatants were used for luciferase assays using a dual luciferase reporter assay system. StAR promoter activities were expressed as relative light units (RLUs). *, Significantly different from controls ($P < 0.05$, n = 4). **, Highly significantly different from that of pMSCV/COX2-infected cells without NS398 ($P < 0.01$, n = 4).

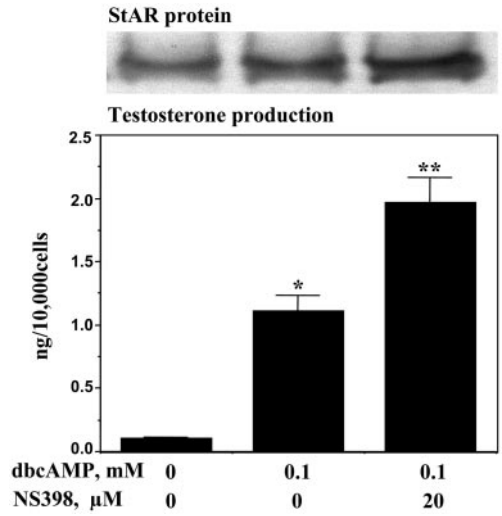

FIG. 5. Inhibition of COX2 activity increased cAMP-stimulated StAR expression and testosterone production in aged Leydig cells. Leydig cells were isolated from 24-month-old male Brown Norway rats. The cells were incubated with 20 μM of the COX2 inhibitor NS398 for 30 min and then stimulated with 0.1 mM dbcAMP for 4 h. The cells and culture medium were collected and stored at −80 C. Testosterone concentrations in the medium were determined by RIA. StAR protein in the cells was analyzed by Western blot. *, Significantly different from controls ($P < 0.05$, n = 6). **, Highly significantly different from controls ($P < 0.01$, n = 6).

0.05). Both of these AA metabolites are derived through the action of 5-lipoxygenase and have been previously reported to enhance StAR gene expression and steroidogenesis (35). To determine whether a 5-lipoxygenase metabolite of AA is able to increase testosterone synthesis in aged Leydig cells, 5-HETE was added to cultured Leydig cells isolated from 24-month-old male Brown Norway rats, and then the cells were stimulated with human chorionic gonadotropin (hCG). As shown in Fig. 8, incubation with 5-HETE significantly increased hCG-stimulated testosterone production ($P < 0.05$).

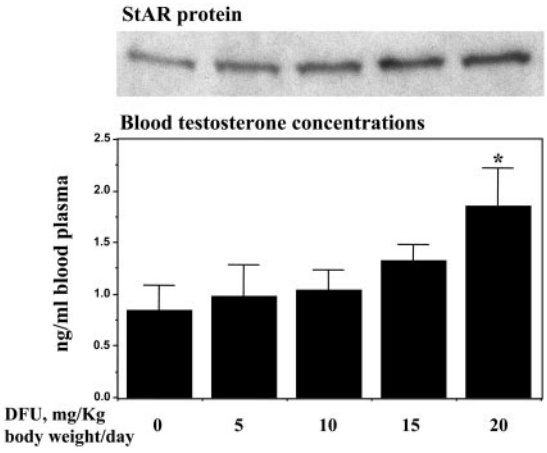

FIG. 6. Dietary supplementation of a COX2 inhibitor increased blood testosterone levels and StAR protein in Leydig cells in aged rats. Forty 16-month-old male Brown Norway rats were divided into five groups of eight rats each. The selective COX2 inhibitor, DFU, was mixed in their diet every day for individual rats to give dosages of 0 (control), 5, 10, 15, or 20 mg DFU per kilogram body weight per day, respectively, based on daily food intake and body weight of each rat. After 30 d, the rats were killed in a chamber filled with carbon dioxide. Blood was collected by heart puncture. Testes were collected for Leydig cell isolation. StAR protein in their Leydig cells was analyzed by Western blot. Testosterone concentrations in the blood plasma were determined by RIA. *, Significantly different from control ($P < 0.05$, n = 8).

Discussion

The decrease in testosterone biosynthesis in aging Leydig cells involves multiple factors. The present study focuses on age-related increases in COX2 protein in Leydig cells and its inhibitory effect on StAR-mediated testosterone production.

Previous studies have indicated that the primary reason for the age-related decline in blood testosterone is the decrease in steroidogenic sensitivity of aging Leydig cells to LH stimulation (1, 10). Our recent studies suggested that COX2 produces a tonic inhibition of StAR gene expression in Leydig cells, thereby reducing their sensitivity to cAMP stimulation (23). Those observations encouraged us to analyze the levels of COX2 protein in aged Leydig cells, following the hypothesis that increased COX2 protein that may occur as a consequence of aging could be an important factor in attenuating the LH responsivity of Leydig cells. Mirroring observations in other tissues (25, 26), the results from the present study demonstrated an age-related increase in COX2 protein in Leydig cells isolated from Brown Norway male rats (Fig. 1). Statistical analysis of the data indicated a significant difference in COX2 proteins among the different age groups ($P < 0.01$). Also, recent studies by another group (27) reported an increase in COX2 mRNA levels in aged Leydig cells, an observation in keeping with our present findings. Importantly, we also observed an inverse relationship in which the increase in COX2 protein that accompanies aging was closely linked to decreases in StAR protein, Leydig cell testosterone synthesis, and blood testosterone levels.

The correlation between COX2 levels and steroidogenesis was substantially strengthened by our observation that increasing COX2 protein expression in Leydig cells artificially by infection resulted in decreased StAR gene transcription, StAR protein expression, and steroid hormone biosynthesis. Because COX2 protein was overexpressed in MA-10 Leydig cells, their sensitivity to cAMP stimulation was markedly decreased as indicated by the sharp reductions in dbcAMP-induced StAR protein and steroid production. This indicated that COX2 is involved in regulating Leydig cell sensitivity to cAMP, an observation strengthened by our experimental results that a selective COX2 inhibitor could reverse or block the restraints imposed by COX2 on StAR gene expression and steroidogenesis. Whereas it is still unknown as to how COX2 achieves its inhibitory effect, taken together, our data indicate that a COX2-dependent tonic inhibition of StAR gene expression increases during the course of male aging and reduces the sensitivity of Leydig cells to LH or cAMP stimulation. Consequently, the age-related increase in COX2 expression results in a decline in testosterone biosynthesis. Because COX2 gene expression is up-regulated by many pathological and stress-related factors, including reactive oxygen (26, 36), the present study provides important information for understanding the roles of these factors in the aging process.

On the basis of the above observations, we reasoned that the functional decline in LH- or cAMP-stimulated testoster-

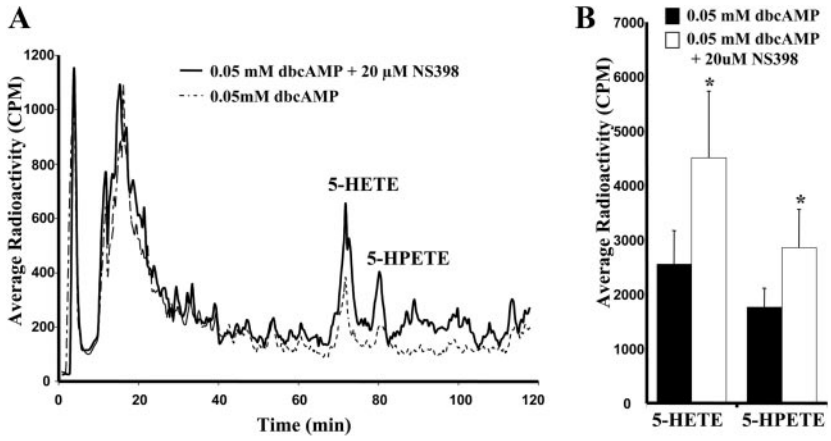

FIG. 7. Inhibition of COX2 activity increased AA metabolites produced by 5-lipoxygenase in MA-10 mouse Leydig cells. MA-10 cells preloaded with ^{3}H-AA were incubated with 20 μM NS398 for 30 min and then stimulated with 0.05 mM dbcAMP for 6 h. AA metabolites were extracted and separated by HPLC. The column eluate was collected directly into scintillation vials, and the radioactivities of the fractions were measured using a Beckman LS 6500 scintillation counter. A, A radioactivity chromatogram of ^{3}H-AA metabolites separated by HPLC shown as the average of five individual experiments. B, Averages of the total radioactivity for the samples in the peak areas of 5-HETE and 5-HPETE from five individual experiments. *, Significantly different from the paired group without NS398 ($P < 0.05$, n = 5).

one biosynthesis in aged Leydig cells could be reversed by inhibition of COX2 activity. This hypothesis is supported by the results from the experiments using Leydig cells isolated from 24-month-old Brown Norway rats. Inhibition of COX2 activity increased cAMP-stimulated StAR protein and testosterone biosynthesis in the aged Leydig cells. Moreover, in the animal experiment, dietary supplementation with the selective COX2 inhibitor DFU increased blood testosterone concentrations and StAR protein in Leydig cells in a dose-dependent manner. These results indicated that inhibition of COX2 activity improved the steroidogenic capacity of individual Leydig cells because an identical number of isolated aged Leydig cells treated with a COX2 inhibitor produced much higher levels of StAR protein and testosterone. Whereas other steroidogenic proteins could affect blood testosterone levels, our previous work suggested that the increased steroidogenesis resulting from inhibition of COX2 activity is mainly due to an increased cholesterol supply because there was no significant difference in steroid production among the experimental groups when 22(R)-hydroxycholesterol was used as substrate (23). This agrees with our results from experiments with isolated aged Leydig cells and also those with aged rats, in which the COX2 inhibitor-increased testosterone production is always concomitant with increased StAR protein, known to play a critical role in cholesterol supply. Thus, results from both *in vitro* and *in vivo* experiments corroborated our observations and suggested that reduction of COX2-dependent tonic inhibition of StAR gene expression may delay or offset the decline in testosterone biosynthesis in aged Leydig cells.

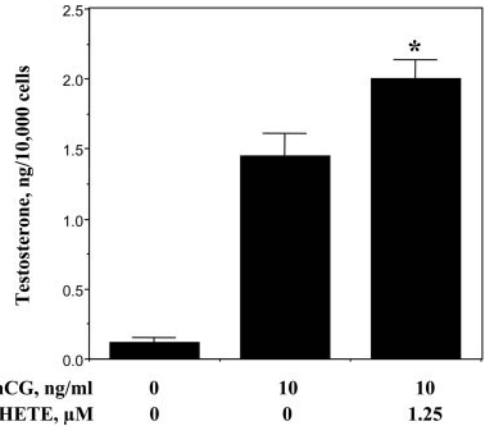

FIG. 8. Incubation of aged Leydig cells with 5-HETE enhanced hCG-stimulated testosterone production. Leydig cells were isolated from 24-month-old male Brown Norway rats. The cells were preincubated with 1.25 μM 5-HETE for 30 min, and then 10 ng/ml hCG were added to the cultures for a further 4 h. Samples of culture medium were collected and stored at −80 C. Testosterone concentrations in the medium were determined by RIA. *, Significantly different from that of the hCG-stimulated group ($P < 0.05$, n = 3).

The mechanism responsible for the COX2-dependent tonic inhibition of StAR expression and steroidogenesis is not clear at this time. The COX2 inhibitor-induced increases in steroid hormone and StAR protein in Leydig cells were not due to an increase in PKA phosphorylation because the COX2 inhibitor, NS398, was unable to increase PKA activity (23). Also, the COX2 inhibitor itself did not induce steroid hormone production but rather reduced the COX2-dependent tonic inhibition of StAR gene expression and steroidogenesis. Reduction of this tonic inhibition dramatically increased the sensitivity of Leydig cells to cAMP stimulation with subthreshold levels of cAMP or PKA activity resulting in maximal StAR gene expression and steroid hormone production in Leydig cells (23).

Earlier studies have reported that an AA metabolite produced by COX2, PGF2α, inhibited StAR gene expression by inducing a negative transcription factor that binds to the StAR promoter to depress StAR gene transcription (37). It is possible that COX2-generated AA metabolites inhibit StAR gene expression by inducing transcriptional repressors. We attempted to discern the AA metabolites that are influenced or controlled by COX2, hoping to reveal how this enzyme might be repressing the StAR gene. Interestingly, the results from HPLC analysis indicated that inhibition of COX2 activity resulted in an AA metabolite profile similar to that from MA-10 Leydig cells treated with the maximally stimulating levels of dbcAMP (35), demonstrating significant increases in 5-HPETE and 5-HETE, two AA metabolites produced by 5-lipoxygenase activity (Fig. 7). We previously reported that these two AA metabolites significantly enhanced cAMP-stimulated StAR gene expression and steroid hormone biosynthesis in MA-10 cells (35). Also, addition of 5-HETE to the culture of aged Leydig cells significantly enhanced hCG-stimulated testosterone production (Fig. 8).

The above observations suggest that inhibition of COX2 activity, although potentially reducing the COX2-generated AA metabolites that inhibit StAR gene expression, increase the 5-lipoxygenase-generated AA metabolites that enhance StAR gene expression, resulting in increased testosterone production. It is also possible that some as-yet-unknown factors might be involved in the COX2-dependent tonic inhibition of testosterone biosynthesis. Further studies on the mechanisms responsible for this tonic inhibition will improve our understanding of the aging process in males.

In summary, our results indicate that a COX2-dependent tonic inhibition of StAR gene expression is involved in the regulation of the steroidogenic sensitivity of Leydig cells to LH or cAMP stimulation. The present study further demonstrates that COX2 protein in Leydig cells increases as rats age, thereby enhancing the tonic inhibition of StAR gene expression and resulting in decreased testosterone biosynthesis. This study also suggests a potential intervention in this mechanism to delay the decrease in testosterone biosynthesis typically accompanying the course of male aging.

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