Inhibition of Cyclooxygenase-2 Down-regulates Aromatase Activity and Decreases Proliferation of Leydig Tumor Cells*

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Our recent studies have revealed that estrogens stimulate an autocrine mechanism determining Leydig tumor cell proliferation. Estrogen overproduction is due to an elevated steroidogenic factor-1 (SF-1) expression and cAMP-response element-binding protein (CREB) phosphorylation, both inducing aromatase overexpression. Although we have shown that increased SF-1 expression depends mainly on higher local insulin-like growth factor I production, the mechanisms and factors determining increased CREB activation in Leydig tumor cells are not completely understood. In this study, we investigated the role of cyclooxygenase-2 (COX-2) in CREB-dependent-aromatase expression in Leydig tumor cells. We found that COX-2 is expressed in rat and human Leydigomas as well as in the rat Leydig tumor cell line R2C, but not in normal testis. Our data indicate that in R2C cells the COX-2-derived prostaglandin E2 (PGE2) binds the PGE2 receptor EP4 and activates protein kinase A (PKA) and ultimately CREB. Inhibitors for COX-2 (NS398), EP4 (AH23848), and PKA (H89) decreased aromatase expression and activity as a consequence of a decreased phosphorylated CREB recruitment to the PI promoter of the aromatase gene. The COX-2/PGE2/PKA pathway also seems to be involved in aromatase post-translational activation, an observation that requires further studies. The reduction in aromatase activity was responsible for a drop in estrogen production and subsequent reduction in cyclin E expression resulting in a decrease in tumor Leydig cell proliferation. Furthermore, COX-2 silencing caused a significant decrease in CREB phosphorylation, aromatase expression, and R2C cell proliferation. These novel findings clarify the mechanisms involved in the growth of Leydig cell tumors and should be taken into account in determining new therapeutic approaches.

Leydig cell tumors are the most common cancers of the gonadal stroma (1). However, their etiology and pathogenesis are poorly defined. Transgenic mice overexpressing aromatase, the enzyme responsible for the conversion of androgens to estrogens (2), present enhancement of circulating 17β-estradiol (E2)3 levels, which have been associated with Leydig cell hyperplasia and tumors (3). These observations and our recent data (4, 5) suggest that estrogens can elicit proliferative effects in human and rat tumor Leydig cells through an autocrine mechanism.

Aromatase activity is regulated primarily at the level of gene expression and is present in testicular somatic cells and during the maturative phases of male germ cells (6). The CYP19 gene that encodes aromatase contains at least eight unique promoters used in a tissue-specific manner (2). The proximal promoter II regulates aromatase expression in human fetal and adult testes, R2C, and H540 rat Leydig tumor cells (7). Specific response elements appear to be involved in rat aromatase expression: a nuclear receptor half site binding SF-1/LRH-1 (8) and CRE-like sequences binding CREB/ATF protein family members (9, 10). In a recent study, we have shown that Leydig cell tumors exhibit increased SF-1 expression and CREB phosphorylation, which is an indicator of elevated transcriptional activity (4). In the same study, we explained that Leydig tumor cells produce high levels of insulin-like growth factor I, which, through the activation of phosphatidylinositol 3-kinase and Akt pathways, leads to increased steroidogenic factor-1 (SF-1) transcription. Moreover, R2C cells are characterized by the absence of DAX-1 (dosage-sensitive sex reversal, adrenal hypoplasia congenita, critical region on the X chromosome, gene I), an SF-1 corepressor. This is probably due to constitutively active PKA signaling, because in a mouse Leydig cell line, a marked decrease of DAX-1 mRNA was observed after addition of LH (luteinizing hormone) and forskolin (11). Increased SF-1 binding to promoter PI, together with pCREB, contribute to constitutive active aromatase expression, which, in turn, is responsible for E2 production involved in Leydig tumor cell proliferation. Although we have elucidated the pathway responsible for increased SF-1 transcription, the molecular mechanisms determining constitutive CREB phosphorylation still remain to be defined.

It has been shown that prostaglandin E2 (PGE2) stimulates cAMP formation and production of promoter II-specific aromatase transcripts in human adipose tissue (12). The first step in prostaglandin (PG) synthesis from arachidonic acid is catalyzed by two isoforms of cyclooxygenase (COX), extracellular regulated kinase 1/2; PGE2, prostaglandin E2; EP, E-series prostanooid receptors; CYP, cytochrome P450; FRRT, Fischer rat normal testis; FRTT, Fischer rat tumor testis; CHIP, chromatin immunoprecipitation; DAX-1, dosage-sensitive sex reversal, adrenal hypoplasia congenita, critical region on the X chromosome gene 1; LH, luteinizing hormone; LHR, LH receptor; siRNA, small interference RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcription.

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§ The abbreviations used are: E2, 17β-estradiol; COX-2, cyclooxygenase-2; CREB, cAMP-response binding protein; pCREB, phosphorylated CREB; SF-1, steroidogenic factor-1; MAP, mitogen-activated protein; ERK1/2, extracellular regulated kinase 1/2; PGE2, prostaglandin E2; EP, E-series prostanooid receptors; CYP, cytochrome P450; FRRT, Fischer rat normal testis; FRTT, Fischer rat tumor testis; CHIP, chromatin immunoprecipitation; DAX-1, dosage-sensitive sex reversal, adrenal hypoplasia congenita, critical region on the X chromosome gene 1; LH, luteinizing hormone; LHR, LH receptor; siRNA, small interference RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcription.
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which are the rate-limiting enzymes in PG synthesis because of their rapid auto-inactivation (13). COX-1 is constitutively present in various tissues and regulates the physiological production of PGs necessary for maintaining normal homeostasis. Conversely COX-2 represents the inducible isofrom, and it is not detectable in most normal tissues but is rapidly induced by mitogens, cytokines, and growth factors (reviewed in Refs. 14, 15).

Recently, overexpression of COX-2 has been observed in several tumor types such as colorectal (16), gastric (17), hepatocellular (18), lung (19), esophageal (20), and pancreatic carcinoma (21). COX-2 was also detected in breast cancer (22), where it was associated with aromatase expression (23). It is worth noting that COX inhibitors decreased both aromatase expression and activity in breast cancer cells (24), and the use of combinations of COX-2 and aromatase inhibitors was more effective than single agents in decreasing estradiol production (25).

COX-2 was not present in normal human testes, but was found in testicular biopsies from men with impaired spermatogenesis (26) or in testicular cancer (27). On the basis of the aforementioned observations, in the present study we evaluated the potential stimulatory action exerted by COX-2-derived PGE2, on aromatase expression, E2 production, and proliferation of Leydig tumor cells. We used, as model systems, rat R2C Leydig cancer cells and Leydig cell tumors from Fisher rats, which were characterized by exceptionally high incidence of spontaneous neoplasms associated with aging (28). Here, we demonstrate that the COX-2/PGE2/PKA transduction pathway is directly involved in CREB phosphorylation, which, in turn, stimulates aromatase expression and E2-dependent Leydig tumor cell proliferation.

EXPERIMENTAL PROCEDURES

Cell Cultures, Animals, and Reagents—Cells were obtained from ATCC (LGC Standards, Teddington, Middlesex, UK), grown for 2 weeks (four passages) before freezing aliquots. Each aliquot was used for no more than ten passages. TM3 cells (an immature mouse Leydig cell line) were cultured in Dulbecco’s modified Eagle’s medium/F-12 medium supplemented with 5% horse serum, 2.5% fetal bovine serum, and antibiotics (Invitrogen); R2C cells (a rat Leydig tumor cell line) were cultured in Ham’s nutrient mixture F-10 supplemented with 15% horse serum, 2.5% fetal bovine serum, and antibiotics (Invitrogen). Six-month-old male Fischer rat normal (FRN) and 24-month-old male Fischer rat with a tumor (FRT) (a generous gift of PerkinElmer Life Sciences) as a substrate (34). Incubations were performed at 37 °C for 2 h under a 95%:5% air/CO2 atmosphere. Results obtained were expressed as picomoles/h and normalized to milligrams of protein (picomoles/h/mg of protein).

Radioimmunoassay—Prior to experiments, TM3 cells were maintained overnight in Dulbecco’s modified Eagle’s medium/F-12 medium and R2C cells in Ham/F-10 without serum or antibiotics (serum-free medium). Cells were then treated as indicated, and the estradiol content of medium recovered from each well determined against standards prepared in serum-free medium using a radioimmunoassay kit (Diagnostic System Laboratories, Webster, TX). Results were normalized to the cellular protein content per well.

Real-time RT-PCR—Prior to experiments, cells were maintained overnight in serum-free medium. Cells were then treated for the indicated times, and RNA was extracted from cells using the TRizol RNA isolation system (Invitrogen). TRizol was also used to homogenize total tissue of normal (FRNT) and tumor (FRTT) Fischer rat testes for RNA extraction. Each RNA sample was treated with DNase I (Ambion, Austin, TX), and purity and integrity of the RNA were confirmed spectrophotometrically and by gel electrophoresis prior to use. One microgram of total RNA was reverse transcribed in a final volume of 30 μl using the ImProm-II Reverse Transcription System Kit (Promega). The cDNA was diluted 1:3 in nuclease-free water, aliquoted, and stored at −20 °C. Primers for the amplification were based on published sequences for rat CYP19. The nucleotide sequences of the primers for CYP19 were as follows: forward, 5’-GAGAAACTGGAAGACTGTA-TGGAT-3’ and reverse, 5’-ACTGATTCAGTTCCTCTTTT-GTCA-3’. PCR reactions were performed in the iCycler iQ Detection System (Bio-Rad), using 0.1 μM of each primer, in a total volume of 30 μl of reaction mixture following the manufacturer’s recommendations. SYBR Green Universal PCR Master Mix (Bio-Rad), with the dissociation protocol, was used for gene amplification. Negative controls contained water instead of cDNA.
of first-strand cDNA. Each sample was normalized on the basis of its 18 S ribosomal RNA content. The 18 S quantification was performed using a TaqMan Ribosomal RNA Reagent kit (Applied Biosystems, Applera Italy, Monza, Milano, Italy) following the method provided in the TaqMan Ribosomal RNA Control Reagent Kit. The relative gene expression levels were normalized to a calibrator that was chosen to be the basal, untreated sample. Final results were expressed as n-fold differences in gene expression relative to 18 S rRNA and calibrator, calculated following the ΔΔCt method, as published previously (4).

**Chromatin Immunoprecipitation (ChIP) Assay**—This assay was performed using the ChIP Assay Kit from Upstate (Lake Placid, NY) with minor modifications in the protocol. R2C cells were grown in 100-mm plates. Confluent cultures (90%) were treated for 24 h with NS398 and H89 (Sigma). Following treatment, DNA-protein complexes were cross-linked with 1% formaldehyde at 37 °C for 10 min. Next, cells were collected and resuspended in 400 µl of SDS lysis buffer (Upstate) and left on ice for 10 min. Then, cells were sonicated four times for 10 s at 30% of maximal power and collected by centrifugation at 4 °C for 10 min at 14,000 rpm. Of the supernatants 10 µl was kept as input (starting material, to normalize results), whereas 100 µl was diluted 1:10 in 900 µl of ChIP dilution buffer (Upstate) and immunocleared with 80 µl of sonicated salmon sperm DNA protein A-agarose (Upstate) for 6 h at 4 °C. The immunocomplex was formed using 2 µl of specific antibody, anti-CREB, or anti-pCREB (Cell Signaling, Celbio, Milano, Italy), overnight at 4 °C. Immunoprecipitation with salmon sperm DNA protein A-agarose was continued at 4 °C for 4 h. DNA-protein complexes were reverse cross-linked overnight at 65 °C. Extracted DNA was resuspended in 20 µl of buffer containing 10 mM Tris and 1 mM EDTA, pH 8.0. A 5-µl volume of each sample and input were used for real-time PCR using CYP19 promoter II-specific primers: forward, 5'-TCAAGGGTAGAAATTGGGA-C-3'; reverse, 5'-GGTGCTGGAATGGGACAGATG-3'. PCR reactions were performed in the iCycler iQ Detection System (Bio-Rad), using 0.1 µM of each primer, in a total volume of 50-µl reaction mixture following the manufacturer’s recommendations. SYBR Green Universal PCR Master Mix (Bio-Rad), with the dissociation protocol, was used for gene amplification. Negative controls contained water instead of DNA. Final results were calculated using the ΔΔCt method as explained above, using input Ct values instead of the 18 S. The basal sample was used as calibrator.

**PGE2 Production**—TM3 and R2C cells were maintained in complete medium for 48 h and in serum-free medium overnight. The following day fresh serum-free medium or, in the case of R2C cells, medium with NS398 was added for 6 h. PGE2 in the culture medium was assayed using an EIA kit following the manufacturer’s instructions (Cayman).

**Western Blot Analysis**—Methods for protein extraction and blot preparation have been previously published (4). Blots were incubated overnight at 4 °C with (a) anti-COX-2 (1:1000, Cell Signaling Technology), (b) anti-COX-1 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), (c) anti-human P450 aromatase (1:50, Serotec, Oxford, UK), (d) anti-CREB (1:1000, 48H2, Cell Signaling Technology), (e) anti-pCREB Ser-133 (87G3, 1:1000, Cell Signaling Technology), (f) anti-cyclin E (M-20, 1:1000, Santa Cruz Biotechnology), (g) anti-EP4 (1:200, Santa Cruz Biotechnology), and (h) anti-GAPDH (1:1000, Santa Cruz Biotechnology). Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences), and immunoreactive bands were visualized with the ECL Western blotting detection system (Amersham Biosciences). To assure equal loading of proteins, membranes were stripped and incubated overnight with GAPDH antibody.

**In Vitro Assay for PKA Activity**—This assay was performed using the PepTag for Non-Radioactive Detection of cAMP-dependent Protein Kinase Assay kit (Promega). R2C cells were grown in 100 × 60-mm plates to 100% confluence. After treatments (1 h) cells were washed with phosphate-buffered saline (5 ml/100-mm dish) and lysed in cold PKA extraction buffer (0.5 ml/plate) containing 25 mEq Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, 1 µg/ml leupeptin, 1 µg/ml aprotinin.

The lysates were cleared by centrifugation at 14,000 × g, 5 min, and 5 µl of cleared lysates was subjected to a kinase reaction with the fluorescence-labeled PKA substrate, Leu-Arg-Ala-Ser-Leu-Gly (Kemptide), following the manufacturer’s protocol. The reaction was stopped by boiling the samples for 10 min. The samples were separated on 0.8% agarose gel by electrophoresis at 100 volts for 15 min. Phosphorylated peptide migrated toward the anode (+), while non-phosphorylated peptide migrated toward the cathode (−). The gel was photographed on a transilluminator. The quantitative differences in the amount of phosphorylated and non-phosphorylated peptide species were detected by spectrophotometric method reading the absorbance at 570 nm.

**RNA Interference**—COX-2 and EP4 stealth siRNA and scrambled siRNA were purchased from Invitrogen. Forty-eight hours after plating cells into 100-mm dishes at 7 × 106 cells, siRNAs were transfected in Ham/F-10 to a final concentration of 100 nM using the Lipofectamine 2000 Transfection Reagent (Invitrogen) according to manufacturer’s instructions. Forty-eight hours after transfection cells were lysed for protein extraction. COX-2- and EP4-specific knockdown and effects on aromatase were analyzed by Western analysis.

**Cell Proliferation Assay**—For proliferative analysis a total of 1 × 105 cells was seeded onto 12-well plates in complete medium and grown for 2 days. Prior to experiments, cells were maintained for 24 h in Ham/F-10 medium and then treated with NS398, AH23848, and H89 (Sigma) or transfected with COX-2 siRNA (Invitrogen) as described above. Control (basal) cells were treated with the same amount of vehicle alone (DMSO) that never exceeded the concentration of 0.01% (v/v). [3H]Thymidine incorporation was evaluated after a 24-h incubation period with 1 µCi of [3H]thymidine (PerkinElmer Life Sciences) per well. Cells were washed once with 10% trichloroacetic acid, twice with 5% trichloroacetic acid, and lysed in 1 ml of 0.1 M NaOH at 37 °C for 30 min. The total suspension was added to 10 ml of Optifluor fluid and was counted in a scintillation counter.
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FIGURE 1. Expression of COX-2 in Leydig tumor cells. A, Western blot analysis was performed using 50 μg of total proteins extracted from TM3 and R2C cells or from total tissue of normal (FRNT) and tumor (FRTT) Fischer rat testes. GAPDH was used as a loading control. Results are representative of three independent experiments. B and C, immunodetection of COX-2 in Leydig cell tumor (B) and normal human testis (C). Inset: negative control. Arrow: Leydig cell. Scale bars = 12.5 μm.

Data Analysis and Statistical Methods—Pooled results from triplicate experiments were analyzed using one-way analysis of variance with Student-Newman-Keuls multiple comparison methods, using SigmaStat version 3.0 (SPSS, Chicago, IL).

RESULTS

COX-2 Is Highly Expressed in Leydig Cell Tumors—Using Western analysis we investigated COX-2 expression in normal and Leydig tumor cells (Fig. 1A). Both R2C cells in basal condition as well as testes from Fischer rats with a developed tumor (FRTT) express COX-2, which is absent in normal controls. On the other hand, COX-1 appears to be expressed at similar levels in all the samples (Fig. 1A). Moreover, by immunohistochemistry, we found similar results in human samples; the cytoplasm of neoplastic human Leydig cells showed a strong COX-2 immunoreactivity (Fig. 1B), while human control testis showed immunonegative reaction for COX-2 (Fig. 1C).

COX-2 Silencing and COX-2 Inhibitor, NS398, Decrease pCREB and Aromatase Expression—We have previously shown that expression of aromatase, SF-1, and the phosphorylated form of CREB are elevated in R2C and in rat Leydig tumor cells (4). The altered activation of CREB, together with high SF-1 expression, may explain the increase in aromatase levels observed in tumor Leydig cells. Knockdown of COX-2 in R2C cells using siRNA caused a significant decrease in aromatase expression together with a decrease in CREB phosphorylation (Fig. 2A). We also measured aromatase activity and found that the presence of COX-2 siRNA resulted in a 60% decrease in enzyme activity (Fig. 2B); similarly aromatase mRNA levels were decreased by 70% (Fig. 2C). Western blot analyses, using a specific COX-2 inhibitor NS398 (NS), showed that addition of increasing doses of NS (5, 25, and 50 μM) decreased both phosphorylated CREB and aromatase levels without affecting CREB expression (Fig. 3A). A drop in aromatase expression was also reflected by a change in enzymatic activity, which was dramatically reduced at all investigated NS doses (Fig. 3B). CREB is a transcription factor necessary for aromatase transcription via the PII promoter. As a consequence, the observed changes in pCREB levels induced changes in aromatase mRNA expression. As shown by real-time RT-PCR, the addition of different doses of NS resulted in a decrease in aromatase mRNA (Fig. 3C) without affecting CREB mRNA levels (data not shown), indicating a specific effect of COX-2 inhibition on CREB phosphorylation and pCREB binding to the aromatase PII promoter. We confirmed this hypothesis using a ChIP assay, which showed a specific decrease in pCREB binding (Fig. 3D, left panel) without any change in the amount of total CREB protein present on the aromatase PII promoter (Fig. 3D, right panel).

PGE2-activated Pathway Regulates Aromatase Expression—We first measured the ability of R2C cells to produce PGE2. We found that tumor cells were able to produce 650 ± 47 pg of PGE2/mg of protein (mean ± S.E. from three independent experiments; data not shown), an amount that is 5-fold higher than levels produced by TM3 cells. PGE2 levels in R2C cells were decreased by 60% in the presence of 50 μM NS398 (data not shown). Considering the high endogenous production of PGE2, only the addition of relatively high exogenous amounts of PGE2 to R2C cells were able to increase aromatase expression and CREB phosphorylation (Fig. 4A). When COX-2 was silenced, induction of aromatase expression was observed with 100-fold lower concentration of PGE2 (Fig. 4B), confirming that this prostaglandin is involved in aromatase regulation in R2C cells. Because PGE2 binds four different receptor isoforms, named EP (prostaglandin E receptor), we tested the effect of selective inhibitors for the different isoforms on aromatase and pCREB levels (Fig. 4C). Only the selective antagonist for the EP4 isoform, AH23848, resulted in a decrease in pCREB and in aromatase levels as well as a dose-dependent inhibition in aromatase activity (Fig. 4D). We then evaluated expression of the EP4 receptor isoform and found higher expression in R2C cells as well as in testes from Fischer rats with a developed tumor (FRTT) compared with the normal controls (Fig. 4E). To con-
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PKA Inhibitor H89 Decreases Aromatase Expression and Activity as a Consequence of Reduced pCREB Activation—Because EP4 receptor activates the PKA transduction pathway, we wanted to test the effect of a specific PKA inhibitor, H89, on aromatase expression. H89 decreased both phosphorylated CREB and aromatase levels in a dose-dependent manner (Fig. 5A), concomitantly with enzymatic activity (Fig. 5B) and aromatase mRNA (Fig. 5C, left panel), due to a lower recruitment of pCREB to the P1I promoter (Fig. 5C, right panel), and to increased DAX-1 expression that progressively blocks SF-1 action (data not shown).

PKA involvement can be further demonstrated by the direct measurement of kinase A activity after treatment with the three inhibitors (Fig. 5D). As expected H89 caused an 80% inhibition of PKA activity after 4-h treatment. Similarly, NS398 and AH23848 resulted in 40 and 63% inhibition, respectively.

Short Term Inhibition of COX-2/PGE2/PKA Pathway Affects Aromatase Activity at the Post-transcriptional Level—The observation that NS398 almost completely decreases aromatase activity (Fig. 3B), with fewer effects on aromatase mRNA levels (Fig. 3C), suggests that part of the effects of NS398 on aromatase may be a post-translational modification. To investigate whether COX-2 could regulate aromatase at post-translational levels, we treated R2C cells with NS398, AH23848, and H89 for 1 h and measured aromatase activity (Fig. 6). NS398 resulted in a 53% inhibition, whereas AH23848 and H89 inhibited R2C cells by 25 and 33%, respectively.

Inhibition of the COX-2/PGE2/PKA Pathway Decreases E2 Production and Cyclin E Expression in R2C Cells—The involvement of COX-2 through PGE2 production and PKA activation in controlling aromatase activity in tumor Leydig cell is further supported by the ability of NS398, AH23848, and H89 to inhibit basal E2 production (Fig. 7A). As previously demonstrated, E2 treatment activates target genes involved in cell cycle regulation such as cyclin E. For this reason we investigated the effects of PGE2 pathway inhibitors on cyclin E expression. NS398 (Fig. 7B), AH23848 (Fig. 7C), and H89 (Fig. 7D) resulted in a decrease in cyclin E expression as a consequence of reduced E2 production.

Inhibition of the COX-2/PGE2/PKA Pathway Decreases R2C Cell Proliferation—As expected, the decreased E2 production resulted in a significant reduction in cell proliferation. Increasing doses of NS398 (Fig. 8A), AH23848 (Fig. 8B), or H89 (Fig. 8C) were associated with a dose-dependent reduction of cell growth. An additional demonstration of the involvement of the COX-2-dependent pathway in controlling E2 production in R2C cells is provided by the evaluation of cell proliferation after knocking down COX-2 in these cells with a specific siRNA (Fig. 8D). Thymidine incorporation was reduced by a similar percentage with all amounts of transfected COX-2 siRNA. These findings led us to propose that in tumor Leydig cells the PGE2 effects of NS398, AH23848, and H89 to inhibit basal E2 production (Fig. 7A).

Discussion—In the present study, we have demonstrated that COX-2 overexpression in Leydig tumor cells activates the PGE2/PKA transduction pathway, which stimulates consecutive events such as CREB phosphorylation, aromatase expression, and E2-dependent proliferation. In this regard, we have first demon-
FIGURE 3. Effects of NS98 on CREB phosphorylation and aromatase expression in R2C cells. R2C cells were untreated (basal) or treated for 24 h with the indicated doses of NS398. A, Western blot analysis of pCREB, CREB, and aromatase were performed on 50 μg of total proteins extracted from R2C cells. Blots are representative of three independent experiments with similar results. GAPDH was used as a loading control. B, aromatase activity was assessed using the modified tritiated water method. Results obtained were calculated as picomoles of [3H]H2O released per hour normalized to the protein content per well (picomoles/h/mg of protein) and expressed as percent of basal. Columns, mean of three independent experiments each performed with triplicate samples expressed as percent of basal; bars, ± S.E. C, total RNA was extracted and real-time RT-PCR was used to analyze mRNA levels of CYP19. Data represent the mean ± S.E. of values from three separate RNA samples expressed as percent of basal. Each sample was normalized to its 18 S ribosomal RNA content. D, effects of COX-2 inhibitor on pCREB (left) or CREB (right) binding to aromatase PII promoter in R2C cells. ChIP assays were performed on R2C cells untreated (basal) or treated as indicated. Immunoprecipitated (IP pCREB) and total (10% input) DNA were subject to real-time PCR using specific primers. Ct values from immunoprecipitated sample were normalized to the input Ct values. Columns, mean of three independent experiments expressed as percent of basal; bars, ± S.E. *, p < 0.01 compared with basal.
strated, by Western analysis, that both R2C cells and tumor testes from Fischer rats express higher COX-2 levels than normal cell lines and tissues. Thereafter, we have shown, by immunohistochemistry, that COX-2 is highly expressed in human Leydigioma, but is not present in normal testes. Interestingly, COX-2 silencing in R2C cells decreased aromatase expression and CREB phosphorylation, which is involved in the regulation of the aromatase PII promoter in Leydig cells. Moreover, the inhibitory effects observed by knocking down COX-2 were also obtained using a selective COX-2 antagonist, which reduced aromatase activity. These findings together with our previous report (5), showing an increased expression of aromatase in human Leydigioma, provide support for a correlation between COX-2 expression and increased aromatase activity in Leydig cell tumors.

Recent studies have suggested that COX-2 exerts an important role in breast cancer progression. In this respect, it should be noted that COX-2 protein was found to be overexpressed in ~40% of breast tumors (35). Additionally, high COX-2 levels were sufficient to induce mammary tumorigenesis in transgenic mice (36), while the inhibition of COX-2 activity exerted protective effects following the tumorigenesis process in animal models of breast cancer (37). A recent report demonstrated a strong positive correlation between COX-2 and aromatase mRNA expression in human breast cancer tissues (38). In postmenopausal subjects with breast cancer, aromatase activity within the tumor and/or surrounding adipose tissue allows local estrogen production through conversion of androgen precursors (39). Consequently, E2 concentrations within tumor breast tissue can be more than 10 times higher with respect to those measured at circulating levels (40). It is worth noting that elevated aromatase expression within the malignant tissue and/or surrounding

**FIGURE 4.** PGE2 activated pathway regulates aromatase expression. A, R2C cells maintained in serum-free medium for 24 h were then left untreated (basal) or treated for 24 h with the indicated doses of PGE2. Western blot analysis of aromatase (Arom), pCREB, and CREB were performed on 50 μg of total proteins extracted from R2C cells. Blots are representative of three independent experiments with similar results. GAPDH was used as a loading control. B, R2C cells were grown for 2 days in the presence of siRNA for COX-2 (100 nM) or scrambled siRNA (100 nM), 24 h after transfection cells were treated with 0.1 μM PGE2 and then lysed for protein extraction. Western blot analysis of aromatase (Arom), pCREB, and CREB were performed on 50 μg of total proteins extracted from R2C cells. Blots are representative of three independent experiments with similar results. GAPDH was used as a loading control. C, R2C cells were untreated (basal) or treated for 24 h with 10 μM of EP inhibitors: SC19220, AH6809, AH23848. Western blot analysis of aromatase (Arom), pCREB, and CREB were performed on 50 μg of total proteins extracted from R2C cells. Blots are representative of three independent experiments with similar results. GAPDH was used as a loading control. D, R2C cells were untreated (basal) or treated for 48 h with increasing doses of AH23848 (1, 3, and 10 μM). Aromatase activity was assessed using the modified tritiated water method. Results obtained were calculated as picomoles of [3H]H2O released per hour normalized to the well protein content (picomoles/h/mg of protein) and expressed as percent of basal. Columns, mean of three independent experiments each performed with triplicate samples expressed as percent of basal; bars, ± S.E. *, p < 0.01 compared with basal. E, Western blot analysis of EP4 was performed using 50 μg of total proteins extracted from TM3 and R2C cells or from total tissue of normal (FRNT) and tumor (FRTT) Fischer rat testes. GAPDH was used as a loading control. Results are representative of three independent experiments. F, R2C cells were grown for 2 days in the presence of siRNA for EP4 (100 nM) or scrambled siRNA (100 nM). Western blot analysis for aromatase (Arom), pCREB, CREB, and EP4 was performed on 50 μg of total proteins extracted from R2C cells following siRNA gene silencing. GAPDH was used as a loading control. Results are representative of three independent experiments.
FIGURE 5. PKA is involved in COX-2/PGE2/pCREB-dependent aromatase expression in R2C cells. R2C cells were untreated (basal) or treated for 24 h with the indicated doses of H89. A, Western blot analysis of aromatase (Arom), pCREB, and CREB were performed on 50 μg of total proteins extracted from R2C cells. Blots are representative of three independent experiments with similar results. GAPDH was used as a loading control. B, aromatase activity was assessed by using the modified tritiated water method. Results obtained were calculated as picomoles of [3H]H2O released per hour normalized to the protein content per well (picomoles/h/mg of protein) and expressed as percent of basal. Columns, mean of three independent experiments each performed with triplicate samples expressed as percent of basal; bars, ±S.E. In C, left panel, total RNA was extracted, and real-time RT-PCR was used to analyze mRNA levels of CYP19. Data represent the mean ± S.E. of values from three separate RNA samples expressed as percent of basal. Each sample was normalized to its 18 S ribosomal RNA content. Right panel, effects of COX-2 inhibitor on pCREB binding to aromatase PII promoter in R2C cells. ChIP assays were performed on R2C cells untreated (bs) or treated as indicated. Immunoprecipitated (IP pCREB) and total (10% input) DNA were subject to real-time PCR using specific primers. C, values from immunoprecipitated samples were normalized to the input C, values. Data represent the mean ± S.E. of three independent experiments expressed as percent of basal. D, R2C cell lysates were analyzed for PKA activity by non-radioactive in vitro PKA assay as described under “Experimental Procedures.” R2C cells were treated for 4 h with NS398 (50 μM), AH23848 (10 μM), and H89 (20 μM). Absorbance of phosphorylated peptide was quantified spectrophotometrically. Columns, mean of three independent experiments samples expressed as percent of basal; bars, ±S.E. *, p < 0.01 compared with basal.
stroma may occur in response to tumor-derived stimulatory factors (41) in combination with a switch in CYP19 gene promoter usage from the specific adipose promoter I.4 to the promoter II active in gonadal tissues (42). In this context, it has been shown that COX-2-derived PGE2 strongly stimulates aromatase promoter II activity (12). These findings suggest that PGE2 may also regulate aromatase activity in other cell types such as Leydig tumor cells where PII drives aromatase expression.

Leydig, Sertoli, and spermatogenic rat testicular cells synthesize PGE2, which has been implicated in the control of steroidogenesis, spermatogenesis, and local immune response (43). Moreover, it has been shown that, in various species, such as the newt, PGE2 increases 17β-estradiol, cAMP, and aromatase activity, while it decreases testosterone, probably due to an increased conversion into estrogens (44). Consistent with findings related to COX-2 and cancer, PGE2 exhibited biological properties facilitating tumor development such as angiogenesis, invasiveness, and inhibition of immune surveillance (45). Importantly R2C cells are able to produce high levels of PGE2 when compared with TM3 normal Leydig cells. In this study we demonstrated the specific role of

![FIGURE 6. Regulation of aromatase activity by COX-2/PGE2/PKA pathway occurring at the post-transcriptional level. R2C cells were untreated (basal) or treated for 1 h with NS398 (50 μM), AH23848 (10 μM), or H89 (20 μM). Aromatase activity was assessed using the modified tritiated water method. Results obtained were calculated as picomoles of [3H]H2O released per hour normalized to the protein content per well (picomoles/h/mg of protein) and expressed as percent of basal. Columns, mean of three independent experiments each performed with duplicate samples expressed as percent of basal; bars, ± S.E. *, p < 0.01 compared with basal.](image)

![FIGURE 7. Inhibition of COX-2/PGE2/PKA pathway decreases E2 production and cyclin E expression in R2C cells. A, R2C cells were maintained for 24 h in HAM-F10 medium in the absence (basal) or presence of NS398 (50 μM), AH23848 (10 μM), or H89 (10 μM). E2 content in culture medium was determined by radioimmunoassay and normalized to the cell culture protein content per well (picomoles/mg of protein). Data represent the mean ± S.E. of values from three separate cell culture wells expressed as percent of basal. *, p < 0.01 compared with basal. B–D, cells were treated in serum-free medium in the absence (basal) or presence of increasing doses of NS398 (5, 25, and 50 μM) (B), AH23848 (1, 3, and 10 μM) (C), or H89 (5, 10, and 20 μM) (D) for 24 h after 24-h starvation. Western blot analysis of cyclin E was performed on 50 μg of total proteins. Blots are representative of three independent experiments with similar results. GAPDH was used as a loading control.](image)
PGE2 in mediating COX-2 effects on aromatase silencing COX-2 (to block synthesis of any PG) and then treated cells with PGE2, which resulted in a clear induction of aromatase expression.

PGs, produced through COX activity, act by binding to transmembrane G-protein-coupled receptors termed prostanoid receptors, which are expressed as different splice variants (46). Specifically, the expression of the PGE2 receptors EP1, EP2, EP3, and EP4 occurs through modulation of adenylyl cyclase activity, inositol phospholipid hydrolysis, and calcium mobilization (47). EP1 activates PKC while EP2 and EP4 are coupled to PKA. EP3 can either couple negatively to adenylyl cyclase through binding a Gi protein or associate with inositol phospholipid hydrolysis and calcium mobilization. Importantly, in adipose stromal cells the stimulatory effects on aromatase exerted by PGE2 were inhibited using selective antagonists of EP1 and EP2 (48), suggesting that both PKA and protein kinase C transduction pathways are required for aromatase regulation.

In the current study, using selective antagonists for the EP subtypes we show that only the EP4 inhibitor AH23848 decreased aromatase expression and activity. We confirmed the involvement of EP4 in regulating aromatase expression using a specific EP4 siRNA. Considering that EP4 signals through PKA, we also blocked this transduction cascade demonstrating a consequent reduction in CREB phosphorylation. In particular, treating R2C cells with the COX-2 inhibitor NS398, the EP4 inhibitor AH23848, and the specific PKA inhibitor H89, we observed that decreased kinase A activity was paralleled by a reduction in CREB phosphorylation and aromatase mRNA and protein levels. Aromatase transcription in Leydig cells is mainly controlled by transcription factors pCREB and SF-1. In a previous study (4) we showed that SF-1 transcriptional regulation is controlled by a transduction pathway that does not involve PKA directly. This indicates that, even in the presence of very low levels of pCREB, as is the case after treating cells with H89, SF-1 can still drive aromatase transcription maintaining the gene expression. This is confirmed by results shown in Fig. 5 (A and C) demonstrating the presence of aromatase protein and mRNA expression in R2C even after 24 h of treatment with H89.

All these findings imply a regulatory role for the COX-2/PGE2/PKA signaling pathway in aromatase expression. We
also showed that the same pathway could be involved in short term aromatase activation.

It has been postulated that aromatase regulation can occur not only by altering gene expression but also through post-translational modifications (49–51). The observation that NS398, AH23848, and H89 are also able to decrease PKA activity suggests that aromatase activation could occur through PKA-dependent phosphorylation of specific amino acid residues. NS398 showed a more potent effect on aromatase inhibition compared with the other compounds utilized. This could be explained by the observation that NS398, independently of COX-2 inhibition, can also affect the enzymatic activity of kinases involved in post-translational aromatase regulation (52). In a previous study (4), using the same cell system, we showed that PD98089, an inhibitor of MAP kinase, can decrease aromatase activity, without changing mRNA or protein levels. NS398 can also potentially affect MAPK in R2C cells (51, 53), indicating the existence of multiple pathways involved in aromatase activation. This preliminary observation needs to be supported by further studies.

Moreover, it should be taken into account that LH binding to its cognate receptor (LHR) activates a cAMP-PKA transduction cascade that primarily controls Leydig cell function (54). Several observations indicate that constitutively active mutants of LHR could be involved in Leydig cell transformation (55). Indeed, the constitutive activation of the cAMP-PKA pathway in R2C Leydig tumor cells allows for a steroidogenic phenotype (56), which could contribute to aromatase overexpression in Leydig cell tumors. For these reasons we believe that other molecular mechanisms could be involved in constitutive activation of CREB-dependent aromatase activation. However, in the current study all tested inhibitors blocking COX-2, EP4, or PKA reduced the ability of R2C cells to produce E2 resulting in the decreased expression of the estrogen-regulated cyclin E and a reduction in cell proliferation. The same effect was also observed knocking down COX-2 expression, demonstrating that COX-2 overexpression is important for Leydig tumor cell proliferation.

Targeted inhibition of COX-2 and/or PGE2 has been indicated as a potential strategy to stop cancer development. Selective COX-2 inhibitors are used in treatment of colorectal polyps (57) and in women with high grade cervical dysplasia (58). Furthermore, experimental studies have shown that the inhibition of COX-2 or PGE2 leads to in vivo tumor reduction in murine lung cancer models (59). Our data in human Leydiggioma, demonstrating that COX-2 overexpression is associated with elevated aromatase expression, support the hypothesis that the PGE2/PKA/CREB pathway is also activated in human Leydig cell tumors and suggest the use of COX-2 inhibitors for the treatment of Leydig cell tumors.

In regards to the molecular mechanisms determining COX-2 overexpression in Leydig tumor cells, a recent study has suggested that LH stimulates COX-2 but not COX-1 expression in rat Leydig cells through the cAMP-PKA signaling pathway (60). These findings could explain the COX-2 overexpression observed in Leydig tumor cells. However, it has been demonstrated that COX-2 expression can also be regulated by testicular growth factors such as transforming growth factor-α and/or cytokines from macrophages or Sertoli cells (60). Moreover, E2 is also able to induce COX-2 expression in vascular endothelial cells (61). The latter observation, if confirmed in tumor Leydig cells, will open a new interesting perspective regarding a possible autocrine loop triggered by E2 via COX-2/PGE2 determining an increase of aromatase activity and E2 production. Our studies are currently investigating this hypothesis.

In conclusion, the present and our previous study (4) show that Leydig cell tumors are characterized by overexpression of COX-2 and constitutively active insulin-like growth factor I signaling resulting in CREB phosphorylation and SF-1 expression, respectively. Both transcription factors, by binding to promoter II, increase aromatase transcription and, consequently, activity. The excessive local E2 production is able to stimulate the expression of genes involved in cell cycle regulation thereby sustaining Leydig tumor cell proliferation. The observations that both COX-2 and insulin-like growth factor I signaling inhibitors decrease R2C cell proliferation suggest their potential application as new adjuvant therapies in Leydig tumor cell treatment.

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