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Cyclooxygenase-2 and Prostaglandin F2α in Syrian Hamster Leydig Cells: Inhibitory Role on Luteinizing Hormone/Human Chorionic Gonadotropin-Stimulated Testosterone Production

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We have previously found that cyclooxygenase-2 (COX-2), a key enzyme in the biosynthesis of prostaglandins (PGs), is present in the testicular interstitial cells of infertile men, whereas it is absent in human testes with no evident morphological changes or abnormalities. To find an animal model for further investigating COX-2 and its role in testicular steroidogenesis, we screened testes from adult species ranging from mice to monkeys. By using immunohistochemical assays, we found COX-2 expression only in Leydig cells of the reproductively active (peripubertal, pubertal, and adult) seasonal breeder Syrian hamster. COX-2 expression in hamster Leydig cells was confirmed by RT-PCR. In contrast, COX-1 expression was not detected in hamster testes. Because COX-2 expression implies PG synthesis, we investigated the effect of various PGs on testosterone production and found that PGF2α stood out because it significantly reduced human chorionic gonadotropin-stimulated testosterone release from isolated hamster Leydig cells in a dose-dependent manner. This mechanism involves a decreased expression of testicular steroidogenic acute regulatory protein and 17β-hydroxysteroid dehydrogenase. Testicular concentration and content of PGF2α in reproductively active hamsters as well as production of PGF2α from isolated hamster Leydig cells were also determined. Moreover, PGF2α receptors were localized in Leydig cells of hamsters and testicular biopsies from patients with Sertoli cell only and germ arrest syndromes. Thus, in this study, we described a COX-2-initiated pathway that via PGF2α production, PGF2α receptors, steroidogenic acute regulatory protein, and 17β-hydroxysteroid dehydrogenase represents a physiological local inhibitory system of human chorionic gonadotropin-stimulated testosterone production in the Syrian hamster testes. (*Endocrinology* 147: 4476–4485, 2006)

PROSTAGLANDINS (PGs) ARE a group of bioactive substances derived from arachidonic acid by action of the cyclooxygenase (COX) isoenzymes type 1 (COX-1) and type 2 (COX-2) as well as by that of other PG synthetic enzymes (1–3) (Fig. 1). Nevertheless, COX-1 and COX-2 are the key enzymes in the biosynthetic pathway of PGs. There are processes in which each isoenzyme is uniquely involved and others in which both isoenzymes function coordinately, and there are also physiological events in which one COX isoenzyme normally functions but for which the other can compensate when the first is lacking (4). When both COX-1 and COX-2 are expressed in the same cells, it appears that their activities are controlled differentially by regulating the amount of arachidonic acid and lipid peroxide available to the enzymes (1). Moreover, COX-2 but not COX-1 can use esterified fatty acids as alternative substrates (5).

The constitutively expressed COX-1 is found in most cell types. In contrast, the inducible isoenzyme COX-2 is thought to be expressed during early stages of cell differentiation or replication in response to different stimuli such as cytokines and mitogenic factors (1, 6). COX-2 expression has been described in both physiological and pathophysiological states, including inflammation, angiogenesis, bone absorption, gastric ulcer, and colon cancer as well as kidney, brain, and female reproductive organ diseases (6).

The development of mice deficient in COX-1 and COX-2 has pointed out the role exerted by COX isoenzymes in reproduction. Early studies have demonstrated that COX-1-null female mice produce litters of normal size but have difficulty with parturition, a physiological process related to COX-1 and PGF2α (7, 8). COX-2–null female mice are infertile (9). In this context, ovulation seems to depend solely on COX-2 and PGE2 (9–11).

In marked contrast, male fertility is not affected in COX-1 or COX-2 mutant mice from knockout experiments (12, 13), suggesting that PGs may not be important for the functioning of the normal testis, at least in mice. This early general view is being challenged by recent observations. We have previously reported that whereas COX-2 is not detected in human testes with no evident morphological changes or abnormalities, it is expressed in testicular biopsies of men with im-

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Abbreviations: COX, Cyclooxygenase; GA, germ arrest; 3β-HSD, 3β-hydroxysteroid dehydrogenase; LD, long-day; PG, prostaglandin; P450scc, P450 side chain cleavage; 5α-R1, 5α-reductase isoform 1; SCO, Sertoli cell only; SD, short-day; 16-SD, 16 wk in SD; StAR, steroidogenic acute regulatory.

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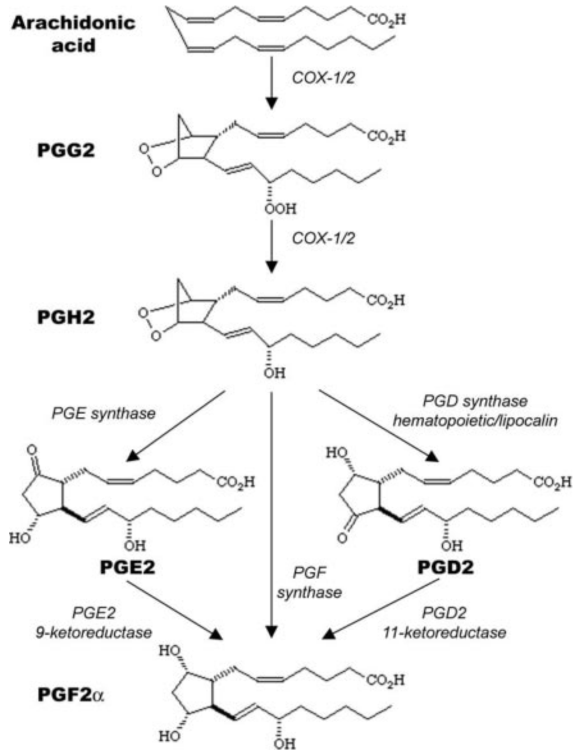

FIG. 1. Schematic representation of the COX pathway illustrating the synthesis of the major PGs. This process is initiated when COX-1 and COX-2 catalyze both a reaction in which arachidonic acid is converted to PGG2 and a subsequent peroxidase reaction in which PGG2 is reduced to PGH2, which serves as the common precursor for all of the terminal PGs.

paired spermatogenesis and male infertility (14). Moreover, in another pathological condition such as testicular cancer, both COX-1 and COX-2 are induced (15). Thus, testicular COX and PGs may be of relevance in most male fertility disorders and could play a key role in the regulation of testicular function. Bearing this in mind, we examined the expression of COX-2 in testes from several species including adult Rhesus monkeys, pigs, Wistar and Sprague Dawley rats, BALBc mice, and Syrian hamsters. Unexpectedly, we found COX-2 expression only in Leydig cells of reproductively active Syrian hamsters (*Mesocricetus auratus*). Thus, we used Syrian hamsters, a thoroughly studied seasonal breeder, for investigating the action of COX-2 and PGs on testosterone production and the expression of key testicular steroidogenic enzymes.

Materials and Methods

Animals

Male Syrian hamsters (*M. auratus*) were raised in our animal care unit (Charles River descendants; Animal Care Laboratory, Instituto de Biología y Medicina Experimental, Buenos Aires) and maintained from birth to adulthood in rooms at 23 ± 2 C under a long-day (LD) photoperiod (14 h light, 10 h dark; lights on 0700–2100 h). Hamsters aged 12, 18, 36, 45, 60, and 90 d exposed to LD photoperiod were used in the present study. In addition, adult hamsters aged 90 d were transferred to a short-day (SD) photoperiod (6 h light, 18 h dark; lights on 0900–1500 h) for 16 wk. It is important to mention that hamsters from our colony reach the maximum testicular regression after 16 wk of SD photoperiod [see additional information in Frungieri *et al.* (16)]. Animals had free access to water and Purina formula chow. Hamsters were killed by asphyxia with CO_2 according to protocols for animal laboratory use, approved by the Institutional Animal Care and Use Committee [Instituto de Biología y Medicina Experimental-Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET)], following the National Institutes of Health guidelines. At the time of killing, left testes were dissected, fixed for at least 48 h in Bouin's fluid followed by 70% ethanol, and then embedded in paraffin wax for histological and immunohistochemical studies. Right testes were rapidly removed and either used for quantification of testicular PGF2α concentration and content by immunoassay or frozen at −80 C until RT-PCR assays were performed.

In other groups of 12 LD adult animals, testes were dissected and used for Leydig cell purification. *In vitro* incubations of Leydig cells were performed followed by determination of mRNA expression (by RT-PCR), measurement of protein expression (by immunoblotting), transmission electron microscopy studies, or quantification of testosterone and PGF2α levels in the incubation media (by RIA and immunoassay, respectively).

Human biopsies

Diagnostic records of testicular biopsies from adult men with fertility disorders (age range, 28–37 yr old) were assigned to the following groups: specimens from men with cases of idiopathic infertility revealing normal spermatogenesis with no evident morphological changes or abnormalities (n = 4), specimens from patients with Sertoli cell-only (SCO) syndrome (n = 5), and specimens from patients with severe hypospermatogenesis and germ-arrest (GA) syndrome (n = 5). The records were used to examine the existence of PGF2α receptors by immunohistochemistry. The etiology of testicular failure was heterogenic; most patients presented cryptorchidism in early childhood or idiopathic infertility. The evaluation of human specimens was approved by the local ethics committee of the Instituto de Biología y Medicina Experimental, CONICET, Argentina, and by the appropriate local ethics committee of the University in Munich, Germany.

Hamster Leydig cell purification and in vitro incubations

For all the experiments, Leydig cells were isolated from a pool of 24 testes obtained from 12 adult hamsters (90 d old) maintained in LD photoperiod. Isolation was carried out under sterile conditions using a discontinuous Percoll density gradient as previously described by Frungieri *et al.* (17). Cells that migrated to the 1.06–1.12 g/ml density fraction were collected and suspended in medium 199. An aliquot was incubated for 5 min with 0.4% Trypan blue and used for cell counting and viability assay in a light microscope. Viability of Leydig cell preparations was 97.5–98.5%. To evaluate enrichment in Leydig cells, the activity of 3β-hydroxysteroid dehydrogenase (3β-HSD) was measured as previously described by Levy *et al.* (18). Cell preparations were 85–90% enriched with hamster Leydig cells. Less than 1.7 and 1.2% of the contaminating cells were positive macrophages for ED-1 and ED-2 antigens, respectively. The remaining cell types had the morphology of either peritubular cells or endothelial cells. Petri dishes with 1.5 ml medium 199 containing 2.5×10^5 cells (for RT-PCR, immunoblotting, and *in vitro* testosterone production) or 7.5×10^5 cells (for PGF2α production and transmission electron microscopy) were incubated at 37 C under a humid atmosphere with 5% CO_2 in the presence of 0.1 mM 3-isobutyl-1-methylxanthine, a phosphodiesterase inhibitor (Sigma Chemical Co., St. Louis, MO), and in the presence of the following chemicals: 100 mIU/ml human chorionic gonadotropin (hCG) (Ayerst, Princeton, NJ; specific activity, 59 UI/mg), 100 pM to 1 μM PGD2, PGE2, or PGF2α (Sigma), and/or 1 μM meloxicam (Calbiochem, La Jolla, CA). Meloxicam is a well-known nonsteroidal antiinflammatory drug classified as a preferential COX-2 inhibitor taking into account data obtained by using different experimental models to investigate drug selectivity [see additional information in Pairet and van Ryn (19)]. In this study, meloxicam stock solution was prepared in dimethylsulfoxide (Sigma) and for those experiments in which meloxicam effect was tested, control incubations received the same vehicle (dimethylsulfoxide) as treated cells. Other chemicals listed above were dissolved in medium 199, and then medium 199 was used as vehicle for control incubations. After incubation, cells in media were transferred to tubes and centrifuged at $1200 \times g$ for 10 min. Cells were used for RNA extraction followed by

RT-PCR and protein extraction, followed by immunoblotting, and for transmission electron microscopy. Media were frozen at −20 C until testosterone and PGF2α concentrations were determined by RIA and immunoassay, respectively.

Histological and immunohistochemical assays

Testes from prepubertal (12 and 18 d old), peripubertal (36 d old), pubertal (45 and 60 d old), and adult (90 d old) LD hamsters, 16-SD adult hamsters, adult (18 yr old) Rhesus monkeys, adult pigs, young adult (60 d old) BALBc mice, young adult (60 d old) Wistar rats, and young adult (60 d old) Sprague Dawley rats as well as human testicular biopsies from patients with normal testicular morphology with no evident abnormalities, patients with SCO syndrome and patients with GA syndrome were examined by histological and immunohistochemical assays. Eight to 10 animal testes, and four to five human testicular biopsies were evaluated. After fixation, tissues were dehydrated and embedded in paraffin wax, and 5-μm sections obtained from three different levels were used for histological and immunohistochemical studies. Antisera and antibodies (polyclonal goat anti-COX-1 serum, 1:200, from Oxford Biomedical Research, Oxford, MI; polyclonal rabbit anti-COX-2 serum, 1:200, from Oxford Biomedical Research; and polyclonal rabbit anti-PGF2α receptor serum, 1:800, from Cayman Chemical, Ann Arbor, MI) and an avidin-biotin-peroxidase system (Vector Laboratories, Burlingame, CA) were used for COX-1, COX-2, and PGF2α receptor detections. Hamster stomach tissue was used as positive control for COX-1 detection. For control purposes, the first antiserum was omitted, or incubation with normal nonimmune sera was carried out. For the case of PGF2α receptor, an additional control was performed by preabsorption of the antiserum with a 2-fold-concentrated specific blocking peptide (Cayman Chemical) for 2 h at room temperature.

RT-PCR analysis

RNA was extracted from total testicular tissue (LD adult hamster, LD prepubertal hamster, and 16-SD adult hamster) or isolated Leydig cells from adult hamsters kept under LD photoperiod conditions using the Purescript kit (Biozym, Hessisch Oldenburg, Germany). Hamster stomach tissues were used as positive controls for COX-1 expression.

For human testicular biopsies, tissue sections were scratched from the slides, and RNA was extracted using the Purescript kit. Then, RT reaction using oligo-dT_{15} primers followed by PCR amplification was performed (14). Information about oligonucleotide primers used and cDNAs isolated is detailed in Table 1. When information about exon structure was available at GenBank, oligonucleotide primers were designed as homologous to regions of different exons: COX-1, COX-2, steroidogenic acute regulatory (StAR) protein, P450 side chain cleavage (P450scc), 3β-HSD, 1β-HSD, 5α-reductase isoform 1 (5α-R1), and α-tubulin.

PCR products were separated on 2% agarose gels and visualized with ethidium bromide. The identity of PCR products was confirmed by sequencing, using a fluorescence-based dideoxy-sequencing reaction and an automated sequence analysis on an ABI 373A DNA sequencer.

Testosterone assay

Testosterone levels were determined in the incubation media by RIA according to the method described by Frungieri *et al.* (20) without extraction using antibodies obtained from Immunotech Diagnostic (Montreal, Canada). Testosterone was measured using an antibody to testosterone-7α-butyrate-BSA, which is known to have 35% cross-reactivity with dihydrotestosterone. The minimal detectable assay concentration was 0.215 pmol/ml. Intra- and interassay coefficients of variation were less than 12% and less than 15%, respectively.

In vitro testosterone production from Leydig cells is expressed in terms of picomoles per 10^6 Leydig cells.

Transmission electron microscopy

For ultrastructural studies, LD hamster Leydig cells were incubated for 3 h either with or without 100 mIU/ml hCG and 1 μM or 100 pM PGF2α and were then fixed with 5% glutaraldehyde in 0.1 M cacodylate acid (pH 7.4). After embedding, thin sections were cut and examined with an EM10 electron microscope (Carl Zeiss, Oberkochen, Germany) as previously described (21, 22).

PGF2α immunoassay

For assessment of testicular PGF2α concentration and content, testes were thawed, cut, and homogenized in 0.2 M perchloric acid. After centrifugation at 20,000 × *g* for 30 min, supernatants were concentrated and extracted through C18 Sep-columns (Peninsula Laboratories, Belmont, CA) and finally eluted with ethyl acetate.

Approximately 7.5×10^5 Leydig cells were used to determine PGF2α levels in the incubation media. After 3 h incubation, media were acidified using 2 N HCl (pH 3.5), injected into a 200-mg C18 column and eluted with ethyl acetate.

Eluted fractions from testis as well as Leydig cell incubation media were evaporated to dryness under a nitrogen stream and reconstituted in assay buffer.

PGF2α was assayed by using a commercially available kit (R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany) according to the method described by Frungieri *et al.* (14) The minimal detectable immunoassay concentration was 3.05 pmol/ml. Intra- and interassay co-

TABLE 1. Oligonucleotide primers used for PCR amplification of cDNAs obtained after reverse transcription from total testicular tissue or isolated Leydig cells from mice, rats, and hamsters

Target	Sense primers (5′–3′)	Antisense primers (5′–3′)	Expected cDNA length (bp)	Annealing temperature (C)
COX-2				
First set	GCAAATCCTTGCTGTTCC	GGAGGAAGGGCCCTGGTG	368	60
Second nested set	TGTATGTATGAGTGTGGGA	GGCTTCCCAGCTTTTGTA	292	54
COX-1				
First set	CTTTCCAGGAGCTCACAG	CAGTTTCTTCAGTGAGGC	271	54
Second nested set	AGAAAGAGATGGCCGCTG	CACATCACCACCGAATGT	211	54
PGF2α receptor				
First set	GATGGCCATTGAGCGGTG	GGAGACACACATTATCGC	391	54
Second nested set	CAAACATGTGAAAATGATG	TATCTTCCCAGTCTTTGATGT	149	54
StAR	GAGTGGAACCCCAATGTC	GCACCATGCAAGTGGGAC	243	56
P450scc	GAGTCCCAGCGGTTCAT	CCTCCTGCCAGCATCTC	291	54
3β-HSD	TCAATGTGAAAGGTACCC	ATCATAGCTTTGGTGAGG	499	51
17β-HSD	GCCGGACACTGGAGAAGC	TGACTACGGAGGTGACGT	252	55
5α-R1	CAGGAGCTGCCCTCGATG	CCTGGTTTTCTCAGATTCC	361	56
α-Tubulin				
First set	GGCAAGGAGATCATTGAC	TCTCAGGGAAGCAGTGAT	398	54
Second nested set	GTCTCCAGGGCTTCTTG	GTCTACCATGAAGGCACA	223	54

GenBank accession numbers are as follows: COX-2, AY426532; COX-1, AF414605; PGF2α receptor, AY426533 and AF004021; StAR, U66490; P450scc, AF323965; 3β-HSD, L38710; 17β-HSD, AY426534; 5α-R1, NM_175283, J05035, and BT006834; α-tubulin, M12252.

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efficients of variation were less than 10% and less than 8%, respectively. PGF2α levels were expressed as femtomoles per testis, femtomoles per gram tissue, and femtomoles per 10^6 Leydig cells.

Immunoblotting

Cells and tissues were homogenized in 62.5 mM Tris-HCl buffer (pH 6.8) containing 10% sucrose and 2% SDS by sonication. Samples were heated (at 95 C for 5 min) under reducing conditions (10% mercaptoethanol), loaded on tricine-SDS-polyacrylamide gels (15%), electrophoretically separated, and blotted onto nitrocellulose (14). Blots were incubated with rabbit polyclonal anti-StAR (1:2000) protein or mouse monoclonal antiactin (1:5000; Calbiochem) and subsequently with peroxidase-labeled secondary antibodies (donkey antirabbit IgG, 1:1000, from Amersham Pharmacia Biotech AB, Uppsala, Sweden; and goat antimouse IgM, 1:2000, from Calbiochem). Signals were detected with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech). StAR and actin have been detected in two consecutive exposures of the same membrane. The membrane was not stripped between the immunodetection for StAR and the consecutive immunodetection for actin.

StAR polyclonal rabbit antiserum was kindly provided by Dr. Tesone (Instituto de Biología y Medicina Experimental, Argentina) and Dr. Stocco (Texas Tech University Health Sciences Center) and raised against a peptide fragment (amino acid 88–98) of the mouse StAR protein [see additional information in Clark *et al.* (23)].

Statistical analysis

Statistical analyses were performed using ANOVA followed by Student's *t* test for two comparisons or Student-Newman-Keuls test for multiple comparisons. Data are expressed as mean ± SEM.

For semiquantitative RT-PCR and immunoblotting studies, bands were quantified by densitometry and normalized to α-tubulin or actin housekeeping genes using Scion IMAGE (Scion Corp., Frederick, MD).

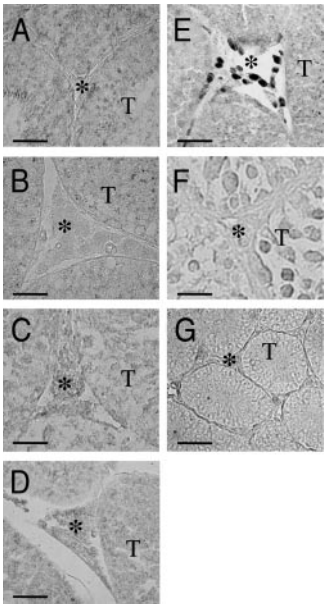

FIG. 2. Immunohistochemical analyses of COX-2 in testes. Immunolocalization of COX-2 in testes from adult (18-yr-old) Rhesus monkey (A), adult pig (B), adult (60 d old) BALBc mouse (C), adult (60 d old) Wistar rat (D), LD adult (90 d old) Syrian hamster (E), 16-SD adult Syrian hamster (F), and LD prepubertal (18 d old) Syrian hamster (G). No reaction was observed in testis sections incubated only with the conjugated antibody (controls, data not shown). *Bar*, 40 μm. *, Interstitium; T, tubule.

Results

Identification of COX-2 in LD hamster testes by immunohistochemistry

The immunoperoxidase technique revealed the presence of COX-2 in the cytoplasm of interstitial cells showing the characteristic punctuate chromatin pattern of Leydig cells in LD adult Syrian hamster testes (Fig. 2E). Similar results were observed in testes from LD peripubertal (36 d old) and pubertal (45 and 60 d old) hamsters (data not shown). Nevertheless, COX-2 was not found either in testis from 16-SD adult hamsters (Fig. 2F) or in testes from LD prepubertal (12 and 18 d old) hamsters (Fig. 2G). Moreover, immunoreactivity was not observed in testes from adult (18 yr old) Rhesus monkeys (Fig. 2A), adult pigs (Fig. 2B), young adult (60 d old) BALBc mice (Fig. 2C), young adult (60 d old) Wistar rats (Fig. 2D), and young adult (60 d old) Sprague Dawley rats (data not shown).

Identification of COX-2 in testes and freshly isolated Leydig cells from LD hamsters by RT-PCR

We detected mRNA expression of COX-2 in testes from LD adult (90 d old) Syrian hamster testes (Fig. 3A) but not in those from 16-SD adult or LD prepubertal (12 and 18 d old) Syrian hamsters (Fig. 3A). Moreover, the addition of 100 mIU/ml hCG to the incubation media of Leydig cells isolated from LD adult hamsters significantly induced the expression of COX-2 (Fig. 3B). Novel sequence information about COX-2 obtained from the analysis of LD hamster Leydig cells (representing four independently derived identical sequences) was submitted to GenBank (accession no. AY426532). This partial sequence showed 99.0% homology with human, 82.9% homology with rat, and 85.6% homology with mouse COX-2 at the nucleotide level.

Evaluation of COX-1 expression in hamster testes

Testicular COX-1 immunoreactivity was not detected in LD adult (90 d old) (Fig. 4A), 16-SD adult (Fig. 4B), or LD prepubertal (12 and 18 d old) Syrian hamsters (Fig. 4C). However, COX-1-immunoreactive cells were found when stomach tissue from LD adult (90 d old) Syrian hamsters was used as positive control (Fig. 4D).

Although cDNA fragments (271 bp) corresponding to COX-1 were detected in the positive control, namely in the LD adult (90 d old) Syrian hamster stomach (Fig. 4E), COX-1 was not found in testes from LD adult (90 d old), 16-SD adult or LD prepubertal (12 and 18 d old) Syrian hamsters even after a second PCR amplification using nested primers (Fig. 4E).

Inhibitory effect of PGF2α on hCG-stimulated in vitro production of testosterone from freshly isolated LD hamster Leydig cells

After 3 h incubation, neither PGD2 nor PGE2 within a range of 100 pM and 1 μM altered hCG-stimulated (100 mIU/ml) *in vitro* production of testosterone from LD adult hamster Leydig cells (data not shown). In contrast, in the presence of 100 mIU/ml hCG in the incubation media, testosterone production was significantly inhibited by PGF2α within a range of 100 pM and 1 μM (Fig. 5A).

On the other hand, basal testosterone production in LD

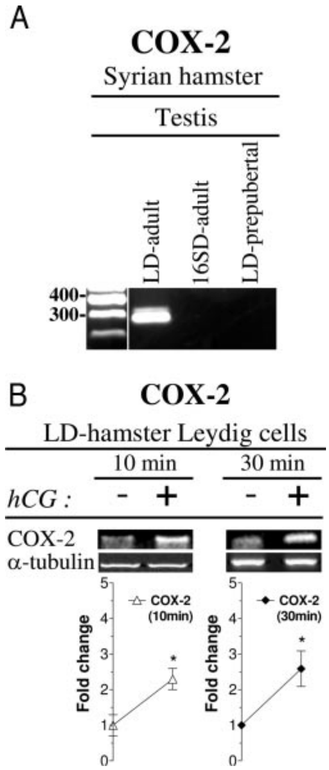

FIG. 3. Gene expression analyses of COX-2 in hamster testes. Ethidium-bromide-stained agarose gels showing 292-bp cDNA fragments that, after sequencing, were shown to correspond to COX-2. A, Testes from LD adult (90 d old), 16-SD adult, and LD prepubertal (12 and 18 d old) Syrian hamsters were examined. B, COX-2 expression was examined in LD adult (90 d old) hamster Leydig cells incubated in the presence or absence of 100 mIU/ml hCG. Incubation times were 10 and 30 min. These representative ethidium-bromide-stained agarose gels show results obtained from one of the three experiments that showed comparable results. In all experiments, PCR bands were quantified by densitometry. Results are expressed as fold change relative to the control (basal conditions), which was assigned a value of 1, and normalized to α-tubulin gene. *Line plots* show the mean ± SEM from three independent experiments (three replicates per experiment) performed in different cell preparations. Where SEM *lines* are not present, the area of the *symbol* encompassed them. *, $P < 0.05$ compared with the control group.

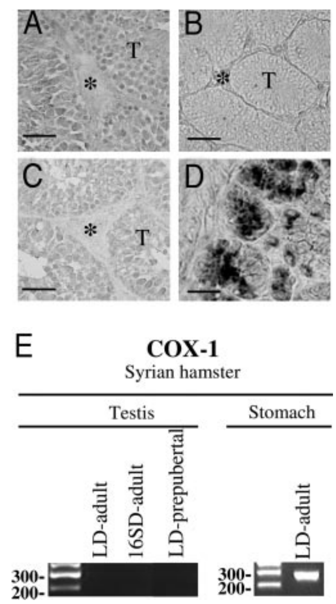

FIG. 4. Analyses of COX-1 expression in hamster testes. A–D, COX-1 immunohistochemical analyses in testes from LD adult (90 d old) (A), 16-SD adult (B), and LD prepubertal (18 d old) Syrian hamsters (C). Stomach tissue from LD adult (90 d old) Syrian hamsters was used as positive control for COX-1 immunostaining (D). No reaction was observed in testis sections incubated only with the conjugated antibody (controls, data not shown). *Bar*, 40 μm. *, Interstitium; T, tubule. E, Ethidium-bromide-stained agarose gels showing 271-bp cDNA fragments that, after sequencing, were shown to correspond to COX-1. Testes from LD adult (90 d old), 16-SD adult, and LD prepubertal (12- and 18 d old) Syrian hamsters were examined. Stomachs from LD adult (90 d old) Syrian hamsters were used as positive controls for COX-1 gene expression analysis.

adult hamster Leydig cells showed a significant increase in the presence of 1 μM PGD2 (data not shown). Neither PGE2 (data not shown) nor PGF2α (Fig. 5A) altered the production of testosterone in the absence of hCG in the incubation media.

No evidence for morphological changes was detected when electron microscopic studies of cellular morphology were performed in LD adult hamster Leydig cells after 3 h incubation with hCG (100 mIU/ml) in the presence or absence of either 1μM or 100 pM PGF2α (Fig. 5B and data not shown, respectively).

Testicular PGF2α concentration and content in Syrian hamsters: evidence for PGF2α production from freshly isolated LD hamster Leydig cells

Testicular PGF2α concentration and content were determined in LD adult hamsters (Fig. 6A). Moreover, PGF2α production from LD hamster Leydig cells showed a 2.5-fold decrease after 3 h incubation in the presence of 1 μM meloxicam, a preferential COX-2 inhibitor (Fig. 6B).

Identification of PGF2α receptors in freshly isolated LD hamster Leydig cells and human testicular biopsies by immunohistochemistry and RT-PCR

The immunohistochemical technique in adult LD hamster testis sections revealed the presence of PGF2α receptors in interstitial cells (Fig. 7A). PGF2α receptor immunoreactivity was also found in the interstitial compartment of human pathological biopsies from patients with SCO and GA syndromes (Fig. 7A). In contrast, immunoreactivity was not observed in four human biopsies from patients showing normal spermatogenesis with no evident morphological abnormalities (data not shown). Nevertheless, evaluation of a higher number of human testicular biopsies is required to establish the lack of PGF2α receptor expression in testes with no evident morphological changes or abnormalities.

When the PGF2α receptor antibody was preabsorbed with a specific blocking peptide to test specificity, PGF2α receptors were not seen either in hamster or in human testes (Fig. 7A).

By using RT-PCR, we amplified cDNA fragments (149 bp) that, after sequencing, were shown to correspond to PGF2α receptors from LD adult hamster testes and Leydig cells isolated from adult animals maintained under LD conditions (Fig. 7B). Novel sequence information about the PGF2α re-

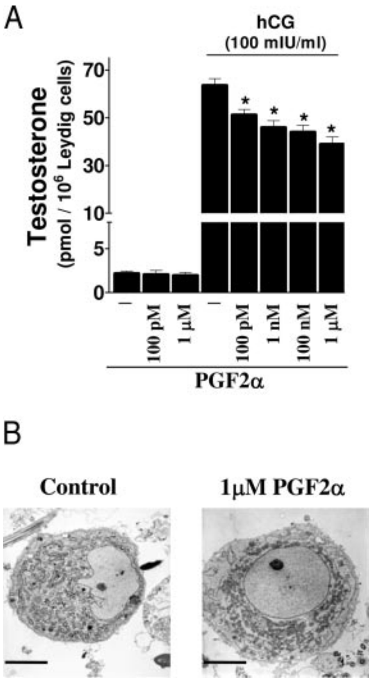

FIG. 5. Effects of PGF2α on *in vitro* production of testosterone from freshly isolated LD adult hamster Leydig cells. A, Effect of different concentrations (100 pM to 1 μM) of PGF2α on basal and maximally hCG-stimulated (100 mIU/ml) testosterone production in Leydig cells isolated from LD adult (90 d old) hamsters. Incubation time was 3 h. *Bar plots* show the mean ± SEM from three independent experiments (five to six replicates per experiment) performed in different cell preparations. Where SEM *lines* are not present the area of the *bar* encompassed them. *, $P < 0.05$ compared with the control group. B, Transmission electron micrographs. Electron microscopic studies of cellular morphology were performed in maximally hCG-stimulated LD adult (90 d old) hamster Leydig cells incubated in the presence or absence of 1 μM PGF2α. *Bar*, 5 μm.

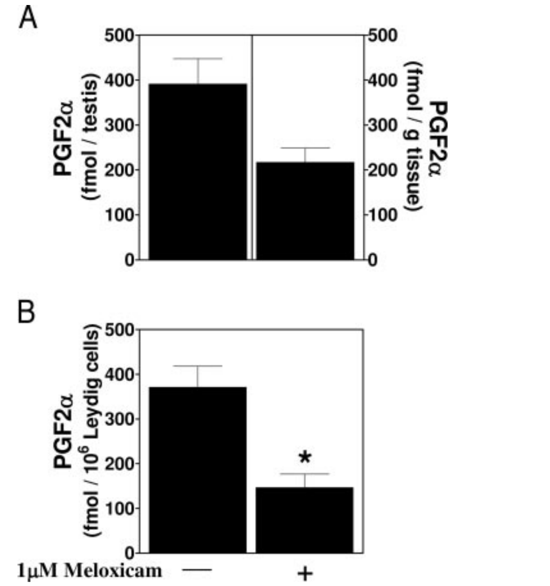

FIG. 6. PGF2α in hamster testes. A, Testicular concentration (fmol/g tissue) and content (fmol/ testis) of PGF2α in LD adult (90 d old) Syrian hamsters. *Bar plots* show the mean ± SEM from two independent experiments (six replicates per experiment). B, *In vitro* production of PGF2α from freshly isolated LD adult (90 d old) hamster Leydig cells. The effect of 1 μM meloxicam, a preferential COX-2 inhibitor, on PGF2α production from LD adult (90 d old) hamster Leydig cells is shown. Incubation time was 3 h. *Bar plots* show the mean ± SEM from three independent experiments (four replicates per experiment) performed in different cell preparations. *, $P < 0.05$ compared with the control group.

ceptor obtained from the analysis of LD adult hamster testes and LD hamster Leydig cells (representing five independently derived identical sequences) was submitted to GenBank (accession no. AY426533). This partial sequence showed 97.5 and 83.2% homology at the nucleotide level with the human and rat PGF2α receptor, respectively.

We also amplified cDNA fragments corresponding to the PGF2α receptor in pathological testicular biopsies from patients with SCO and GA syndromes (Fig. 7B).

Inhibitory effect of PGF2α on gene expression of StAR and 17β-HSD in freshly isolated LD hamster Leydig cells

The addition of hCG (100 mIU/ml) alone to LD hamster Leydig cells resulted in an increased expression of StAR, P450scc, 3β-HSD, 17β-HSD, and 5α-R1 (Fig. 8, A–C).

PGF2α did not affect StAR expression after 30 and 60 min incubation (Fig. 8, B and C). Nevertheless, after 10 min incubation, 100 mIU/ml hCG-induced StAR expression in LD hamster Leydig cells was markedly inhibited by PGF2α (Fig. 8A). In hCG-stimulated Leydig cells, 1 μM PGF2α significantly reduced mRNA levels of 17β-HSD after 10, 30, and 60 min incubation (Fig. 8, A–C). When 1 μM PGF2α was added into the incubation media of hCG-stimulated Leydig cells, expression of P450scc, 3β-HSD, and 5α-R1 remained unchanged (Fig. 8, A–C).

Novel sequence information about 17β-HSD obtained from the analysis of LD hamster Leydig cells (representing five independently derived identical sequences) was submitted to GenBank (accession no. AY426534). This partial sequence showed 91.3% homology at the nucleotide level with the human 17β-HSD. In addition, this partial sequence showed 88.9% homology at the nucleotide level with the rat and mouse 17β-HSD.

Inhibitory effect of PGF2α on protein expression of StAR in freshly isolated LD hamster Leydig cells

When Western blotting analyses using an anti-StAR antibody were performed, expected immunoreactive bands at approximately 30 kDa were seen in purified LD hamster Leydig cells (Fig. 9A). The addition of hCG (100 mIU/ml) alone to LD hamster Leydig cells resulted in an increased expression of StAR (Fig. 9, A and B). Moreover, when 1 μM PGF2α was added to hCG-stimulated Leydig cells, proteins levels of StAR were partially reduced after 1 and 2 h incubation and significantly diminished after 3 h incubation (Fig. 9, A and B).

Discussion

This study provides novel evidence for testicular COX-2 expression and the subsequent local production of PGF2α in the reproductively active seasonal breeder Syrian hamster. Our results indicate that PGF2α, presumably acting through

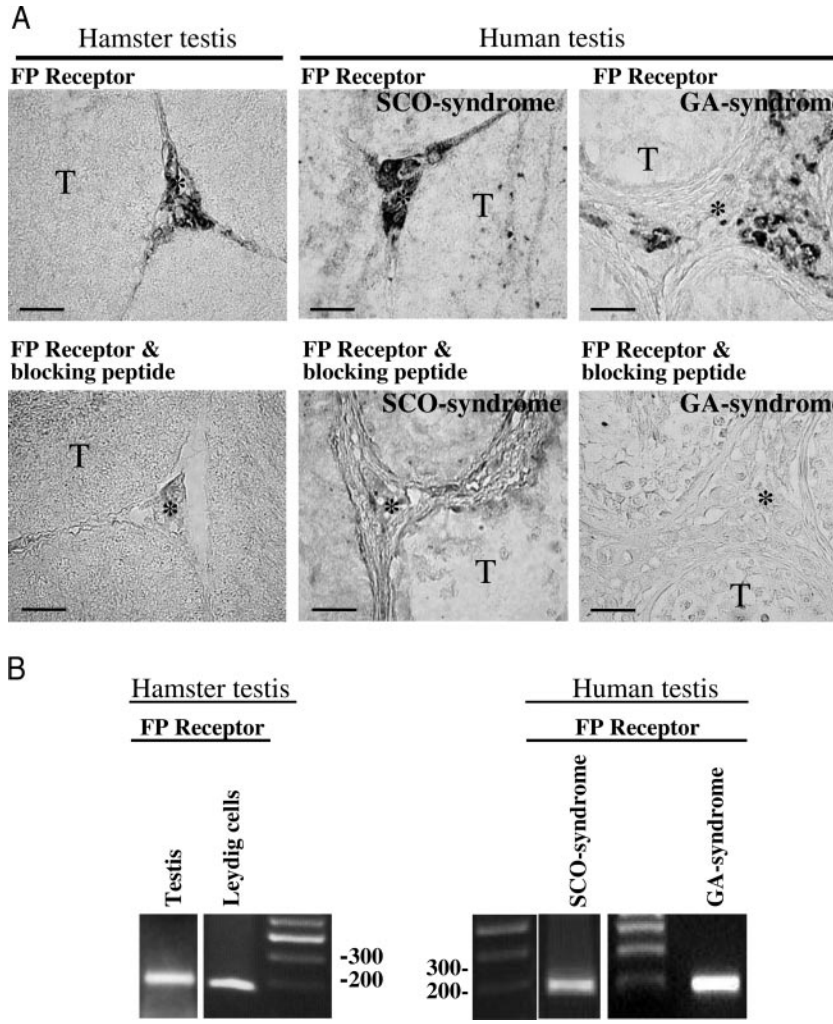

FIG. 7. Identification of PGF2α (FP) receptors in LD adult hamster Leydig cells and human testicular biopsies. A, Testicular immunolocalization of PGF2α receptors in LD adult (90 d old) hamsters and human testicular biopsies from patients with SCO and GA syndromes. Positive reaction for PGF2α receptors was not detected when the antiserum was preabsorbed with a specific blocking peptide (Cayman Chemical) for 2 h at room temperature either in hamster or in human testes. *Bar*, 40 μm. *, Interstitium; T, tubule. B, Ethidium-bromide-stained agarose gels showing 149-bp cDNA fragments that, after sequencing, were shown to correspond to PGF2α receptor in testes and isolated Leydig cells from LD adult (90 d old) hamsters and testicular biopsies of patients with SCO or GA syndrome.

PGF2α receptors located in Leydig cells and through a mechanism involving down-regulation of StAR and 17β-HSD expression, leads to the inhibition of LH/hCG-stimulated testosterone production. Thus, the testicular PGF2α system working in concert with the primary effect of gonadotropins on the hypothalamic-pituitary axis represents a local inhibitory control of steroidogenesis in Syrian hamsters. Besides this novel aspect, we also found PGF2α receptors in human pathological biopsies from patients with SCO and GA syndromes, *i.e.* in samples in which we had previously described the existence of testicular COX-2 expression (14). However, the relevance of our results to understanding the events leading to male infertility should be further investigated.

There is growing evidence suggesting that arachidonic acid and its oxygenated metabolites regulate physiological and pathological processes in reproduction, mainly in the ovary (24–27). In testes, however, there are few and controversial reports, and consequently, the possible role of PGs in testicular activity is not yet well understood. According to early reports, COX-1 and COX-2, key isoenzymes in the biosynthesis of PGs, may be not expressed in testes. This hypothesis is in line with results from knockout experiments showing that fertility is not affected in COX-1 and COX-2 mutant male mice. In striking contrast, female COX mutant mice are infertile (13). Thus, it may be concluded that PGs may not be important for testis functioning. However, recent reports have shown that COX-2 could play a role in the regulation of testicular activity mainly in fertility disorders and aging. We have previously described that although COX-2 is not detected in human testicular biopsies with no evident morphological changes or abnormalities, it is expressed in testes from men with impaired spermatogenesis and male infertility (14). COX is also induced in testicular cancer (15). Moreover, COX-2 represents a potential key factor in the age-related reduction of testosterone production because an increased COX-2 expression in Brown-Norway rats during aging, concomitantly with the decreased testicular production of testosterone, has been recently described (28). In this context, COX-2 inhibition enhances steroidogenesis and StAR gene expression in MA-10 mouse Leydig cells, whereas its overexpression leads to the opposite (29).

To further characterize the role of COX and PGs in the modulation of testicular function, we initially examined COX-2 expression in testes from different adult species. Immunohistochemical assays failed to detect COX-2 expression in adult BALBc mice, Wistar rats, Sprague Dawley rats, Rhesus monkeys, and pigs. However, immunohistochemical studies confirmed by RT-PCR assays revealed the presence

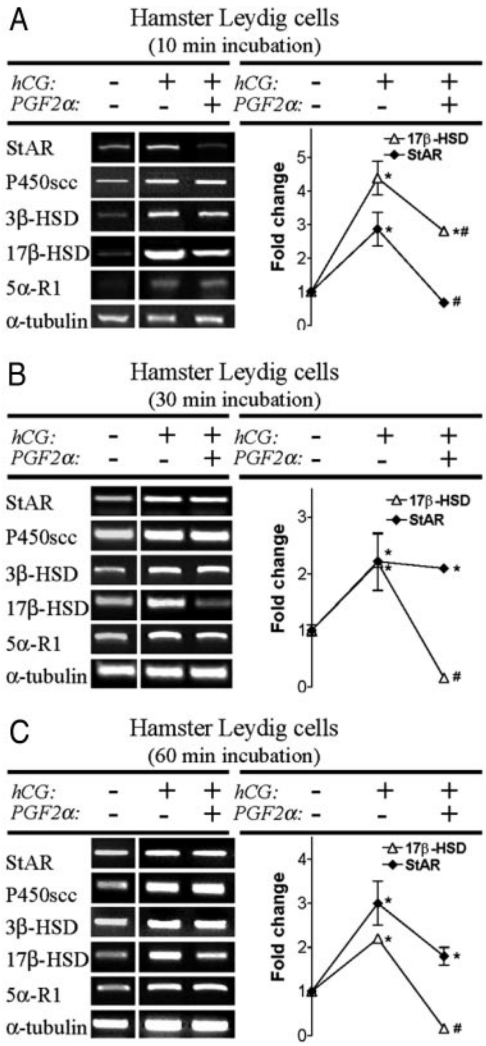

FIG. 8. Effect of PGF2α on gene expression of StAR and steroidogenic enzymes in freshly isolated LD adult hamster Leydig cells. Ethidium-bromide-stained agarose gels showing cDNA fragments that, after sequencing, were shown to correspond to the testicular StAR protein, P450scc, 3β-HSD, 17β-HSD, 5α-R1, and α-tubulin in Leydig cells isolated from LD adult (90 d old) hamsters. RNA expression was examined in the presence or absence of 100 mIU/ml hCG and 1 μM PGF2α. Incubation times were 10 min (A), 30 min (B), and 60 min (C). These representative ethidium-bromide-stained agarose gels show results obtained from one of the three experiments that showed comparable results. In all experiments, PCR bands were quantified by densitometry. Results are expressed as fold change relative to the control (basal conditions), which was assigned a value of 1, and normalized to α-tubulin gene. *Line plots* show the mean ± SEM from three independent experiments (three replicates per experiment) performed in different cell preparations. Where SEM *lines* are not present, the area of the *symbol* encompassed them. *, $P < 0.05$ compared with the control group; #, $P < 0.05$ compared with the 100 mIU/ml hCG-stimulated group.

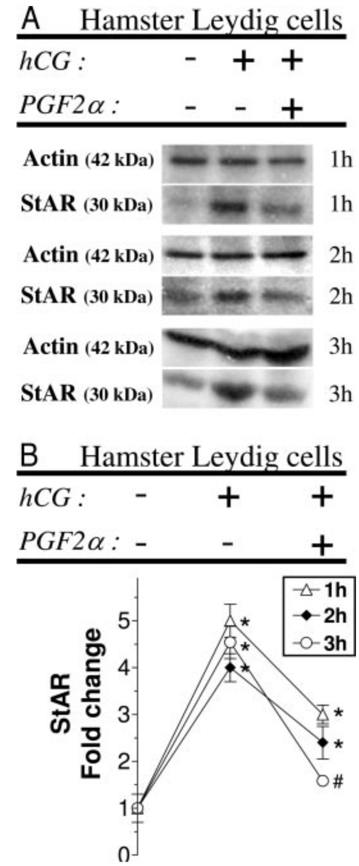

FIG. 9. Effect of PGF2α on protein expression of StAR protein in freshly isolated LD adult hamster Leydig cells. A, Western blot analysis of hamster Leydig cells isolated from adult (90 d old) hamsters kept in LD photoperiod. StAR expression was probed in the presence or absence of 100 mIU/ml hCG and 1 μM PGF2α with anti-StAR (dilution 1:2000) and antiactin (1:5000). Incubation times were 1, 2, and 3 h. The enhanced chemiluminescence method was used. Fifty micrograms from Leydig cell homogenates were loaded on the gel. This representative immunoblotting shows results obtained from one of the two experiments that showed comparable results. B, In all experiments, bands were quantified by densitometry. Results are expressed as fold change relative to the control (basal conditions), which was assigned a value of 1, and normalized to actin. *Line plots* show the mean ± SEM. Where SEM *lines* are not present, the area of the *symbol* encompassed them. *, $P < 0.05$ compared with the control group; #, $P < 0.05$ compared with the 100 mIU/ml hCG-stimulated group.

of COX-2 in Leydig cells of reproductively active Syrian hamsters. The evolutionary divergence in testicular coding sequences (30), the existence of a marked variation between different species in the photoperiodic regulation of GnRH/gonadotropin secretion (31), and/or the differential physiological role of PRL on testes as a consequence of its molecular heterogeneity (32, 33), might be responsible for the species-specific testicular COX-2 expression described in the current study.

We did not observe COX-2 expression either in reproductively inactive testes from adult Syrian hamsters kept under SD photoperiod for 16 wk or in testes from prepubertal LD hamsters. Circulating levels of LH are markedly decreased in prepubertal Syrian hamsters when compared with peripubertal, pubertal, and adult animals, and serum LH levels show a severe decline when adult hamsters are exposed to a SD photoperiod for 16 wk (16, 34–39). In this study, we found that incubation of Leydig cells isolated from reproductively active hamsters with hCG significantly induces COX-2 mRNA expression. Thus, we speculate that LH could be involved in the regulation of testicular COX-2 expression and, in consequence, one of the potential multiple factors responsible for the differences observed between reproductively active hamsters and inactive prepubertal/regressed adult animals.

We did not detect expression of COX-1, a constitutive isoenzyme, in Syrian hamster testes. Despite the lack of expression of COX-1, expression of COX-2 in hamster Leydig cells implies the production and eventually the action of PGs in the testis. In this context, several reports describe actions of PGs on adrenal, ovarian, and testicular steroidogenesis in different species including bovines, rats, fishes, monkeys, and humans (24–26, 40–47). Because the plethora of PGs could act on the testis via multiple receptors, we tested the effect of some PGs on the functionality of adult LD hamster Leydig cells. PGF2α was the only PG that significantly altered hCG-stimulated testosterone release from isolated hamster Leydig cells in a dose-dependent manner. These results suggest a potential role of PGF2α as local negative modulator of the primary effect of LH on testicular androgen production. Our findings in Syrian hamsters are supported by previous reports in rats. In this context, repeated administration of PGF2α (46–48), as well as induction of testicular PGF2α by cadmium (49), cause a significant reduction in testosterone levels in rats. Moreover, in agreement with our findings in hamsters, Romanelli *et al.* (42) have previously described that PGF2α does not modify *in vitro* basal testosterone production but reduces hCG-stimulated testosterone secretion in rat Leydig cells. In contrast, a single dose of PGF2α has been shown to increase serum testosterone concentrations in monkeys (45).

Quantification of testicular PGF2α levels (concentration and content) in Syrian hamsters demonstrated that physiological concentrations of PGF2α (within a range of 100 pM and 1 μM) were assayed in the *in vitro* incubations of hamster Leydig cells performed in this study. In addition, when hamster Leydig cells were incubated in the presence of meloxicam, a COX-2 preferential inhibitor, a 2.5-fold decrease of PGF2α levels was evident. These data point out the key role played by COX-2 in the testicular production of PGF2α.

We also detected expression of PGF2α receptors in isolated hamster Leydig cells. Reports on PG receptors in testis are scarce, but at least one previous study in rats (50) has described the existence of PGF2α receptors in progenitor rat Leydig cells, whereas no PGF2α receptors have been found in adult rat Leydig cells.

To better understand the mechanisms underlying the inhibitory action of PGF2α on testosterone production, we performed semiquantitative RT-PCR analysis, which showed that PGF2α inhibition of hCG-induced testosterone production in hamster Leydig cells is accompanied by down-regulation of StAR (the protein involved in the regulation of cholesterol transport to the inner mitochondrial membrane) and 17β-HSD (enzyme that converts androstenedione into testosterone). Western blotting studies allowed us to confirm, at protein level, the inhibitory role exerted by PGF2α on StAR expression. Unfortunately, we failed to find antibodies that recognize 17β-HSD protein in hamster tissues. There is evidence from other studies that support our results in hamster Leydig cells. For instance, PGF2α treatment has been reported to significantly decrease StAR expression in porcine, rat, and human ovaries (51–54). Moreover, cadmium can cause a strong induction of testicular PGF2α production that is accompanied by the inhibition of StAR expression in adult rats (49). In MA-10 Leydig cells that constitutively express COX-2, inhibition of COX activity by indomethacin and NS398 enhances StAR gene transcription and progesterone production (29, 55), and the age-related increase in COX-2 activity in Brown Norway rat Leydig cells exerts a tonic inhibition of StAR gene expression (28). Nevertheless, changes in steroid production by COX/PGF2α without alteration of StAR expression have also been reported in bovine adrenal gland (41) and rat corpus luteum (52).

In conclusion, our current findings show the existence of a COX-2/PGF2α system in the Leydig cells of the Syrian hamster and provide insights in how this previously unknown physiological system up-regulated during puberty/adulthood, together with the exposure to optimal light conditions for reproduction, may serve as a local inhibitor of testicular testosterone production by setting up a brake on stimulatory endocrine mechanisms.

Therefore, the adult hamster is a readily available animal model to study the testicular role of COX-2/PGs. We have also identified the expression of COX-2 (10) and the existence of PGF2α receptors in testicular biopsies from infertile men. Nevertheless, whether our findings for the Syrian hamster can be extrapolated to states of male infertility and whether COX-2 and its signaling mechanism may be targets for new therapeutic approaches are possibilities that remain to be clarified and further investigated.

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