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Prolactin (PRL) induction of cyclooxygenase 2 (COX2) expression and prostaglandin (PG) production in hamster Leydig cells

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ABSTRACT

Serum prolactin (PRL) variations play a crucial role in the photoperiodic-induced testicular regression–recrudescence transition in hamsters. We have previously shown that cyclooxygenase 2 (COX2), a key enzyme in the biosynthesis of prostaglandins (PGs), is expressed mostly in Leydig cells of reproductively active hamsters with considerable circulating and pituitary levels of PRL. In this study, we describe a stimulatory effect of PRL on COX2/PGs in hamster Leydig cells, which is mediated by IL-1β and prevented by P38-MAPK and JAK2 inhibitors. Furthermore, by preparative isoelectric focusing (IEF), we isolated PRL charge analogues from pituitaries of active [isoelectric points (pI): 5.16, 4.61, and 4.34] and regressed (pI: 5.44) hamsters. More acidic PRL charge analogues strongly induced COX2 expression, while less acidic ones had no effect.

Our studies suggest that PRL induces COX2/PGs in hamster Leydig cells through IL-1β and activation of P38-MAPK and JAK2. PRL microheterogeneity detected in active/inactive hamsters may be responsible for the photoperiodic variations of COX2 expression in Leydig cells.

1. Introduction

Prolactin (PRL) is synthesized and secreted mainly by the lactotroph cells of the pituitary (Freeman et al., 2000), but also by extrapituitary sources such as the mammary gland, the placenta, the uterus and T lymphocytes (Ben-Jonathan et al., 1996). This hormone was initially named for its stimulatory action on lactation. However, it is now recognized that PRL has over 300 different biological activities that include actions on behavior, metabolism, immune responses, and electrolyte balance (for reviews see Bole-Feysot et al., 1998; Ben-Jonathan et al., 2008).

In terms of reproduction, PRL plays a critical role in the reproductive neuroendocrine function controlling LH release (Steger et al., 1998). In females, PRL participates in the regulation of the ovarian corpus luteum function, the development of the mammary gland and the control of maternal behavior (Bachelot and Binart, 2007). Studies performed in PRL-deficient mice have shown that females are infertile because of luteal failure (Bartke, 1999). Initial studies performed in adult dwarf mice genetically deficient in PRL pointed out the ability of this hormone to affect the growth of male accessory reproductive glands (Bartke, 1978; Gonzalez et al., 1991). However, PRL knock-out males are fertile (Steger et al., 1998), suggesting that PRL plays a modest role in male testicular function contrary to that played in female reproduction. Nevertheless, it has been demonstrated that under pathological conditions, such as the existence of prolactinomas, PRL may cause male-factor infertility by producing hypogonadism (Biller, 1999).

In most seasonally breeding mammals, the photoperiod influences PRL secretion via its effects on the release of the pineal hormone melatonin (Curlewis, 1992). The Syrian hamster (*Mesocricetus auratus*) is a seasonal breeder that under a natural photoperiod restricts its reproductive activity to spring and summer, while, in autumn and winter, undergoes a severe gonadal involution. Under artificial light conditions of less than 12.5 h light per day, the hamster experiences a drastic morphological and physiological testicular regression accompanied by a marked decrease in the serum levels of FSH, LH, PRL and testosterone. This quiescence period is followed by a spontaneous gonadal recrudescence (Bartke, 1985). Interestingly, a significant increase in testicular weight can be observed after treatment of regressed animals

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with pituitary grafts, ovine PRL and sulpiride (a drug known to stimulate PRL release). This complete reversal of testicular atrophy is accompanied by normalization of serum testosterone levels, a significant increase in the number of testicular LH receptors and reappearance of fertility (Czyba et al., 1971; Bex and Bartke, 1977; Bex et al., 1978; Bartke et al., 1979). Unexpectedly, treatment of regressed animals with LHRH, ovine LH or ovine FSH has little, if any, effect on testicular function (Reiter et al., 1975; Bex and Bartke, 1977). Therefore, the excellent gonadal response to exogenous PRL and to sulpiride-induced stimulation of PRL release emphasizes the critical role played by PRL in mediating the testicular photoperiodic regression–recrudescence transition in the Syrian hamster.

Research carried out in recent years has uncovered the potential biological role of PRL molecular microheterogeneity. Glycosylated PRL has been found in the pituitary glands of a wide variety of mammalian, amphibian, and avian species. The linkage of the carbohydrate moiety may be either through nitrogen (*N*-glycosylation) or oxygen (*O*-glycosylation). The carbohydrate residues of the oligosaccharide chain may contain varying ratios of sialic acid, fucose, mannose and galactose, which differ considerably between species, physiological and pathological states (Freeman et al., 2000). In addition to glycosylation, other reported post-translational modifications of the PRL molecule include tyrosine sulfation, phosphorylation and deamination (Ho et al., 1993; Sinha, 1995).

PRL molecular microheterogeneity might represent a key mechanism for creating diversity in the biological actions of this hormone (Sinha, 1992).

To our knowledge, the structural microheterogeneity of PRL and its relevance on molecular bioactivity have not been investigated in seasonal breeder species such as the Syrian hamster.

We have recently found that photoperiodically regressed adult hamsters weakly express cyclooxygenase 2 (COX2), a key enzyme in the biosynthesis of prostaglandins (PGs), in testes. Conversely, Leydig cells of the reproductively active hamster express COX2 and produce PGs, including PGD_2 and $PGF_{2\alpha}$, both involved in the modulation of testosterone production (Frungieri et al., 2006; Schell et al., 2007; Matzkin et al., 2009).

Taking into account the variations observed in PRL serum levels and testicular COX2 expression during the photoperiodic-induced regression–recrudescence in hamsters, the goal of this study was to further investigate the potential action of this pituitary hormone on the induction and maintenance of PG production from Leydig cells. Moreover, charge molecular analogues of pituitary PRL in photoperiodically active and regressed hamsters were isolated and their impact on testicular COX2/PGs was analyzed.

2. Materials and methods

2.1. Animals

Male Syrian hamsters (*Mesocricetus auratus)* were raised in our animal care unit [Charles River descendants, Animal Care Lab., Instituto de Biología y Medicina Experimental (IBYME), Buenos Aires] and kept from birth to adulthood in rooms at 23 ± 2 °C under a long-day (LD) photoperiod (14 h light, 10 h darkness; lights on 0700–2100 h). Animals had free access to water and Purina formula chow.

Young adult hamsters aged 90 days were transferred to a short-day (SD) photoperiod (6 h light, 18 h darkness; lights on 0900–1500 h) for 8, 12, 16, 22 and 28 weeks. It is worth noting that hamsters from our colony reach maximum testicular regression after 16 weeks in a SD photoperiod (see additional information in Frungieri et al., 1996). Hamsters were killed by asphyxia with carbon dioxide (CO_2) according to protocols for animal laboratory use, approved by the Institutional Animal Care and Use Committee (IBYME), following the National Institutes of Health (NIH) guidelines. At the time of sacrifice, trunk blood was collected. Serum was obtained by centrifugation and stored at −20 °C for further PRL determination. Pituitary glands and testes were dissected. Pools of six to ten pituitary glands were weighed, homogenized with a Teflon pestle in 1.2 ml of 10 mM phosphate-buffered saline (PBS, pH 7.4) and centrifuged at 105,000*g* for 1 h. Supernatants were collected and stored at −70 °C until assayed for PRL radioimmunoassay (RIA), preparative size-exclusion chromatography and preparative isoelectric focusing (IEF). Left testes were dissected, fixed for at least 48 h in formaldehyde fluid followed by dehydration, and then embedded in paraffin wax for histological and immunohistochemical studies. Right testes were used for Leydig cell purification. Leydig cells were *in vitro* incubated and mRNA expression [by reverse transcription polymerase chain reaction (RT-PCR) or real-time reverse transcription polymerase chain reaction (RT–qPCR)], protein expression (by immunoblotting), and the levels of PGs and testosterone in the incubation media (by immunoassay and radioimmunoassay, respectively) were determined.

2.2. PRL assay

PRL serum levels, PRL pituitary content, and PRL pituitary concentration were determined by a homologous RIA (Frungieri et al., 1996). A reference preparation AFP-10302E and a specific antiserum provided by Dr Parlow (Harbor-UCLA Research and Education Institute, Torrance, CA, USA) were used. The sensitivity of the assay was 0.20 ng/tube. The intra-assay coefficient of variation was less than 6.0%. All samples were measured, in duplicate, in the same assay to avoid inter-assay variations.

2.3. Preparative size-exclusion chromatography

Fractionation of pituitary extracts was performed in columns (1.6 × 80 cm) packed with medium or superfine Sephadex G-100 size-exclusion gels. The fractionation for globular proteins of Sephadex G-100 ranges from 4 to 100–150 kDa. Samples of 0.1–3 ml were injected into the column and permeated with 0.2 ml/min flow rate of mobile phase [PBS 50 mM, 0.2% bovine serum albumin (BSA)] at 4 °C. Seventy fractions of 1 ml each were collected using an automatic fraction collector (Amersham Pharmacia Biotech Inc., Uppsala, Sweden) and frozen at −20 °C until PRL quantification by RIA.

2.4. Preparative isoelectric focusing (IEF)

Preparative IEF was used to evaluate the relative abundance of the PRL charge analogues. Samples obtained from hamsters exposed to a LD photoperiod or a SD photoperiod for 16 weeks were analyzed in different pools of six to ten pituitaries each in order to reach an appropriate hormone level for the analysis. Alternatively, 66 μg of PRL purified from adult hamsters exposed to a LD photoperiod (AFP-10302E, UCLA Research and Education Institute) were evaluated.

Preparative IEF was carried out using a Rotofor Preparative Cell system (Bio Rad Laboratories Inc., CA, USA) as previously described by Ambao et al. (2009). Before applying the sample, a pre-focusing step was included to establish the pH gradient and avoid protein exposure to extreme pH environments. This step was carried out by filling the focusing chamber with 45 ml of deionized water, ampholytes (3% 3/10 ampholytes and 1% 4/6 ampholytes; Pharmalyte, Sigma–Aldrich, St. Louis, MO, USA) and 0.1% of 3-(3-cholamidopropyl) dimethyl–ammonio-1-propane sulphonate (CHAPS, Sigma–Aldrich). After this procedure, samples were loaded into the chamber (the final volume of the chamber always being

50 ml) and focusing was carried out at 12 W constant power (Power Pac 3000, Bio Rad) for 4 h, keeping the chamber refrigerated (Refrigerated Circulator, Forma Scientific Inc., Marietta, OH, USA). When focusing was completed, 18–20 fractions (2.5 ml each) were harvested and their pH was determined. In order to avoid ampholytes and detergent interaction with sample proteins, 500 μl of 5 M NaCl was added to each tube (final concentration 1 M NaCl). Each individual fraction was exhaustively dialyzed to completely eliminate ampholytes and detergent. Dialysis was carried out using slide A-Lyzer Dialysis cassettes (Thermo Scientific, Meridian Rd, Rockford, IL, USA) against 1 M NaCl at 4 °C in 1500 ml solution for 18 h and replacing this solution three times with a fresh one. Then, the saline solution was replaced two times with H_2O for 1 h, and finally changed to 10 mM NaCl for 1 h. This procedure assured the complete absence of interfering substances in future hormone determinations. After dialysis, PRL levels were determined by RIA.

2.5. Immunohistochemical analyses

Testes from young adult (90 days old) hamsters exposed to a SD photoperiod for 8, 12, 16, 22 and 28 weeks, as well as testes from 90- and 280-day old hamsters kept under a LD photoperiod, were examined by immunohistochemical assays. Groups of eight to ten animal testes were evaluated. After fixation, tissues were dehydrated and embedded in paraffin wax, and 5-μm sections obtained from three different levels were used. In brief, endogenous peroxidase reactivity was quenched by a 20 min pretreatment with 10% methanol, 0.3% H_2O_2 in 0.01 M PBS (pH 7.4). Antigen retrieval was performed by microwave irradiation of hamster testicular sections in Tris-ethylenediaminetetraacetic acid (EDTA) buffer (10 mM Tris Base, 1 mM EDTA Solution, 0.05% Tween 20, pH 9.0). Then, sections were permeabilized by 5 min incubation with 0.5% saponin in PBS and nonspecific proteins were blocked by subsequent incubation with a protein block buffer (5% goat normal serum in PBS) for 30 min. After several washes, sections were incubated overnight in a humidified chamber at 4 °C with a polyclonal rabbit anti-COX2 serum (1:500, Cayman Chemical, Ann Arbor, MI, USA) or a monoclonal mouse anti-IL-1β antibody (1:100, R&D systems, Minneapolis, MN, USA). On the second day, testicular sections were washed and incubated with a biotinylated secondary antiserum (goat anti-rabbit IgG; 1:500, Vector Lab, CA, USA for COX2 or horse anti-mouse IgG; 1:500, Vector Lab, for IL-1β) for 2 h at room temperature. Finally, the immunoreaction was visualized with 0.01% H_2O_2 and 0.05% 3,3-diaminobenzidine (DAB, Sigma–Aldrich) solution in 0.05 M Tris–HCl (pH 7.6) and an avidin–biotin–peroxidase system (Vector Lab).

For control purposes, either the first antiserum was omitted or incubation was carried out with normal nonimmune sera.

The number of COX2-immunoreactive Leydig cells was quantified with a Zeiss microscope (Jena, Germany) with 400× magnification and a gridded eyepiece. In each testicular section, all fields were evaluated. The results were expressed as COX2-immunoreactive Leydig cells/mm^2.

2.6. Hamster Leydig cell purification and "in vitro" incubations

Hamster testes were used to isolate Leydig cells. In brief, decapsulated testes were incubated in a shaking water bath at 34 °C for 5 min in the presence of 0.2 mg/ml collagenase type I (Sigma–Aldrich). At the end of the incubation, collagenase activity was stopped by adding medium 199 (Sigma–Aldrich), and the tubules were allowed to settle for 1 min. Supernatants were transferred to 75 cm^2 sterile flasks, and placed in an incubator at 37 °C under a humid atmosphere with 5% CO_2 for 10 min. The unattached cells were then recovered by swirling, followed by a gentle washing with medium 199, and filtered by a 100 μm Nylon cell strainer (BD Biosciences, Bedford, MA, USA). Attached cells, 95% enriched with macrophages positive for Indian Ink, ED-1 antigen and ED-2 antigen, were discarded.

Filtered cells were used for Leydig cell isolation under sterile conditions using a discontinuous Percoll density gradient as previously described by Frungieri et al. (2006). 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%. In order to evaluate enrichment in Leydig cells, 3β-hydroxysteroid dehydrogenase (3β-HSD) activity was measured as previously described by Levy et al. (1959). Cell preparations were 87–90% enriched with hamster Leydig cells. Less than 0.006% of the contaminating cells were macrophages positive for Indian Ink, ED-1 and ED-2 antigens, whereas no mast cells were detected. The remaining cell types had the morphology of either peritubular cells or endothelial cells. Petri dishes with 1 ml medium 199 containing 2.5×10^5 cells (for RT-PCR, RT-qPCR and testosterone production) or 1.5×10^6 cells (for immunoblotting and PG production) were incubated at 37 °C under a humid atmosphere with 5% CO_2 and in the presence of the following chemicals: 10 and 25 ng/ml PRL purified from adult hamsters kept under a LD photoperiod (AFP-10302E, Dr Parlow, Harbor-UCLA Research and Education Institute), 10 μM SB203580 - a specific P38-mitogen-activated protein kinase (P38-MAPK) inhibitor (Sigma–Aldrich)-, 10 μM AG490 – a JAK2 inhibitor (Calbiochem)-, 10 ng/ml human IL-1β (Sigma–Aldrich), and 25 μg/ml of a neutralizing antibody that prevents IL-1β activity (R&D Systems). Alternatively, 10 ng/ml PRL charge analogues isolated by preparative IEF were added to the incubation media.

In this study, SB203580 and AG490 stock solutions were prepared in dimethyl sulfoxide (DMSO) (ICN Biomedicals Inc., Aurora, OH, USA). These solutions were then further diluted in medium 199. An appropriate volume of DMSO (1 μl DMSO/ml medium 199) was added to control experiments in order to account for possible effects of DMSO. PRL, IL-1β, and a neutralizing antibody that prevents IL-1β activity, were dissolved in medium 199, which was then used as vehicle for control incubations.

After incubations, cells were used either for RNA extraction followed by RT-PCR or RT-qPCR, or for protein extraction followed by immunoblotting. Media were frozen at −70 °C until PG and testosterone levels were determined by immunoassay and radioimmunoassay, respectively.

2.7. RT-PCR and RT-qPCR analyses

Total RNA was prepared from hamster Leydig cells using the QIAGEN RNeasy mini kit (QIAGEN Inc., Valencia, MO, USA). RT-reaction was performed using dN6 random primers as described elsewhere (Frungieri et al., 2002). A cDNA amount corresponding to 400 ng total RNA was used for each RT-PCR and RT-qPCR reaction.

PCR conditions were 95 °C for 5 min, followed by cycles of 94 °C for 1 min, 55–60 °C (annealing temperature) for 1 min and 72 °C for 1 min, and a final incubation at 72 °C for 5 min. PCR products were separated on 2% agarose gels, and visualized with ethidium bromide. The identity of the cDNA products was confirmed by sequencing using a fluorescence-based dideoxy-sequencing reaction and an automated sequence analysis on an ABI 373A DNA sequencer.

Taking into account the published information concerning PRL receptor gene expression in mouse tissues (Davis and Linzer, 1989; Clarke and Linzer, 1993; Ling et al., 2000), PCR studies were performed using one common PCR primer from the extracellular

domain (5′-AAGCCAGACCATGGATACTGGAG) and four specific primers corresponding to the first 23 nucleotides of the unique coding region of the different receptor splice forms: long (PRLR-L: 5′-AGCAGTTCTTCAGACTTGCCCTT), short 1 (PRLR-S1: 5′-AACTGG AGAATAGAACACCAGAG), short 2 (PRLR-S2: 5′-TCAAGTTGCTCT TTGTTGTGAAC), and short 3 (PRLR-S3: 5′-TTGTATTTGCTTGGAGA GCCAGT). Additional PCR studies were performed using oligonucleotide primers for StAR (5′-GCAGCAGGCAACCTGGTG and 5′-GCACCATGCAAGTGGGAC), IL-1β (5′-ACAAGTGGTATTCTCCATG and 5′-TCCACACTCTCCAGCTG), IL-1RI (5′-CTTGTGTGCCCTTATCTG and 5′-TGCTCTTCAGCCACATTC), IL-1RII (5′-GGAATACAACATCACT AGGA and 5′-TTGTGACTGGATCAAAAATC) and IL-1Ra (5′-GCAAG ATGCAAGCCTTCA and 5′-TGTGCAGAGGAACCATCC).

qPCR assays were performed using oligonucleotide primers for COX2 (5′-CTGGCGCTCAGCCATACAG and 5′-ACACTCATACAT ACACCTCGGT), IL-1β (5′-CACCTCTCAAGCAGAGCACAG and 5′-GGGTTCCATGGTGAAGTCAAC), and 18S rRNA (5′-ACACGGACAGGA TTGACAGATT and 5′-CGTTCGTTATCGGAATTAACCA). 18S rRNA was chosen as the housekeeping gene. Reactions were conducted using SYBR Green PCR Master Mix and the ABI PRISM 7500 sequence detector System (Applied Biosystems, Foster City, CA, USA). The reaction conditions were as follows: 10 min at 95 °C (one cycle), followed by 40 cycles of 30 s at 95 °C, 30 s at 55 °C, 1 min at 60 °C and 1 min at 72 °C for COX2, 10 min at 95 °C (one cycle), followed by 40 cycles of 15 s at 95 °C, 30 s at 55 °C and 1 min at 60 °C for 18S, and 10 min at 95 °C (one cycle), followed by 40 cycles of 15 s at 95 °C and 1 min at 58 °C for IL-1β. Following the mathematical model of Pfaffl (2001), the fold change of mRNA COX2 and IL-1β expression was determined for each sample by calculating $(\text{E target})^{\Delta Ct(\text{target})}/(\text{E housekeeping})^{\Delta Ct(\text{housekeeping})}$, where E is the efficiency of the primer set, C_T the cycle threshold, and ΔCt = Ct(normalization cDNA)−Ct(experimental cDNA). The amplification efficiency of each primer set was calculated from the slope of the standard amplification curve of log microlitres of cDNA per reaction *vs* Ct value over at least four orders of magnitude ($E = 10^{-(1/\text{slope})}$; E 18S rRNA = 1.94, E COX2 = 2.05 and E IL-1β = 2.04 *vs* hamster Leydig cells cDNA).

2.8. Immunoprecipitation

Hamster Leydig cells and mice livers (controls) were homogenized at 4 °C in buffer composed of 1% v/v Triton, 0.1 M Hepes, 0.1 M sodium pyrophosphate, 0.1 M sodium fluoride, 0.01 M EDTA, 0.01 M sodium vanadate, 0.002 M phenylmethylsulfonyl fluoride (PMSF), and 0.035 units/ml trypsin inhibitory aprotinin (pH 7.4). Protein concentrations were determined by the method of Bradford (1976).

Aliquots of lysated hamster Leydig cells and solubilized mice livers containing 800 μg and 2 mg protein, respectively, were incubated at 4 °C overnight with 5 μg/ml anti-STAT5 antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA).

After incubation, 20 μl protein A Sepharose (50% v/v) was added to the mixture. The preparation was further incubated at 4 °C with constant rocking for 2 h and then centrifuged at 3000*g* for 1 min. The supernatant that resulted after protein immunoprecipitation was discarded, and the precipitate was washed three times with washing buffer (0.05 M Tris, 0.01 M vanadate, and 1% v/v Triton X-100, pH 7.4). The final pellet was resuspended in 35 μl Laemmli buffer, boiled for 5 min, and stored at −20 °C until immunoblotting.

2.9. Immunoblotting

When immunoprecipitation was not performed, 1.5×10^6 hamster Leydig cells were homogenized in 20 mM Tris–HCl (pH 8), 137 mM NaCl buffer containing 10% glycerol, 1% lysis buffer (NP40, Sigma–Aldrich), and 1% of a pre-formed mixture of protease inhibitors (P8340, Sigma–Aldrich). Protein concentrations were measured by the method of Lowry et al. (1951). Leydig cell protein homogenates (approximately 100 μg) were heated at 95 °C for 5 min under reducing conditions (10% mercaptoethanol), and stored at −20 °C until immunoblotting.

Immunoprecipitated and non-immunoprecipitated samples were loaded onto tricine–SDS–polyacrylamide gels (8–12%), electrophoretically separated, and blotted onto nitrocellulose (Frungieri et al., 2002). Blots were incubated with rabbit polyclonal anti-COX2 antiserum (1:250, Cayman Chemical), mouse monoclonal anti-actin antibody (1:5000, Calbiochem, La Jolla, CA, USA), mouse monoclonal anti-phospho ERK 44/42 MAPK antibody (1:250, Cell Signaling Technology Inc, Beverly MA, USA), rabbit polyclonal anti-phospho P38 MAPK antiserum (1:250, Cell Signaling Technology Inc), mouse monoclonal anti-phospho JNK MAPK antibody (1:200, Santa Cruz Biotechnology Inc.), mouse monoclonal anti-phospho-STAT5a/b Tyr694/699 antibody (1:1000, Upstate Laboratories, Lake Placid, NY,USA), rabbit polyclonal anti-STAT5 antiserum (1:1000, Santa Cruz Biotechnology Inc.), rabbit polyclonal anti IL-1β antiserum (1:250, Cell Signaling Technology Inc), and subsequently with peroxidase-labeled secondary antibodies (1:2000 goat anti-mouse IgM, Calbiochem, for actin; 1:1000 sheep anti-mouse IgG, GE Healthcare, Wauwatosa, WI, USA, for phospho ERK 44/42 MAPK, phospho JNK MAPK and phospho-STAT5a/b Tyr694/699; and 1:2500 goat anti-rabbit IgG, Sigma–Aldrich, for COX2, phospho P38 MAPK, STAT5 and IL-1β). Signals were detected with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech AB).

2.10. PG immunoassays

Approximately 1.5×10^6 hamster Leydig cells were used to determine PGD_2 and $PGF_{2\alpha}$ levels in the incubation media as previously described by our group (Frungieri et al., 2002; Frungieri et al., 2006; Schell et al., 2007). Commercially available kits were used (Cayman Chemical).

For PGD_2 quantification, media were pre-treated with methoxylamine hydrochloride (MOX HCl) in order to prevent PG further chemical degradation and subsequently acidified using 1M citrate buffer (final pH 4.7).

For $PGF_{2\alpha}$ assay, media were acidified using 2 N HCl (final pH 3.5) before being injected into a 200-mg C18 column and then eluted with ethyl acetate.

Eluted fractions of incubation media from hamster Leydig cells were evaporated to dryness under a nitrogen stream and reconstituted in assay buffer. The minimum detectable immunoassay concentrations were 0.28 and 0.64 femtomole (fmol)/tube for PGD_2 and $PGF_{2\alpha}$, respectively. Intra-assay and inter-assay coefficients of variation were less than 10% and less than 15%, respectively. PG levels were expressed as fmol per 10^6 Leydig cells.

2.11. Testosterone assay

Testosterone levels were determined in the incubation media by RIA according to the method described by Frungieri et al. (2006) without extraction using antibodies obtained from Medicorp Inc. (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 215 fmol/ml. Intra- and inter-assay coefficients of variation were less than 12% and less than 15%, respectively.

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

2.12. Statistical analyses

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.

PRL levels in pituitary extracts and sera, and the number of COX2-immunoreactive Leydig cells were analyzed using linear regression. The coefficient (r) was calculated and $p < 0.05$ was considered significant.

For immunoblotting studies, bands were quantified by densitometry and normalized to actin housekeeping gene using SCION IMAGE (SCION Corporation, Frederick, MD, USA).

3. Results

3.1. Determination of pituitary and serum levels of PRL and quantification of COX2-immunoreactive Leydig cells during the photoperiodic regression–recrudescence transition in hamsters

The patterns of PRL pituitary content, PRL pituitary concentration and PRL serum levels in adult hamsters kept under a LD photoperiod or exposed to a SD photoperiod from 8 to 28 weeks are shown in Table 1. The exposure to a SD photoperiod of 6 h light per day resulted in a decline of these parameters showing a nadir after 16 weeks ($p < 0.05$). Nevertheless, under prolonged exposure to a SD photoperiod (28 weeks), pituitary and serum levels of PRL spontaneously returned to values near to those detected in animals exposed to a LD photoperiod.

A 10-fold decrease in the number of COX2-immunoreactive Leydig cells was observed in testes from hamsters exposed to a SD photoperiod for 16 weeks compared to those values detected in testes from adult hamsters kept under a LD photoperiod (Table 1). However, after 22–28 weeks of permanence in a SD photoperiod, the number of COX2-immunoreactive Leydig cells was partially recovered (Table 1).

Immunohistochemical analyses showed the expression of COX2 in the cytoplasm of interstitial cells with the characteristic punctuate chromatin pattern of Leydig cells in adult hamsters kept under a LD photoperiod (Fig. 1A) or transferred to a SD photoperiod for 8 (Fig. 1B), 12 (Fig. 1C), 16 (Fig. 1D), 22 (Fig. 1E) or 28 weeks (Fig. 1F).

COX2 immunostaining was not found when testis sections from adult hamsters kept under a LD photoperiod or transferred to a SD photoperiod were incubated only with normal nonimmune serum and the conjugated antibody (data not shown).

Furthermore, in hamsters kept under a LD photoperiod or exposed to a SD photoperiod from 8 to 28 weeks, the number of COX2-immunoreactive Leydig cells was positively correlated with the PRL pituitary content ($r = 0.903$; $p < 0.005$), PRL pituitary concentration ($r = 0.905$; $p < 0.005$) and PRL serum levels ($r = 0.727$; $p < 0.05$).

3.2. Identification of PRL receptor splice forms in hamster Leydig cells

Leydig cells purified from hamsters exposed to a LD photoperiod or a SD photoperiod for 16 weeks were used to amplify, by RT-PCR, cDNA fragments that, after sequencing, were shown to correspond to the long and short 3 PRL receptor splice forms (PRLR-L and PRLR-S3, respectively) (Fig. 2).

3.3. PRL induction of COX2 expression, PG release to the media, and testosterone production in hamster Leydig cells

When Leydig cells purified from hamsters exposed to a LD photoperiod or a SD photoperiod for 16 weeks were treated with 10 (Fig. 3A and B) and 25 ng/ml PRL (data not shown), a significant induction of mRNA and protein COX2 expression was observed.

Furthermore, the production of PGD_2 and $PGF_{2\alpha}$ in Leydig cells purified from adult hamsters kept under a LD photoperiod was markedly stimulated by PRL. In Leydig cells purified from hamsters exposed to a SD photoperiod for 16 weeks, PGD_2 production remained unchanged whereas PRL increased the levels of $PGF_{2\alpha}$ released to the incubation media (Fig. 3C).

On the other hand, testosterone production was significantly higher in hamster Leydig cells incubated in the presence of 10 ng/ml PRL (9.81 ± 0.73 fmol/10^6 cells, mean ± SEM) or 1 μM PGD_2 (17.82 ± 1.46 fmol/10^6 cells) than control cells under basal conditions (7.35 ± 0.21 fmol/10^6 cells, $P < 0.05$). In contrast, 1 μM $PGF_{2\alpha}$ had no effect on basal testosterone production (6.97 ± 1.01 fmol/10^6 cells).

3.4. Identification of the signaling pathway by which PRL induces COX2 expression

Immunoblotting experiments were performed to determine whether the effect of PRL on COX2 in hamster Leydig cells is mediated via protein kinases.

No significant changes were observed in ERK and JNK phosphorylation when hamster Leydig cells were incubated in the presence or absence of PRL (data not shown). Conversely, the addition of 10 (Fig. 4) and 25 ng/ml PRL (data not shown) concentrations to Leydig cells purified from hamsters kept under a LD photoperiod or exposed to a SD photoperiod for 16 weeks significantly induced not only COX2 but also phosphorylation of P38 after 1 h incubation.

The stimulatory effect of PRL on COX2 expression was prevented by the addition of the P38-MAPK inhibitor SB203580 and the JAK2 inhibitor AG490 (Fig. 5). SB203580 or AG490 alone did not affect protein COX2 expression in hamster Leydig cells (Fig. 5).

3.5. Role of IL-1 system in the PRL-induced expression of COX2 in hamster Leydig cells

Immunohistochemical and RT-PCR analyses showed the expression of IL-1β, its receptor IL-1RI, the antagonist receptor Ra and the

Table 1
Effect of LD and SD photoperiods on PRL pituitary content, PRL pituitary concentration, PRL serum levels and the number of COX2-immunoreactive Leydig cells in adult male Syrian hamsters.

Photoperiod	PRL pituitary content (μg/pituitary)	PRL pituitary concentration (μg/mg protein)	PRL serum levels (ng/ml)	COX2-immunoreactive Leydig cells/mm^2
LD	1.35 ± 0.15^{a}	7.15 ± 0.92^{a}	20.10 ± 1.22^{a}	176.77 ± 11.05^{a}
8 week SD	0.74 ± 0.08^{b}	3.82 ± 0.42^{b}	4.91 ± 0.42^{b}	143.75 ± 28.86^{a,b}
12 week SD	0.17 ± 0.01^{c,d}	0.87 ± 0.91^{c,d}	0.58 ± 0.16^{c}	94.95 ± 10.74^{b}
16 week SD	0.08 ± 0.01^{c}	0.38 ± 0.44^{c}	0.38 ± 0.04^{c}	14.65 ± 7.40^{c}
22 week SD	0.49 ± 0.09^{b,d,e}	3.16 ± 0.12^{b,d}	3.66 ± 0.28^{b,c}	103.50 ± 12.79^{b}
28 week SD	0.68 ± 0.09^{b,e}	3.27 ± 0.29^{b,d}	14.67 ± 1.35^{d}	107.66 ± 22.21^{b}

The data represent the mean ± SEM. For PRL pituitary content and concentration, $n = 6$ pools (each pool contains 6–10 pituitary glands). For PRL serum levels, $n = 6$–8 animals per group. For COX2-immunoreactive Leydig cells, $n = 5$–10 animals per group. Values without the same letter are significantly different at least at $p < 0.05$.

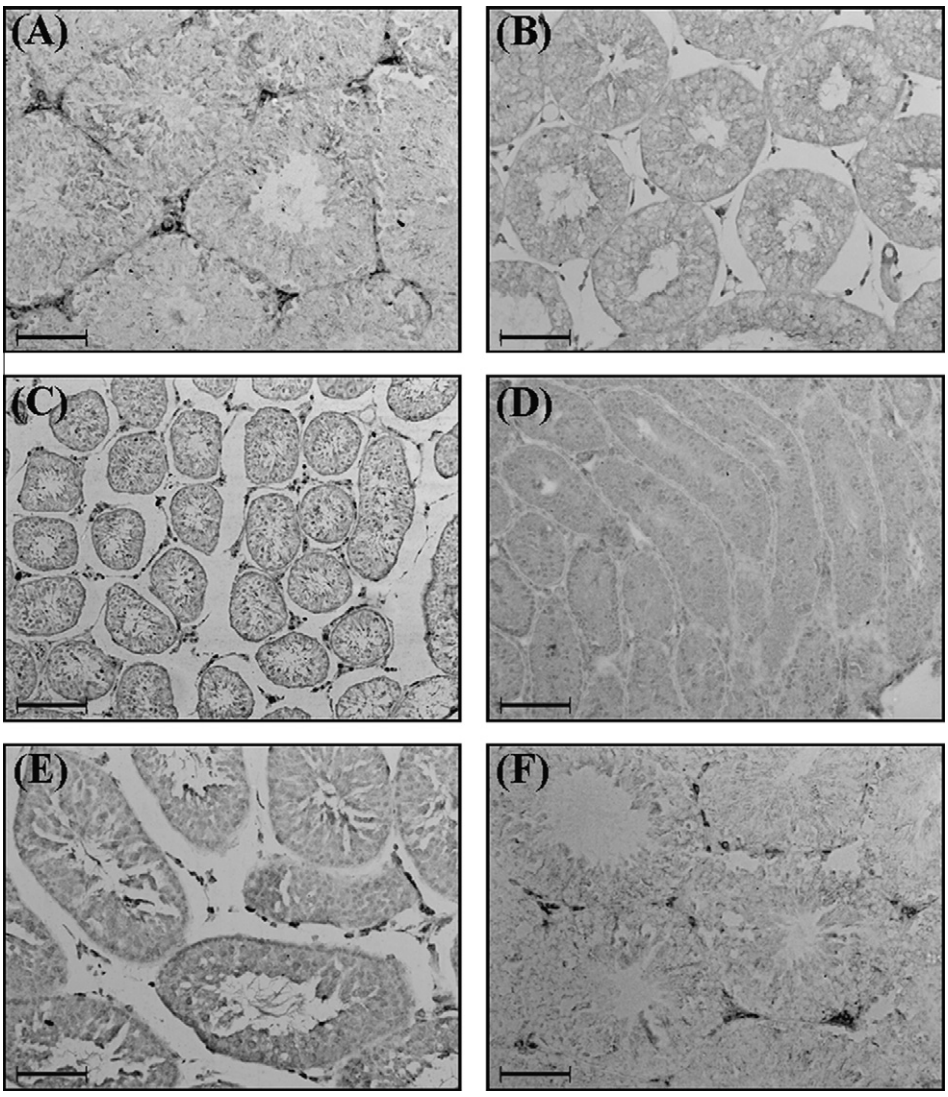

Fig. 1. COX2 immunolocalization in hamster testes: Influence of the photoperiod. These representative images show COX2-positive immunostaining in interstitial cells with the characteristic punctuate chromatin pattern of Leydig cells from adult hamsters kept under a LD photoperiod (14 h light, 10 h darkness) (A) or transferred to a SD photoperiod (6 h light, 18 h darkness) for 8 (B), 12 (C), 16 (D), 22 (E) or 28 weeks (F). Note that only a few cells are COX2-immunoreactive in testes from adult hamsters exposed to a SD photoperiod for 16 weeks (D). Bar, 100 μm.

scavenger receptor IL-1RII in hamster Leydig cells (Fig. 6A). This cytokine markedly stimulated protein COX2 expression. This stimulatory effect of IL-1β on COX2 expression was prevented by the addition of the P38-MAPK inhibitor SB203580 and the JAK2 inhibitor AG490 (Fig. 6B).

On the other hand, PRL significantly induced mRNA and protein IL-1β levels (Fig. 6C). The stimulatory effect of PRL on COX2 expression was avoided by the addition of a neutralizing antibody that prevents IL-1β activity (Fig. 6D).

3.6. Characterization of hamster pituitary PRL molecular microheterogeneity

By preparative size-exclusion chromatography we observed two main peaks in pituitary extracts obtained from both reproductively active and regressed hamsters. The first eluted peak with a molecular weight (MW) between 12 and 34 KDa represented 70% of the total pituitary PRL. The second peak showed a MW of 100–130 kDa and represented only 15% of the total pituitary PRL (data not shown).

By preparative IEF, we isolated PRL charge analogues within a pH range of 4.01–7.00. In pituitary gland homogenates from reproductively active hamsters, PRL charge analogues with isoelectric points (pI) of 5.16 ± 0.21, 4.61 ± 0.16 and 4.34 ± 0.21 were detected. Conversely, in pituitary gland homogenates obtained from hamsters exposed to a SD photoperiod for 16 weeks, PRL charge analogues with a pI of 5.44 ± 0.41 were observed (Fig. 7).

3.7. Evaluation of PRL charge analogues effect on COX2 expression, PG release to the media, and testosterone production in hamster Leydig cells

Incubation of hamster Leydig cells with more acidic PRL charge analogues isolated within a pH range of 4.34–4.74, strongly induced protein COX2 expression (Fig. 8A). In contrast, less acidic PRL charge analogues isolated within a pH range of 5.01–5.59 had no effect (Fig. 8A).

The stimulatory effect of the more acidic PRL charge analogues on COX2 expression was prevented by the addition of the P38-MAPK inhibitor SB203580 and the JAK2 inhibitor AG490 (Fig. 8A).

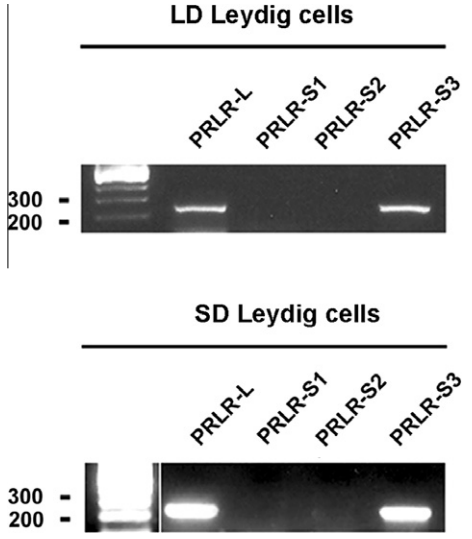

Fig. 2. Identification of PRL receptor splice forms in hamster Leydig cells. mRNA expression of the long (PRLR-L), short 1 (PRLR-S1), short 2 (PRLR-S2), and short 3 (PRLR-S3) PRL receptor splice forms was determined by RT-PCR. Leydig cells were isolated from six adult hamsters kept under a LD photoperiod (14 h light, 10 h darkness) or exposed to a SD photoperiod (6 h light, 18 h darkness) for 16 weeks.

PGD_2 and $PGF_{2\alpha}$ production in Leydig cells purified from adult hamsters kept under a LD photoperiod was markedly stimulated by PRL charge analogues isolated within a pH range of 4.34–4.74. However, less acidic PRL charge analogues isolated within a pH range of 5.01–5.59 did not alter PG secretion to the incubation media (Fig. 8B).

On the other hand, testosterone production was significantly higher in hamster Leydig cells incubated in the presence of 10 ng/ml more acidic PRL charge analogues (10.54 ± 0.87 fmol/10^6 cells, mean ± SEM) than control cells under basal conditions (7.35 ± 0.21 fmol/10^6 cells, $P < 0.05$).

More acidic PRL charge analogues significantly stimulated phosphorylation of STAT5a/b (Fig. 8C) and P38 (Fig. 8D). The stimulatory effect of PRL charge analogues isolated within a pH range of 4.34–4.74 on P38 phosphorylation was prevented by the addition of SB203580 to the incubation media (Fig. 8D).

4. Discussion

This study provides novel evidence for a direct involvement of PRL and its carbohydrate moiety in the regulation of COX2 expression and consequently of PG synthesis in Leydig cells of Syrian hamsters during the photoperiodic regression–recrudescence transition. Our results indicate that COX2 is expressed mainly in Leydig cells of reproductively active adult hamsters kept under a LD photoperiod or exposed to a SD photoperiod for an extended period of time (recrudescence phase). In these animals, considerable circulating and pituitary levels of PRL were detected. In contrast, COX2 was weakly expressed in Leydig cells of photoperiodically regressed adult hamsters showing low pituitary and serum levels of PRL. Furthermore, a physiological dose of homologous PRL significantly induced COX2 expression and PG production in Leydig cells from hamsters exposed to a LD photoperiod or a SD photoperiod for 16 weeks through a mechanism that involves IL-1β, P38 phosphorylation and the activation of JAK2.

We also detected the existence of different PRL charge analogues in pituitaries of reproductive active and regressed hamsters. PRL charge analogues showing a pI of 4.61, 4.34 and 5.16 were found in pituitaries of hamsters kept under a LD photoperiod. In contrast, mainly less acidic PRL charge analogues (pI of 5.44) were present in pituitaries isolated from hamsters exposed to a SD photoperiod for 16 weeks. While acidic PRL charge analogues strongly induced COX2, less acidic PRL charge analogues had no effect, suggesting that PRL molecular microheterogeneity might be responsible for the photoperiodic variations of COX2 expression in hamster Leydig cells.

The possible actions of PGs on testicular activity are not yet well understood. However, recent studies have shown that COX2 might play a role in the regulation of testicular function mainly in pathology, aging, and fertility disorders. COX is induced in human testicular cancer (Hase et al., 2003) and represents a potential key factor in the age-related reduction of testosterone production in rats (Wang et al., 2005). We have previously found that COX2 is not detected by immunohistochemistry in human testicular biopsies with no evident morphological alterations or abnormalities, but is expressed in some interstitial cells (presumably Leydig cells and macrophages) in testes of men suffering from germ cell arrest or Sertoli cell only-syndrome (Frungieri et al., 2002; Matzkin et al., 2010). In addition to their participation in the control of testicular activity under pathological conditions, aging and infertility, COX2 and PGs might play a critical role in the physiological regulation of the testicular function in seasonal breeding mammals. We have recently found COX2 immunostaining in Leydig cells of the reproductively active seasonal breeder Syrian hamster, and established the involvement of testosterone in the regulation of testicular COX2 expression and consequently of PG synthesis (Frungieri et al., 2006; Matzkin et al., 2009).

The first aim of the current study was to further investigate the hormonal regulation of COX2 expression in hamster testes. To this end, we initially evaluated testicular expression of COX2 during the exposure of young adult animals to an inhibitory photoperiod (6 h light:18 h darkness) from 8 to 28 weeks in order to achieve the maximum testicular regression (16 weeks), followed by the spontaneous gonadal recrudescence (22–28 weeks).

By immunohistochemistry, we found that adult hamsters exposed to a SD photoperiod for 16 weeks with low serum and pituitary levels of PRL, weakly express COX2 in testes. In contrast, adult reproductively active hamsters kept under a LD photoperiod or exposed to a SD photoperiod for 22–28 weeks (recrudescence phase) with considerable circulating and pituitary levels of PRL markedly expressed COX2 in Leydig cells. Furthermore, a positive correlation between pituitary and serum PRL levels and COX2 expression in hamster Leydig cells along the photoperiodic-induced inactive–active transition was observed.

Although immunohistochemical analyses showed the expression of COX2 in the cytoplasm of interstitial cells with the characteristic punctuate chromatin pattern of Leydig cells, other testicular population cells (e.g. macrophages and mast cells) might also express COX2. Thus, for our PCR and immunoblotting studies, we purified Leydig cells from testes of reproductively active and regressed hamsters using a discontinuous Percoll density gradient. Cell preparation was enriched in hamster Leydig cells (87–90%). Less than 0.006% of the contaminating cells were macrophages, whereas no mast cells were detected. Even though mast cells are found in the interstitial compartment and the tubular wall in human testes (Meineke et al., 2000), the same does not apply to the hamster testis where mast cells are located only in the capsule (Frungieri et al. 1999) which is initially removed during the procedure used for Leydig cell purification.

A physiological dose of homologous PRL significantly induced mRNA and protein levels of COX2 in Leydig cells isolated from both active and photoperiodically regressed hamsters. Thus, Leydig cells purified from testes of reproductively inactive animals seem to maintain the *in vitro* capability to induce COX2 expression in response to PRL, despite the significant fall in the serum levels of this hormone and the testicular content of PRL receptors observed in

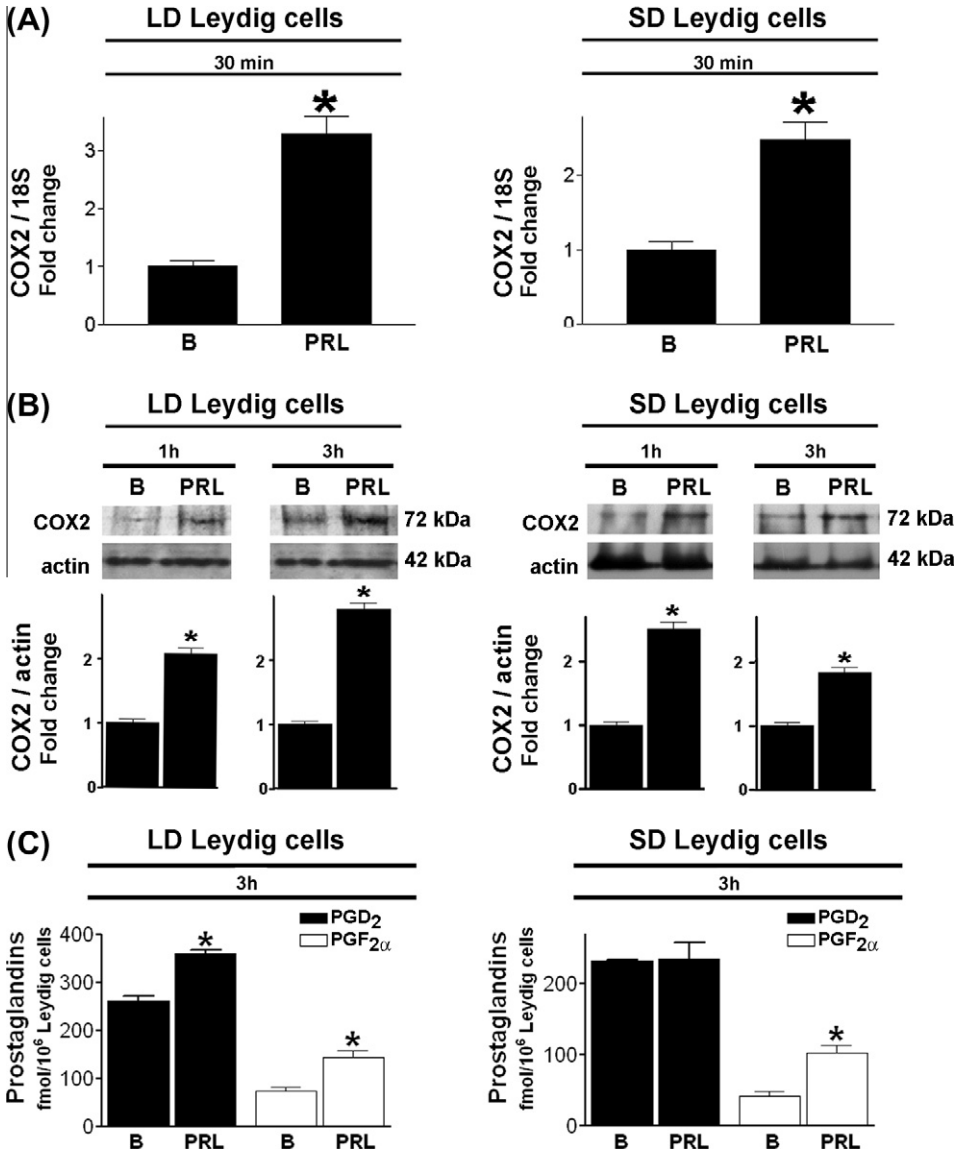

Fig. 3. PRL induction of COX2 expression and PG production in hamster Leydig cells. Leydig cells were incubated in M199 medium for 30 min (A), 1 h (B), and 3 h (B and C) in the presence or absence of homologous PRL (10 ng/ml). For each experiment, cells were isolated from six adult hamsters kept under a LD photoperiod (14 h light, 10 h darkness) or exposed to a SD photoperiod (6 h light, 18 h darkness) for 16 weeks. (A) COX2 mRNA levels were determined by RT-qPCR. 18S rRNA was chosen as the housekeeping gene. The fold change of mRNA COX2 expression was determined as described in Section 2. Bar plot graphs represent the mean ± SEM and depict the fold change of mRNA COX2 expression obtained in three independent experiments. (B) COX2 (72 KDa) and actin (42 KDa) protein levels were determined by immunoblotting. These representative immunoblots show results obtained from one of three experiments performed in different cell preparations that showed comparable results. Bar plot graphs represent the mean ± SEM and depict the quantification by densitometry of the bands obtained in three independent experiments. Results are expressed as fold change relative to the control (basal conditions), which was assigned a value of 1, and normalized to actin. (C) PGD_2 and $PGF_{2\alpha}$ levels in the incubation media were determined by immunoassay. Bar plot graphs represent the mean ± SEM from one of two experiments performed in different cell preparations (six to eight replicates per experiment) that showed comparable results. $^{*}p < 0.05$; Student's t test.

regressed hamsters (Bartke, 1985). Whether receptors are more sensitive to PRL or only a few number of them are required to obtain a maximal response during the quiescence phase remains to be further investigated.

PRL also stimulated PG release from isolated hamster Leydig cells. In agreement with our findings in hamster testes, PRL regulation of PG production has also been described in rodent liver, ovary, uterus and carrageenan-induced pleurisy (Dave et al., 1982; Meli et al., 1993; Prigent-Tessier et al., 1996; Polisseni et al., 2005).

Furthermore, both PRL and PGD_2 stimulated testosterone production in hamster Leydig cells. These results may be of special relevance during the recrudescence period. In this phase, pituitary and circulating PRL levels as well as COX2 expression in hamster Leydig cells are restored, and could contribute to recover testicular and serum testosterone levels.

Although $PGF_{2\alpha}$ had no effect under basal conditions, we have previously described an inhibitory role of $PGF_{2\alpha}$ on testosterone release after hCG stimulation (Frungieri et al., 2006).

In addition to the androgen pathway, estrogen production is also regulated by PRL and PGs. It has been previously described that the addition of PRL to the incubation media of rat Leydig cells regulates basal and LH-stimulated estradiol production (Papadopoulos et al., 1986; Maran et al., 2001). COX2 and PGE2 up-regulates aromatase activity in immature mouse Leydig cells (Sirianni et al., 2009). In addition, a pilot study, applying PCR, showed us a significant inhibition of aromatase expression upon treatment of hCG-induced hamster Leydig cells with $PGF_{2\alpha}$ (data not shown).

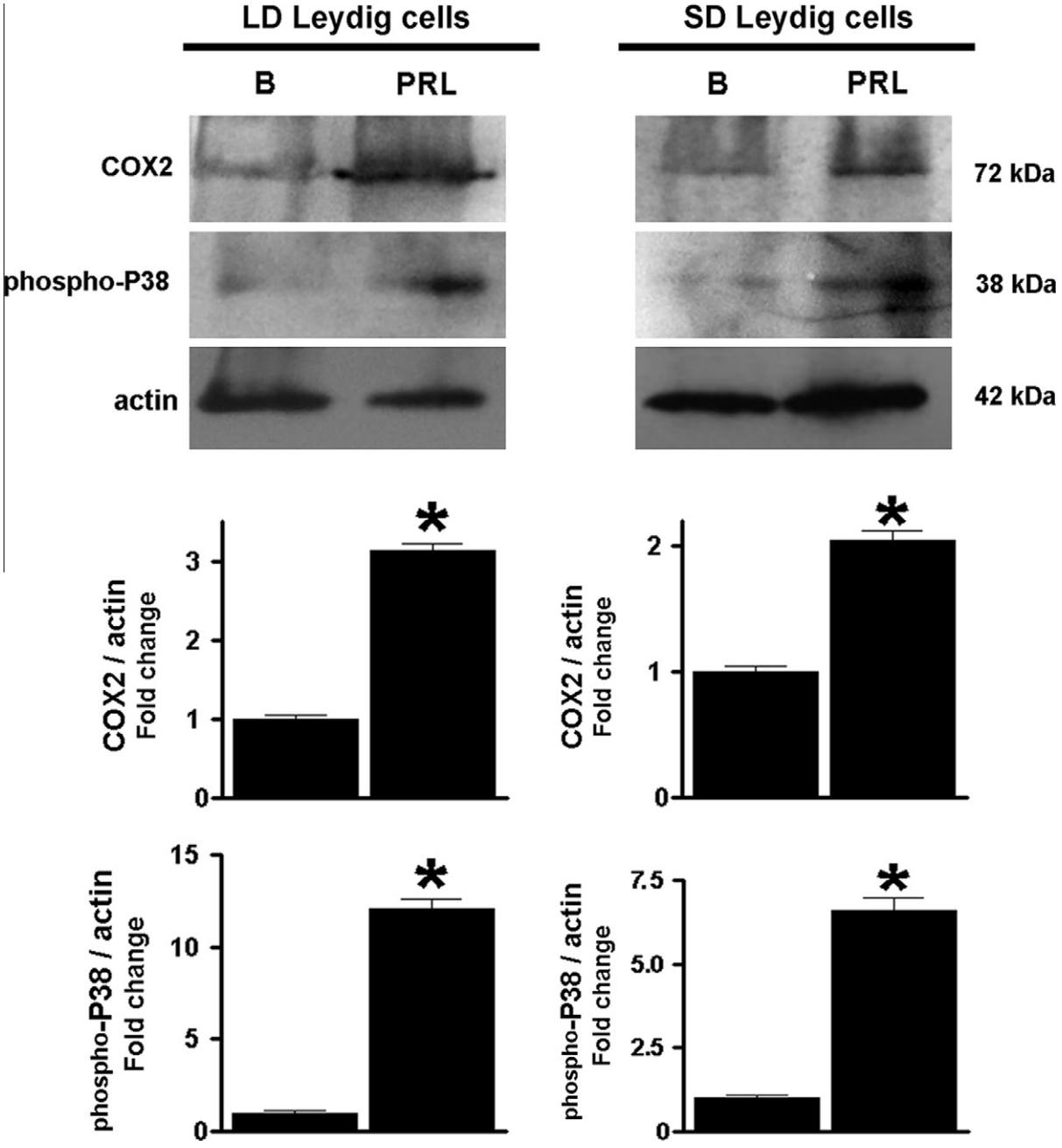

Fig. 4. Effect of PRL on P38 phosphorylation in hamster Leydig cells. Leydig cells were incubated in M199 medium for 1 h in the presence or absence of homologous PRL (10 ng/ml). COX2 (72 kDa), phospho-P38 (38 kDa) and actin (42 kDa) protein levels were determined by immunoblotting. These representative immunoblots show results obtained from one of three experiments performed in different cell preparations that showed comparable results. For each experiment, cells were isolated from six adult hamsters kept under a LD photoperiod (14 h light, 10 h darkness) or exposed to a SD photoperiod (6 h light, 18 h darkness) for 16 weeks. Bar plot graphs represent the mean ± SEM and depict the quantification by densitometry of the bands obtained in three independent experiments. Results are expressed as fold change relative to the control (basal conditions), which was assigned a value of 1, and normalized to actin. *$p < 0.05$; Student's t test.

Multiple isoforms of PRL receptors resulting from alternative splicing of the primary transcript have been identified in a number of cells and tissues of adult mammals (Boutin et al., 1989; Davis and Linzer, 1989; Shirota et al., 1990; Ali et al., 1991; Nagano et al., 1995). These different PRL receptor isoforms differ in the length and composition of their cytoplasmic domain (for a review see Goffin and Kelly, 1996). In rats, the PRL receptor isoforms contain 291 (short), 393 (intermediate), or 591 (long) amino acids, whereas, in mice, one long and three short isoforms have been described (Davis and Linzer, 1989; Clarke and Linzer, 1993; Ling et al., 2000). By RT-PCR, we identified a long and a short PRL receptor splice form in Leydig cells from both reproductively active and regressed hamsters. In accordance with our results, previous studies performed in MA-10 mouse Leydig cells described the existence of two PRL receptor isoforms, a high and a low molecular weight form (Weiss-Messer et al., 1998).

These results allow us to confirm the existence of PRL receptors in hamster Leydig cells. Nevertheless, further studies (i.e. competitive binding assays using PRL receptor antagonists) should be performed before affirming that the stimulatory action of PRL on COX2 expression takes place through specific PRL receptors.

Dimerization of the long receptor isoform leads to transphosphorylation and activation of JAK2, followed by phosphorylation of the signal transducer and activator of transcription 5 (STAT5).

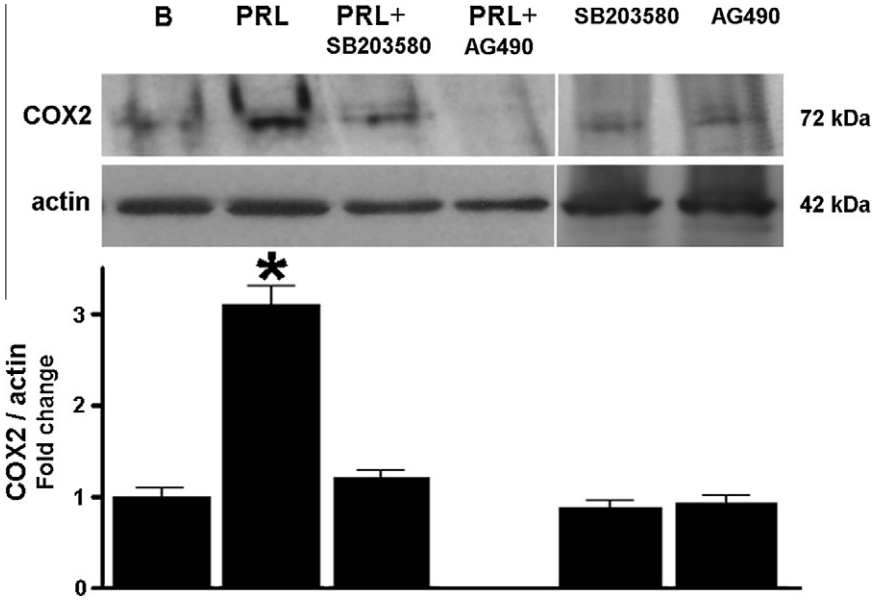

Fig. 5. PRL induction of protein COX2 expression in hamster Leydig cells. Effect of SB203580 and AG490. Leydig cells were incubated in M199 medium for 1 h in the presence or absence of homologous PRL (10 ng/ml), either with or without the P38-MAPK inhibitor SB203580 (10 μM) and the JAK2 inhibitor AG490 (10 μM). COX2 (72 kDa) and actin (42 kDa) protein levels were determined by immunoblotting. This representative immunoblot shows results obtained from one of three experiments performed in different cell preparations that showed comparable results. For each experiment, cells were isolated from six adult hamsters kept under a LD photoperiod (14 h light, 10 h darkness). Bar plot graphs represent the mean ± SEM and depict the quantification by densitometry of the bands obtained in three independent experiments. Results are expressed as fold change relative to the control (basal conditions), which was assigned a value of 1, and normalized to actin. $^{*}p < 0.05$; Student-Newman–Keuls test.

Phosphorylated STAT5 translocates to the nucleus and affects the transcription of genes with STAT5 recognition sites in their promoter (Bole-Feysot et al., 1998). In this context, STAT5 binding sites on the COX2 promoter have been previously described (Yamaoka et al., 1998; Koon et al., 2006). Although both the long and short receptor forms have been shown to dimerize and activate JAK2, only the long PRL receptor splice form is able to activate STAT5 (Ling et al., 2000; Swaminathan et al., 2008). Incubations of hamster Leydig cells with tyrphostin AG-490, a specific and potent JAK2 tyrosine kinase inhibitor, prevented the stimulatory effect of PRL on COX2. In addition to its action on JAK2/STAT5, PRL also induces phosphorylation of ERK 44/42, P38 and JNK in different experimental systems (Olazabal et al., 2000; Cave et al., 2001; Dogusan et al., 2001; Gubbay et al., 2002). Our results indicate that PRL stimulates phosphorylation of P38 in Leydig cells from both reproductively active and regressed hamsters. Furthermore, in the presence of SB203580, a specific P38-MAPK inhibitor, PRL did not alter COX2 expression. No effect of PRL on ERK 44/42 and JNK phosphorylation was observed. These results suggest that PRL induces COX2/PGs through a mechanism that involves JAK2/STAT5 and P38-MAPK activation.

We have previously described a positive correlation between COX-2 expression and IL-1β levels in testes of infertile patients as well as the induction of COX-2 by IL-1β in mouse Leydig cells (TM3) and human macrophages (THP-1) (Matzkin et al., 2010). In this study, a potential action of IL-1β on COX2 expression in hamster testes has been addressed. We found expression of IL-1β, its receptor IL-1RI, the antagonist receptor Ra and the scavenger receptor IL-1RII in hamster Leydig cells. Furthermore, IL-1β significantly induced protein COX2 expression. This effect was prevented by tyrphostin AG-490 and SB203580 suggesting that IL-1β induces COX2 through activation of JAK2/STAT5 and P38-MAPK.

Taking into consideration recent reports describing a stimulatory action of PRL on IL-1β production through a JAK2-dependent mechanism in murine peritoneal macrophages (Sodhi and Tripathi, 2008; Tripathi and Sodhi, 2008), we evaluated a potential effect of this hormone on IL-1β expression in hamster Leydig cells. PRL significantly induced mRNA and protein levels of this cytokine. Therefore, for a better understanding of the mechanisms underlying the action of PRL on testicular COX2 expression, we used a neutralizing antibody to prevent IL-1β activity. Incubations of hamster Leydig cells with this antibody avoided PRL-induced COX2 expression. These findings indicate that PRL action could result from its stimulatory role in IL-1β synthesis and not from a direct mechanism on COX2/PGs.

PRL microheterogeneity has been characterized in many mammals, including humans. Regarding the Syrian hamster, we have previously described that the exposure to an inhibitory photoperiod leads to immunohistochemical, ultrastructural, and biochemical changes in pituitary lactotrophes (Cónsole et al., 2002). Moreover, individual lactotroph cells exhibit a heterogeneous response to changes in the photoperiod. A similar proportion of par distalis cells express low levels of PRL mRNA in both hamsters kept under a LD photoperiod and animals exposed to a SD photoperiod. Nevertheless, the remaining cells that express PRL mRNA exhibit a significant increase in gene expression under a LD photoperiod (Johnston et al., 2003; Johnston, 2004).

In order to analyze PRL microheterogeneity and its potential role in testicular COX2 expression, we studied the molecular structure of pituitary PRL according to its weight and charge. In pituitaries obtained from both reproductively active and regressed hamsters, 70% of the total PRL consisted of a single monomeric form. Nevertheless, variants of higher MW that could result from PRL polymerization or aggregation with binding proteins, such as immunoglobulins, were also detected. In general, these high molecular weight forms have reduced biological activity (Sinha, 1995). Interestingly, different PRL charge analogues were isolated within a pH range of 4.2–5.5 from pituitary gland homogenates of reproductively active and regressed hamsters. In accordance with our results in hamsters, Ishikawa et al. (1985) have previously described that the main PRL charge variants detected by pI in male rat pituitaries are found within a pH range of 4.5–5.4.

Unfortunately, serum PRL charge analogues in reproductively active and regressed hamsters could not be identified due to the

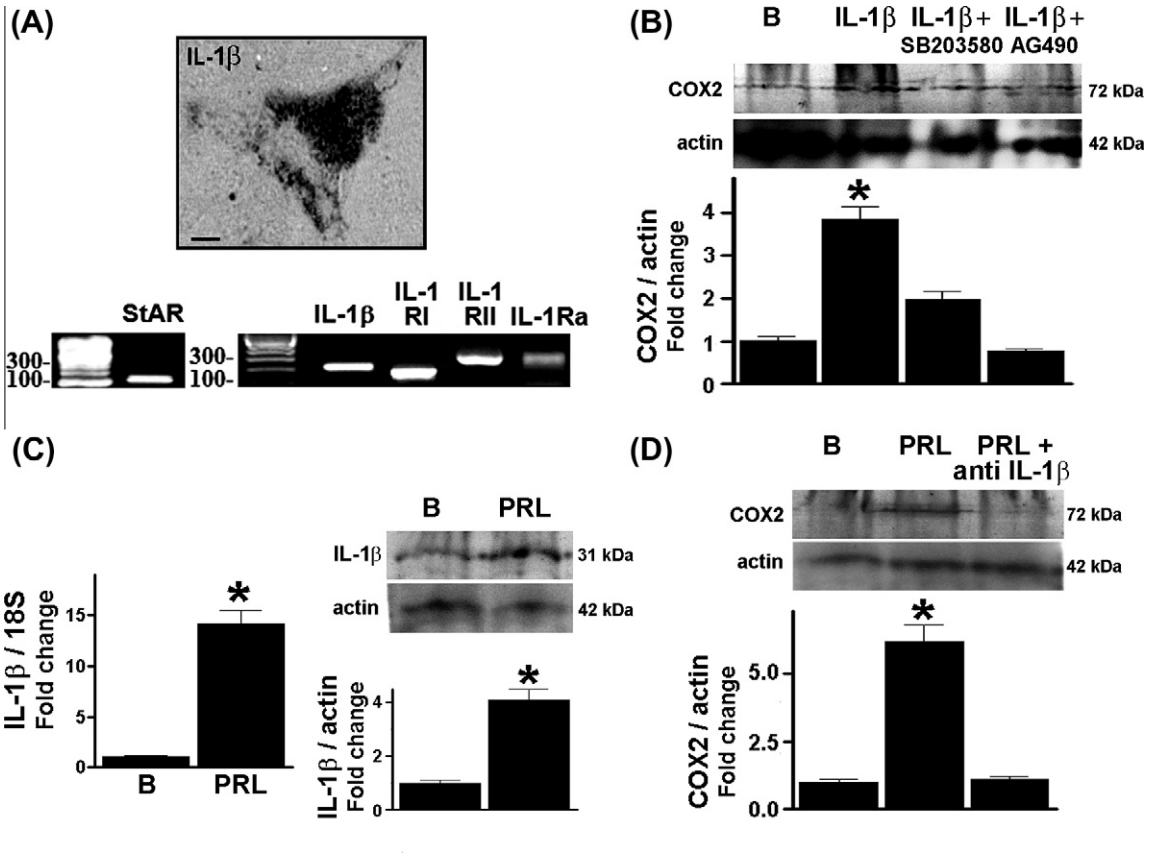

Fig. 6. Role of IL-1β in the PRL-induced expression of COX2 in hamster Leydig cells. Leydig cells isolated from six adult hamsters kept under a LD photoperiod (14 h light, 10 h darkness) were used for characterization of the IL-1β system by immunohistochemistry and RT-PCR (A), or incubated in M199 medium for 1 h in the presence of the following chemicals: IL-1β (10 ng/ml) (B), homologous PRL (10 ng/ml) (C and D), the P38-MAPK inhibitor SB203580 (10 μM) (B), the JAK2 inhibitor AG490 (10 μM) (B), or a neutralizing antibody that prevents IL-1β activity (25 μg/ml) (D). (A) The representative image shows IL-1β-positive immunostaining in interstitial cells from adult hamsters. Bar, 20 μm. mRNA expression levels of StAR, IL-1β, its receptor IL-1RI, the antagonist receptor Ra and the scavenger receptor IL-1RII were determined by RT-PCR. (B) and (D) COX2 (72 kDa) and actin (42 kDa) protein levels were determined by immunoblotting. (C) IL-1β mRNA levels were determined by RT-qPCR. 18S rRNA was chosen as the housekeeping gene. The fold change of mRNA IL-1β expression was determined as described in Section 2. Bar plot graphs represent the mean ± SEM and depict the fold change of mRNA IL-1β expression obtained in three independent experiments. IL-1β (31 kDa) and actin (42 kDa) protein levels were determined by immunoblotting. These representative immunoblots show results obtained from one of three experiments performed in different cell preparations that showed comparable results. Bar plot graphs represent the mean ± SEM and depict the quantification by densitometry of the bands obtained in three independent experiments. Results are expressed as fold change relative to the control (basal conditions), which was assigned a value of 1, and normalized to actin. $^{*}p < 0.05$; Student-Newman–Keuls test.

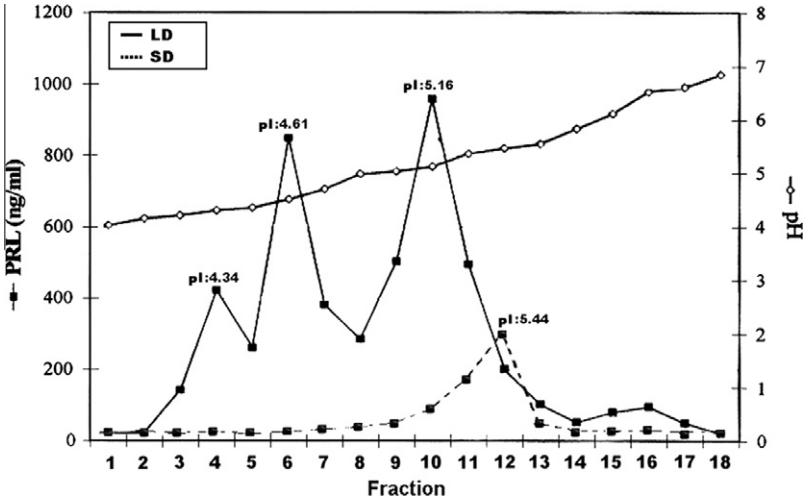

Fig. 7. Characterization of hamster pituitary PRL charge analogues. PRL charge analogues were isolated by preparative IEF within a pH range of 4.01–7.00 (—o—) from adult hamsters kept under a LD photoperiod (14 h light, 10 h darkness) (——) or exposed to a SD photoperiod (6 h light, 18 h darkness) for 16 weeks (- -). Eighteen fractions were collected. After dialysis, PRL levels in each fraction were determined by RIA. The pI of the main PRL charge analogues isolated from pituitaries of reproductively active and regressed hamsters are indicated in the graph.

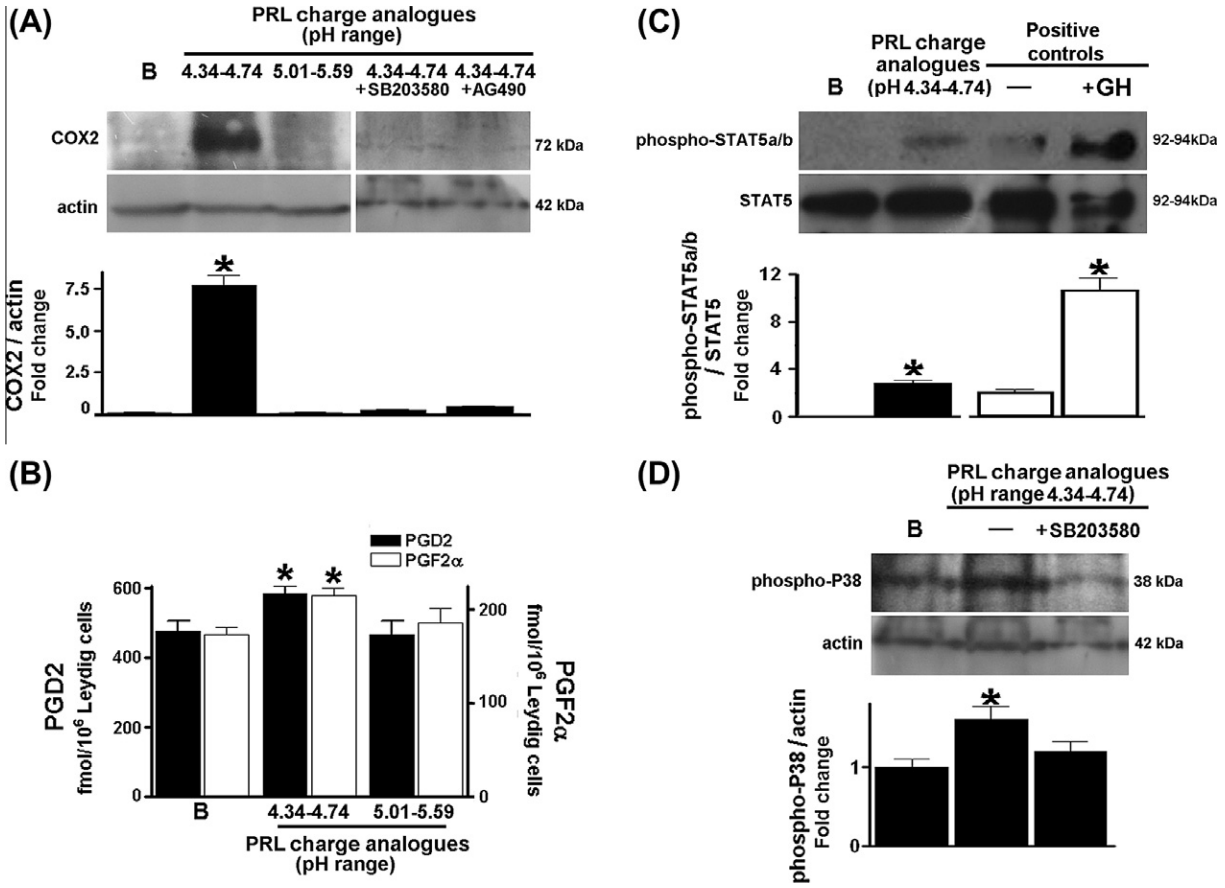

Fig. 8. Effects of PRL charge analogues on protein COX2 expression, PG production, and STAT5a/b and P38 phosphorylation in hamster Leydig cells. For each experiment, Leydig cells were isolated from adult hamsters kept under a LD photoperiod (14 h light, 10 h darkness) and incubated in M199 medium for 1 h (A, C and D) or 3 h (B) in the presence or absence of the following chemicals: more acidic PRL charge analogues (pH range: 4.34–4.74; 10 ng/ml) (A, B, C and D), less acidic PRL charge analogues (pH range: 5.01–5.59; 10 ng/ml) (A and B), the P38-MAPK inhibitor SB203580 (10 μM) (A and D), or the JAK2 inhibitor AG490 (10 μM) (A). (A) COX2 (72 kDa) and actin (42 kDa) protein levels were determined by immunoblotting. (B) PGD_2 and $PGF_{2\alpha}$ levels in the incubation media were determined by immunoassay. Bar plot graph represents the mean ± SEM from one of two experiments performed in different cell preparations (six to eight replicates per experiment) that showed comparable results. (C) Aliquots of lysated hamster Leydig cells containing 800 μg protein were immunoprecipitated with anti-STAT5. For control purposes, normal Swiss-Webster mice were injected i.p. with saline or growth hormone (GH, 2.5 mg/kg body weight), killed after 7.5 min, and livers were removed and homogenized at the ratio 0.1 g/ml in buffer. Solubilized mice livers containing 2 mg protein were then immunoprecipitated with anti-STAT5. Phospho-STAT5a/b (92–94 kDa) and STAT5 (92–94 kDa) protein levels were determined by immunoblotting. (D) phospho-P38 (38 kDa) and actin (42 kDa) protein levels were determined by immunoblotting. These representative immunoblots show results obtained from one of three experiments performed in different cell preparations that showed comparable results. Bar plot graphs represent the mean ± SEM and depict the quantification by densitometry of the bands obtained in three independent experiments. Results are expressed as fold change relative to the control (basal conditions), which was assigned a value of 1, and normalized to actin. $^{*}p < 0.05$; Student-Newman–Keuls test.

low circulating levels of this hormone together with methodological limitations of the IEF technique.

Whereas more acidic pituitary PRL charge analogues isolated within a pH range of 4.34–4.74 strongly induced COX2 expression, PG release to the media and testosterone production in hamster Leydig, less acidic PRL charge analogues isolated within a pH range of 5.01–5.59 had no effect. In the presence of SB203580 and tyrphostin AG-490, more acidic pituitary PRL charge analogues did not alter COX2 expression. Furthermore, PRL charge analogues isolated within a pH range of 4.34–4.74 significantly induced phosphorylation of P38 and STAT5a/b.

These results suggest that the weak expression of COX2 in Leydig cells of regressed hamsters could result not only from the low pituitary and circulating PRL levels but also from the scarcity of more acidic PRL charge analogues which are involved in the induction of COX2/PGs.

In consequence, the biological relevance of the more acidic PRL charge analogues in hamster Leydig cells, might be crucial for a better understanding of the mechanisms underlying the regulation of COX2 expression during the photoperiodic-induced testicular regression–recrudescence transition.

It is known that carbohydrate side-chains and sialylation are essential for biological activity. Although several reports indicate that more acidic/sialylated variants of the pituitary hormones exhibit a lower *in vitro* biological potency than their less acidic counterparts (Cerpa-Poljak et al., 1993; Briski et al., 1996; Zambrano et al. 1996), other authors point out opposite effects (Matzuk et al., 1990; Fares et al., 1992; Lambert et al., 1998). Such discrepancies might be explained by variations in the amount of agonist used, in the density of receptors and/or in the receptor isoforms expressed. In this context, Donadeu and Ascoli (2005) have described different activated signaling pathways and, as a consequence, opposite biological effects triggered by gonadotrophins in granulosa cells expressing a high or a low number of membrane receptors. Furthermore, mutagenesis studies have offered insights into the biology of the prolactin receptor, providing compelling evidence for different isoforms having independent biological activities (Binart et al., 2010).

In summary, our work provides new insights into how PRL modulates COX2 expression in Leydig cells of the Syrian hamster, and points out the biological properties of the different pituitary PRL charge analogues.

Since PGD_2 and $PGF_{2\alpha}$ participate in the modulation of basal and hCG-induced testosterone secretion in hamster Leydig cells (Frungieri et al., 2006), the previously unknown physiological PRL/COX2/PGs system up-regulated during the exposure of hamsters to optimal light conditions for reproduction may serve as a local modulator of the testicular endocrine activity.

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References

Ali, S., Pellegrini, I., Edery, M., Lesueur, L., Paly, J., Djiane, J., Kelly, P.A., 1991. A prolactin-dependent immune cell line (Nb2) expresses a mutant form of prolactin receptor. J. Biol. Chem. 266, 20110–20117.

Ambao, V., Rulli, S.B., Carino, M.H., Cónsole, G., Ulloa-Aguirre, A., Calandra, R.S., Campo, S., 2009. Hormonal regulation of pituitary FSH sialylation in male rats. Mol. Cell. Endocrinol. 309, 39–47.

Bachelot, A., Binart, N., 2007. Reproductive role of prolactin. Reproduction 133, 361–369.

Bartke, A., 1978. Prolactin deficient mice. In: Alexander, N.J. (Ed.), Animals Models for Research on Contraception and Fertility. Hagerston, Harper and Row, pp. 360–365.

Bartke, A., 1985. Male hamster reproductive endocrinology. In: Spiegel, H.I. (Ed.), The Hamster. New York, Plenum, pp. 73–98.

Bartke, A., 1999. Role of growth hormone and prolactin in the control of reproduction: What are we learning from transgenic and knock-out animals? Steroids 64, 598–604.

Bartke, A., Smith, M.S., Dalterio, S., 1979. Reversal of short photoperiod-induced sterility in male hamsters by ectopic pituitary homografts. Int. J. Androl. 2, 257–262.

Ben-Jonathan, N., Lapensee, C.R., Lapensee, E.W., 2008. What can we learn from rodents about prolactin in humans? Endocr. Rev. 29, 1–41.

Ben-Jonathan, N., Mershon, J.L., Allen, D.L., Steinmetz, R.W., 1996. Extrapituitary prolactin: distribution, regulation, functions, and clinical aspects. Endocr. Rev. 17, 639–669.

Bex, F.J., Bartke, A., 1977. Testicular LH binding in the hamster: modification by photoperiod and prolactin. Endocrinology 100, 1223–1226.

Bex, R., Bartke, A., Goldman, B.D., Dalterio, S., 1978. Prolactin, growth hormone, luteinizing hormone receptors, and seasonal changes in testicular activity in the golden hamster. Endocrinology 103, 2069–2080.

Biller, B.M., 1999. Hyperprolactinemia. Int. J. Fertil. Womens Med. 44, 74–77.

Binart, N., Bachelot, A., Bouilly, J., 2010. Impact of prolactin receptor isoforms on reproduction. Trends Endocrin. Met. 21, 362–368.

Bole-Feysot, C., Goffin, V., Edery, M., Binart, N., Kelly, P.A., 1998. Prolactin and its receptor: actions, signal transduction pathways and phenotypes observed in prolactin receptor knockout mice. Endocr. Rev. 19, 225–268.

Boutin, J.M., Edery, M., Shirota, M., Jolicoeur, C., Lesueur, L., Ali, S., Gould, D., Djiane, J., Kelly, P.A., 1989. Identification of a cDNA encoding a long form of prolactin receptor in human hepatoma and breast cancer cells. Mol. Endocrinol. 3, 1455–1461.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Briski, K.P., Swanson, G.N., Sylvester, P.W., 1996. Size and charge heterogeneity of pituitary and plasma prolactin in the male rat. Neuroendocrinology 63, 437–445.

Cave, B.J., Wakerley, J.B., Luckman, S.M., Tortonese, D.J., 2001. Hypothalamic targets for prolactin: assessment of c-Fos induction in tyrosine hydroxylase- and proopiomelanocortin-containing neurones in the rat arcuate nucleus following acute central prolactin administration. Neuroendocrinology 74, 386–395.

Cerpa-Poljak, A., Bishop, L.A., Hort, Y.J., Chin, C.K., DeKroon, R., Mahler, S.M., Smith, G.M., Stuart, M.C., Schofield, P.R., 1993. Isoelectric charge of recombinant human follicle-stimulating hormone isoforms determines receptor affinity and in vitro bioactivity. Endocrinology 132, 351–356.

Clarke, D.L., Linzer, D.I.H., 1993. Changes in prolactin receptor expression during pregnancy in the mouse ovary. Endocrinology 133, 224–232.

Cónsole, G.M., Jurado, S.B., Petruccelli, M., Carino, M., Calandra, R.S., Gómez Dumm, C.L.A., 2002. Influence of photoinhibition on the morphology and function of pituitary lactotropes in male Golden hamsters. Neuroendocrinology 75, 316–325.

Curlewis, J.D., 1992. Seasonal prolactin secretion and its role in seasonal reproduction: a review. Reprod. Fertil. Dev. 4, 1–23.

Czyba, J.C., Cottinet, D., Dams, R., Curé, M., 1971. Effets du sulpiride sur l'appareil genital du Hamster doré mâle en phase d'involution hivernale. CR Seances Soc. Biol. Ses. Fil. 165, 1624–1626.

Dave, R.J., Brown, N.V., Knazek, R.A., 1982. Prolactin modifies the prostaglandins synthesis, prolactin binding and fluidity of mouse liver membranes. Biochem. Biophys. Res. Commun. 108, 193–199.

Davis, J.A., Linzer, D.I.H., 1989. Expression of multiple forms of the prolactin receptor in mouse liver. Mol. Endocrinol. 3, 674–680.

Dogusan, Z., Hooghe, R., Verdood, P., Hooghe-Peters, E.L., 2001. Cytokine-like effects of prolactin in human mononuclear and polymorphonuclear leukocytes. J. Neuroimmunol. 120, 58–66.

Donadeu, F.X., Ascoli, M., 2005. The differential effects of the gonadotropin receptors on aromatase expression in primary cultures of immature rat granulosa cells are highly dependent on the density of receptors expressed and the activation of the inositol phosphate cascade. Endocrinology 146, 3907–3948.

Fares, F.A., Suganuma, N., Nishimori, K., LaPolt, P.S., Hsueh, A.J., Boime, I., 1992. Design of a long-acting follitropin agonist by fusing the C-terminal sequence of the chorionic gonadotropin beta subunit to follitropin beta subunit. Proc. Natl. Acad. Sci. USA 89, 4303–4308.

Freeman, M.E., Kanyicska, B., Lerant, A., Nagy, G., 2000. Prolactin: structure, function, and regulation of secretion. Physiol. Rev. 80, 1523–1631.

Frungieri, M.B., Gonzalez-Calvar, S.I., Calandra, R.S., 1996. Polyamine levels in testes and seminal vesicles from adult golden hamsters during gonadal regression–recrudescence. J. Androl. 17, 683–691.

Frungieri, M.B., Gonzalez-Calvar, S.I., Parborell, F., Albrecht, M., Mayerhofer, A., Calandra, R.S., 2006. Cyclooxygenase-2 (COX-2) and Prostaglandin F2α ($PGF2\alpha$) in Syrian Hamster Leydig Cells: inhibitory role on LH/hCG-Stimulated Testosterone Production. Endocrinology 147, 4476–4485.

Frungieri, M.B., Gonzalez-Calvar, S.I., Rubio, M., Lustig, L., Calandra, R.S., 1999. Serotonin in golden hamster testes: testicular levels, immunolocalization, and role during sexual development and photoperiodic regression–recrudescence transition. Neuroendocrinology 69, 299–308.

Frungieri, M.B., Weidinger, S., Meineke, V., Köhn, F.M., Mayerhofer, A., 2002. Proliferative action of mast-cell tryptase is mediated by PAR2, COX2, prostaglandins, and PPARgamma: possible relevance to human fibrotic disorders. Proc. Natl. Acad. Sci. USA 99, 15072–15077.

Goffin, V., Kelly, P.A., 1996. The prolactin/growth hormone receptor family: structure/function relationships. J. Mammary Gland. Biol. Neopl. 2, 7–17.

Gonzalez, S.I., Chandrashekar, V.J., Shire, J.G., Luthy, I.A., Bartke, A., Calandra, R.S., 1991. Effects of hyperprolactinemia on ornithine decarboxylase activity and polyamine levels in seminal vesicles of genetically prolactin-deficient adult dwarf mice. Biol. Reprod. 44, 321–326.

Gubbay, O., Critchley, H.O.D., Bowen, J.M., King, A., Jabbour, H.N., 2002. Prolactin induces ERK phosphorylation in epithelial and CD56(+) natural killer cells of the human endometrium. J. Clin. Endocr. Metab. 87, 2329–2335.

Hase, T., Yoshimura, R., Matsuyama, M., Kawahito, Y., Wada, S., Tsuchida, K., Sano, H., Nakatani, T., 2003. Cyclooxygenase-1 and -2 in human testicular tumours. Eur. J. Cancer 39, 2043–2049.

Ho, T.W., Leong, F.S., Olaso, C.H., Walker, A.M., 1993. Secretion of specific nonphosphorylated and phosphorylated rat prolactin isoforms at different stages of the estrous cycle. Neuroendocrinology 58, 160–165.

Ishikawa, J., Wakabayashi, K., Igarashi, M., 1985. Charge heterogeneity of rat pituitary prolactin in relation to the estrous cycle, gonadectomy, and estrogen treatment. Endocrinol. Jpn. 32, 725–739.

Johnston, J.D., 2004. Photoperiodic regulation of prolactin secretion: changes in intra-pituitary signaling and lactotroph heterogeneity. J. Endocrinol. 180, 351–356.

Johnston, J.D., Stirland, J.A., White, M.R.H., Davis, J.R.E., Loudon, A.S.I., 2003. Heterogeneous regulation of individual lactotroph cells by photoperiod in the Syrian hamster (*Mesocricetus auratus*). Gen. Comp. Endocrinol. 134, 182–186.

Koon, H.-W., Zhao, D., Zhan, Y., Rhee, S.H., Moyer, M.P., Pothoulakis, Ch., 2006. Substance P Stimulates Cyclooxygenase-2 and Prostaglandin E_2 Expression through JAK-STAT Activation in Human Colonic Epithelial Cells. J. Immunol. 176, 5050–5059.

Lambert, A., Talbot, J.A., Anobile, C.J., Robertson, W.R., 1998. Gonadotrophin heterogeneity and biopotency: implications for assisted reproduction. Mol. Hum. Reprod. 4, 619–629.

Levy, H., Deane, H.W., Rubin, B.L., 1959. Visualization of steroid 3beta-o1-dehydrogenase activity in tissues of intact and hypophysectomized rats. Endocrinology 65, 932–943.

Ling, C., Hellgren, G., Gebre-Medhin, M., Dillner, K., Wennbo, H., Carlsson, B., Billig, H., 2000. Prolactin (PRL) receptor gene expression in mouse adipose tissue: increases during lactation and in PRL-Transgenic mice. Endocrinology 141, 3564–3572.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Maran, R.R., Arunakaran, J., Aruldhas, M.M., 2001. Prolactin and Leydig cells: biphasic effects of prolactin on LH-, T3- and GH-induced testosterone/oestradiol secretion by Leydig cells in pubertal rats. Int. J. Androl. 24, 48–55.

Matzkin, M.E., Gonzalez-Calvar, S.I., Mayerhofer, A., Calandra, R.S., Frungieri, M.B., 2009. Testosterone induction of prostaglandin-endoperoxide synthase 2 expression and prostaglandin $F_{2\alpha}$ production in hamster Leydig cells. Reproduction 138, 163–175.

Matzkin, M.E., Mayerhofer, A., Rossi, S.P., Gonzalez, B., Gonzalez, C.R., Gonzalez-Calvar, S.I., Terradas, C., Ponzio, R., Puigdomenech, E., Levalle, O., Calandra, R.S., Frungieri, M.B., 2010. Cyclooxygenase-2 (COX-2) in testes of infertile men: evidence for the induction of prostaglandins (PGs) synthesis by interleukin-1β (IL-1β). Fertil. Steril. 94, 1933–1936.

Matzuk, M.M., Hsueh, A.J.W., Lapolt, P., Tsafriri, A., Keener, J.L., Boime, I., 1990. The biological role of the carboxyl-terminal extension of human chorionic gonadotrophin beta subunit. Endocrinology 126, 376–383.

Meineke, V., Frungieri, M.B., Jessberger, B., Vogt, H., Mayerhofer, A., 2000. Human testicular mast cells contain tryptase: increased mast cell number and altered distribution in the testes of infertile men. Fertil. Steril. 74, 239–244.

Meli, R., Gualillo, O., Raso, G.M., di Carlo, R., 1993. Further evidence for the involvement of prolactin in the inflammatory response. Life Sci. 53, 105–110.

Nagano, M., Chastre, E., Choquet, A., Bara, J., Gespach, C., Kelly, P.A., 1995. Expression of prolactin and growth hormone receptor genes and their glycoforms in the gastrointestinal tract. Am. J. Physiol. 268, G431–G442.

Olazabal, I., Muñoz, J., Ogueta, S., Obregón, E., García Ruiz, J.P., 2000. Prolactin (PRL)-PRL receptor system increases cell proliferation involving JNK (c-Jun amino terminal kinase) and AP-1 activation: inhibition by glucocorticoids. Mol. Endocrinol. 14, 564–575.

Papadopoulos, V., Drosdowsky, M.A., Carreau, S., 1986. In vitro effects of prolactin and dexamethasone on rat Leydig cell aromatase activity. Andrologia 18, 79–83.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acid Res. 29, e45.

Polisseni, F., Faletti, A.G., Pereira, V.M., Reis, A.M., Camargos, A.F., Reis, F.M., 2005. Prolactin inhibits oocyte release after gonadotropin stimulation in the rat: putative mechanism involving ovarian production of beta-endorphin and prostaglandin. Fertil. Steril. 83, 1119–1124.

Prigent-Tessier, A., Pageaux, J.F., Fayark, J.M., Lagarde, M., Laugier, C., Cohen, H., 1996. Prolactin up-regulates prostaglandin E2 production through increased expression of pancreatic-type phospholipase A2 (type I) and prostaglandin G/H synthase 2 in uterine cells. Mol. Cell. Endocrinol. 122, 101–108.

Reiter, J.R., Vaughan, M.K., Blask, D.E., Johnson, L.Y., 1975. Pineal methoxyindoles: new evidence concerning their function in the control of pineal-mediated changes in the reproductive physiology of male golden hamsters. Endocrinology 96, 206–213.

Schell, C., Frungieri, M.B., Albrecht, M., Gonzalez-Calvar, S.I., Kohn, F.M., Calandra, R.S., Mayerhofer, A., 2007. A prostaglandin D2 system in the human testis. Fertil. Steril. 88, 233–236.

Shirota, M., Banville, D., Ali, S., Jolicoeur, C., Boutin, J.-M., Edery, M., Djiane, J., Kelly, P.A., 1990. Expression of two forms of prolactin receptor in rat ovary and liver. Mol. Endocrinol. 4, 1136–1143.

Sinha, Y.N., 1992. Prolactin variants. Trends Endocrinol. Metab. 3, 100–106.

Sinha, Y.N., 1995. Structural variants of prolactin: occurrence and physiological significance. Endocr. Rev. 16, 354–369.

Sirianni, R., Chimento, A., De Luca, A., Zolea, F., Carpino, A., Rago, V., Maggiolini, M., Andò, S., Pezzi, V., 2009. Inhibition of cyclooxygenase-2 down-regulates aromatase activity and decreases proliferation of Leydig tumor cells. J. Biol. Chem. 284, 28905–28916.

Sodhi, A., Tripathi, A., 2008. Prolactin induced production of cytokines in macrophages involves Ca++ and p42/44 MAP kinase signaling pathway. Growth Factors 26, 212–219.

Steger, R.W., Chandrashekar, V., Zhao, W., Bartke, A., Horseman, N.D., 1998. Neuroendocrine and reproductive functions in male mice with targeted disruption of the prolactin gene. Endocrinology 139, 3691–3695.

Swaminathan, G., Varghese, B., Fuchs, S.Y., 2008. Regulation of prolactin receptor levels and activity in breast cancer. J. Mammary Gland. Biol. Neopl. 13, 81–91.

Tripathi, A., Sodhi, A., 2008. Prolactin-induced production of cytokines in macrophages in vitro involves JAK/STAT and JNK MAPK pathways. Int. Immunol. 20, 327–336.

Wang, X., Shen, C.-L., Dyson, M.T., Eirmerl, S., Orly, J., Hutson, J.C., Stocco, D.M., 2005. Cyclooxygenase-2 regulation of the age-related decline in testosterone biosynthesis. Endocrinology 146, 4202–4208.

Weiss-Messer, E., Ber, R., Amit, T., Barkey, R.J., 1998. Characterization and regulation of prolactin receptors in MA-10 Leydig cells. Mol. Cell. Endocrinol. 143, 53–64.

Yamaoka, K., Otsuka, T., Niiro, H., Arinobu, Y., Niho, Y., Hamasaki, N., Izuhara, K., 1998. Activation of STAT5 by lipopolysaccharide through granulocyte-macrophage colony-stimulating factor production in human monocytes. J. Immunol. 160, 838–845.

Zambrano, E., Barrios-de-Tomasi, J., Cárdenas, M., Ulloa-Aguirre, A., 1996. Studies on the relative in-vitro biological potency of the naturally-occurring isoforms of intrapituitary follicle stimulating hormone. Mol. Hum. Reprod. 2, 563–571.