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Testosterone induction of prostaglandin-endoperoxide synthase 2 expression and prostaglandin $F_{2\alpha}$ production in hamster Leydig cells

María E Matzkin[1,2], Silvia I Gonzalez-Calvar[1,2], Artur Mayerhofer[3], Ricardo S Calandra[1,4] and Mónica B Frungieri[1,2]

[1]*Laboratorio de Esteroides, Instituto de Biología y Medicina Experimental, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Vuelta de Obligado 2490, Buenos Aires 1428, Argentina,* [2]*Departamento de Bioquímica Humana, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires 1121, Argentina,* [3]*Institut für Zellbiologie, Universität München, München D-80802, Germany and* [4]*Instituto Multidisciplinario de Biología Celular, La Plata 1900, Argentina*

Correspondence should be addressed to M B Frungieri at Laboratorio de Esteroides, Instituto de Biología y Medicina Experimental, CONICET, Vuelta de Obligado 2490, Buenos Aires 1428, Argentina; Email: mfrung@dna.uba.ar

Abstract

We have previously observed expression of prostaglandin-endoperoxide synthase 2 (PTGS2), the key enzyme in the biosynthesis of prostaglandins (PGs), in reproductively active Syrian hamster Leydig cells, and reported an inhibitory role of $PGF_{2\alpha}$ on hamster testicular steroidogenesis. In this study, we further investigated PTGS2 expression in hamster Leydig cells during sexual development and photoperiodic gonadal regression. Since PTGS2 is mostly expressed in pubertal and reproductively active adult hamsters with high circulating levels of LH and androgens, we studied the role of these hormones in the regulation/maintenance of testicular PTGS2/$PGF_{2\alpha}$. In active hamster Leydig cells, LH/hCG and testosterone induced PTGS2 and $PGF_{2\alpha}$ production, and their actions were abolished by the antiandrogen bicalutamide (Bi). These results indicate that LH does not exert a direct effect on PG synthesis. Testosterone also stimulated phosphorylation of the mitogen-activated protein kinase isoforms 3/1 (MAPK3/1) within minutes and hours, but the testosterone metabolite dihydrotestosterone had no effect on PTGS2 and MAPK3/1. Because Bi and U0126, an inhibitor of the MAP kinase kinases 1 and 2 (MAP2K1/2), abolished testosterone actions on MAPK3/1 and PTGS2, our studies suggest that testosterone directly induces PTGS2/$PGF_{2\alpha}$ in hamster Leydig cells via androgen receptors and a non-classical mechanism that involves MAPK3/1 activation. Since $PGF_{2\alpha}$ inhibits testosterone production, it might imply the existence of a regulatory loop that is setting a brake on steroidogenesis. Thus, the androgen environment might be crucial for the regulation of testicular PG production at least during sexual development and photoperiodic variations in hamsters.

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Introduction

Prostaglandins (PGs) are bioactive substances derived from arachidonic acid by the action of the prostaglandin-endoperoxide synthase (PTGS) enzyme as well as by that of other PG synthesizing enzymes (Frungieri *et al.* 2006). Nevertheless, PTGS is the key enzyme in the biosynthetic pathway of PGs. The constitutively expressed PTGS isoenzyme type 1 (PTGS1) is found in most cell types. By contrast, expression of the inducible isoenzyme type 2 (PTGS2) has been described in early stages of cell differentiation or replication in response to different stimuli such as cytokines and mitogenic factors. In addition, PTGS2 has been associated with both physiological and pathological states (Katori & Majima 2000, Smith *et al.* 2000).

The development of mice deficient in PTGS1 and PTGS2 has pointed out the roles exerted by PTGS isoenzymes in female reproduction. Early reports have demonstrated that PTGS1 null female mice have difficulty in parturition and PTGS2 null female mice are infertile (Langenbach *et al.* 1995, Lim *et al.* 1997, Davis *et al.* 1999). By contrast, male fertility is not affected in PTGS1 or PTGS2 mutant mice from knockout experiments (Dinchuk *et al.* 1995, Langenbach *et al.* 1999). Thus, these early studies suggest that PGs may not be important for the functioning of the testis. However, several reports have shown that PTGS is up-regulated in testicular cancer (Hase *et al.* 2003) and aging (Wang *et al.* 2005), and that some PGs (i.e. PGD_2, PGE_2, $PGF_{2\alpha}$) participate in the regulation of testicular testosterone production (Saksena *et al.* 1973, Kimball *et al.* 1979, Didolkar *et al.* 1981, Sawada *et al.* 1994, Romanelli *et al.* 1995, Gunnarsson *et al.* 2004). In this context, we have reported that although PTGS2 is not detected in

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normal human testes, it is expressed in interstitial cells, presumably Leydig cells, in testicular biopsies of men with impaired spermatogenesis and male infertility (Frungieri *et al.* 2002). In fact, we have described that 15d-PGJ_2 might participate in the development of human testicular fibrosis (Frungieri *et al.* 2002). Therefore, these reports suggest that PGs might actually be of relevance in male fertility physiology and/or pathology.

By immunohistochemistry, we have recently found PTGS2 expression in Leydig cells of the reproductively active Syrian hamster (*Mesocricetus auratus*; Frungieri *et al.* 2006). The Syrian hamster is a seasonal breeder that undergoes a morphological and physiological testicular regression when exposed to a short-day (SD) photoperiod. As a consequence, this species provides an excellent opportunity to study the mechanisms involved in the control of reversible infertility, in which PTGS2 and PGs may be among the molecules involved. Therefore, in this study, we used Leydig cells from young adult Syrian hamsters to investigate the potential action of LH, testosterone and dihydrotestosterone (DHT) on the induction and maintenance of PTGS2 expression and $PGF_{2\alpha}$ production.

Results

Testicular expression of PTGS2 in Syrian hamsters

By immunohistochemistry, PTGS2 was not found in testes from 18-day-old prepubertal animals kept under a long-day (LD) photoperiod (Fig. 1A). By contrast, immunohistochemical analyses showed the expression of PTGS2 in the cytoplasm of interstitial cells with the characteristic punctuate chromatin pattern of Leydig cells in early pubertal (46-day-old; Fig. 1B), late pubertal (60-day-old; Fig. 1C), young adult (90-day-old; Fig. 1D) and adult (200-day-old; Fig. 1E) LD Syrian hamster testes. PTGS2 immunostaining was not detected in testis sections from young adult (90-day-old) Syrian hamsters transferred to a SD photoperiod for 16 weeks (Fig. 1G). Furthermore, PTGS2 immunostaining was not found when testis sections from young adult hamsters kept under a LD photoperiod or transferred to a SD photoperiod for 16 weeks were incubated only with normal nonimmune serum and the conjugated antibody (controls, Fig. 1F and H respectively).

In order to detect PTGS2 expression by western blot analyses in Leydig cells from prepubertal hamsters and young adult animals exposed to a SD photoperiod for 16 weeks, 300 μg protein extracted from approximately two million Leydig cells were necessary to be loaded onto SDS acrylamide gels (Fig. 2). PTGS2 protein levels in Leydig cells from reproductively active adult hamsters were 12-fold higher than those detected in 18-day-old animals and photoperiodically regressed adult hamster Leydig cells (Fig. 2).

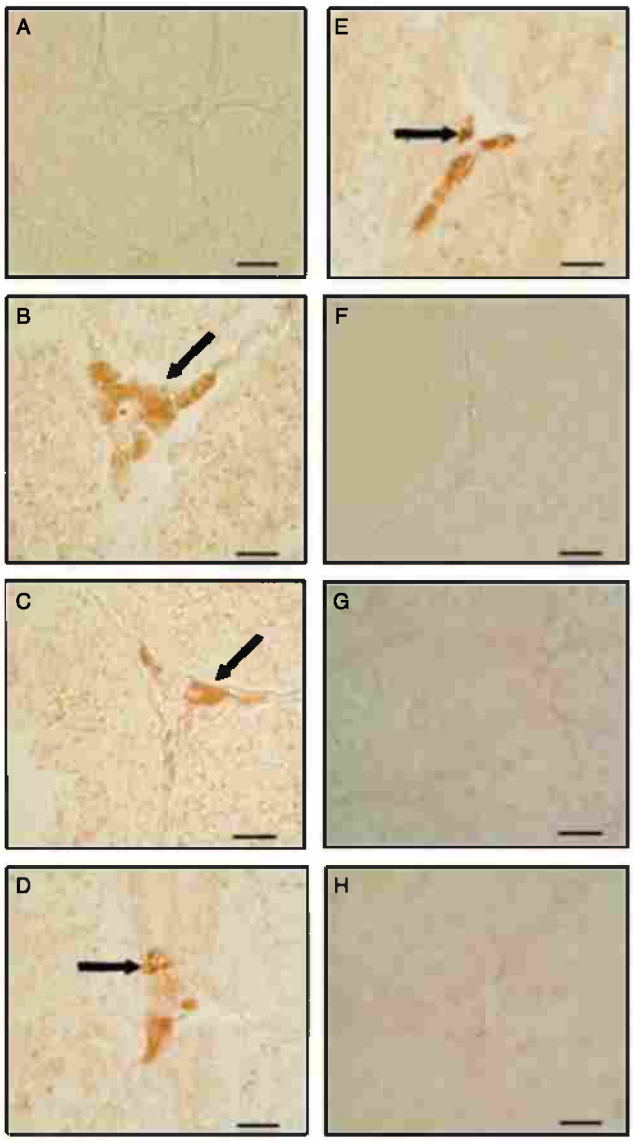

Figure 1 PTGS2 immunolocalization in Syrian hamster testes: influence of age and photoperiod. PTGS2 immunostaining was not detected in testes from prepubertal (18-day-old) hamsters (A), or young adult hamsters transferred to a short-day (SD) photoperiod (6 h light:18 h darkness) for 16 weeks (G). By contrast, positive reaction was found in interstitial cells with the characteristic punctuate chromatin pattern of Leydig cells (black arrows) from early pubertal (46-day-old) (B), late pubertal (60-day-old) (C), young adult (90-day-old) (D) and adult (200-day-old) (E) male Syrian hamsters kept under a long-day (LD) photoperiod (14 h light:10 h darkness). No reaction was observed in testis sections from young adult hamsters kept under a LD photoperiod (F) or transferred to a SD photoperiod for 16 weeks (H) incubated only with normal nonimmune serum and the conjugated antibody. Bar, 20 μm.

Stimulatory role of LH/hCG and testosterone in mRNA and protein PTGS2 expression in hamster Leydig cells: effect of bicalutamide

After 1- (Fig. 3A) and 2-h (data not shown) incubations in the presence of a maximum hCG concentration (100 mIU/ml), mRNA expression of *PTGS2* was

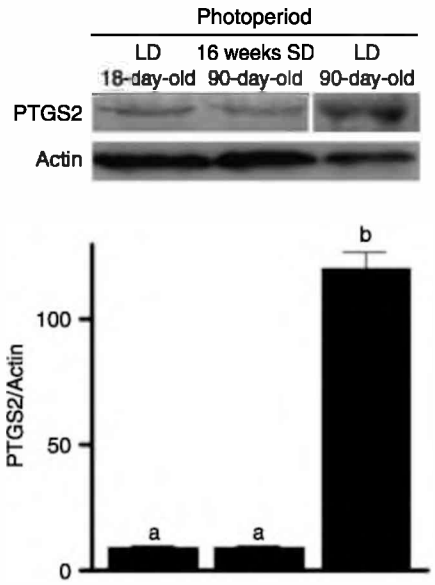

Figure 2 PTGS2 protein expression by immunoblotting in Syrian hamster Leydig cells: influence of age and photoperiod. PTGS2 protein was detected in Leydig cells from prepubertal (18-day-old) hamsters, young adult (90-day-old) hamsters kept under a long-day (LD) photoperiod (14 h light:10 h darkness) and young adult hamsters transferred to a short-day (SD) photoperiod (6 h light:18 h darkness) for 16 weeks. PTGS2 (72 kDa) and actin (42 kDa) protein levels were determined by western blot. These representative immunoblots show results obtained from one of two experiments performed on different cell preparations. For each experiment, approximately two million cells were isolated from four hamsters and 300 μg protein extracts were loaded onto the gels. Bar plot graph represents the mean ± S.E.M. and depicts the quantification by densitometry of PTGS2 bands obtained in two independent experiments and normalized to actin. All groups were compared; different letters above the bars denote a statistically significant difference between the groups ($P<0.05$).

significantly increased in LD hamster Leydig cells. Furthermore, the mRNA expression of *PTGS2* was significantly induced in LD hamster Leydig cells incubated for 1 (Fig. 3B) and 2 h (data not shown) in the presence of a physiological testosterone dose (1 μM). Nevertheless, neither testosterone nor hCG had an effect on *PTGS2* mRNA expression in hamster Leydig cells when bicalutamide (Bi) (10 μM), a pure antiandrogen, was added to the incubation media (Fig. 3B and C). Bi alone had no effect on *PTGS2* mRNA levels (Fig. 3D).

The expression of PTGS2 protein in hamster Leydig cells showed a significant increase after 2- (data not shown) and 3-h incubations (Fig. 3E) in the presence of a maximum hCG dose (100 mIU/ml). Moreover, a physiological testosterone concentration (1 μM) markedly increased protein PTGS2 expression after 2- (data not shown) and 3-h incubations (Fig. 3E). The stimulatory effect of testosterone and hCG on protein PTGS2 expression was reversed by Bi (10 μM; Fig. 3E). Bi alone had no effect on PTGS2 protein expression (Fig. 3F).

Stimulatory role of LH/hCG and testosterone in $PGF_{2\alpha}$ production in hamster Leydig cells: effect of bicalutamide

After 2- (Fig. 4A) and 3-h (Fig. 4B) incubations, testosterone (1 μM) significantly increased $PGF_{2\alpha}$ production from LD hamster Leydig cells. The stimulatory effect of testosterone on $PGF_{2\alpha}$ production was reversed when Bi (10 μM) was added to the incubation media (Fig. 4A and B).

No significant changes were observed in the production of $PGF_{2\alpha}$ from hamster Leydig cells after 2 h incubation in the presence of hCG (100 mIU/ml; Fig. 4A). Nevertheless, after 3 h (Fig. 4B) incubations, hCG significantly increased $PGF_{2\alpha}$ production, and this stimulatory effect was prevented by Bi (Fig. 4A and B). Bi alone had no effect on $PGF_{2\alpha}$ production (Fig. 4A and B).

Role of DHT in PTGS2 expression in hamster Leydig cells

After 2- (data not shown) and 3-h incubations (Fig. 5A and B), both a physiological DHT concentration (0.1 μM, Fig. 5A) and a supraphysiological DHT dose (1 μM, Fig. 5B) did not alter the expression of PTGS2 protein in hamster Leydig cells.

Identification of the signalling pathway by which testosterone induces PTGS2

Western blot experiments were performed to determine whether the effect of testosterone on PTGS2 in hamster Leydig cells is mediated via protein kinases.

No significant changes were observed in AKT phosphorylation and total AKT levels when hamster Leydig cells were incubated for 3 h in the presence or absence of 1 μM testosterone, a physiological DHT dose or a supraphysiological DHT concentration (data not shown).

Addition of a physiological testosterone concentration (1 μM) to hamster Leydig cells induced phosphorylation of MAPK3 and MAPK1 within 1–3 min (Fig. 6A and B), but did not change total protein levels of MAPK3/1 (Fig. 6C and D). After 2 (data not shown) and 3 h (Fig. 7) of the initial testosterone stimulation, phospho-MAPK3/1 increased eight- to tenfold (Fig. 7A and B), but total MAPK3/1 remained unchanged (Fig. 7C and D). Furthermore, PTGS2 induction followed similar patterns than those observed for MAPK3/1 phosphorylation (Fig. 7E and F). The stimulatory effects of testosterone on MAPK3/1 phosphorylation and PTGS2 expression were reversed when U0126 (10 μM; Fig. 7A and E respectively) and Bi (10 μM; Fig. 7B and F respectively) were added to the incubation media. U0126 alone (Fig. 7A, C and E) and Bi alone (Fig. 7B, D and F) had no

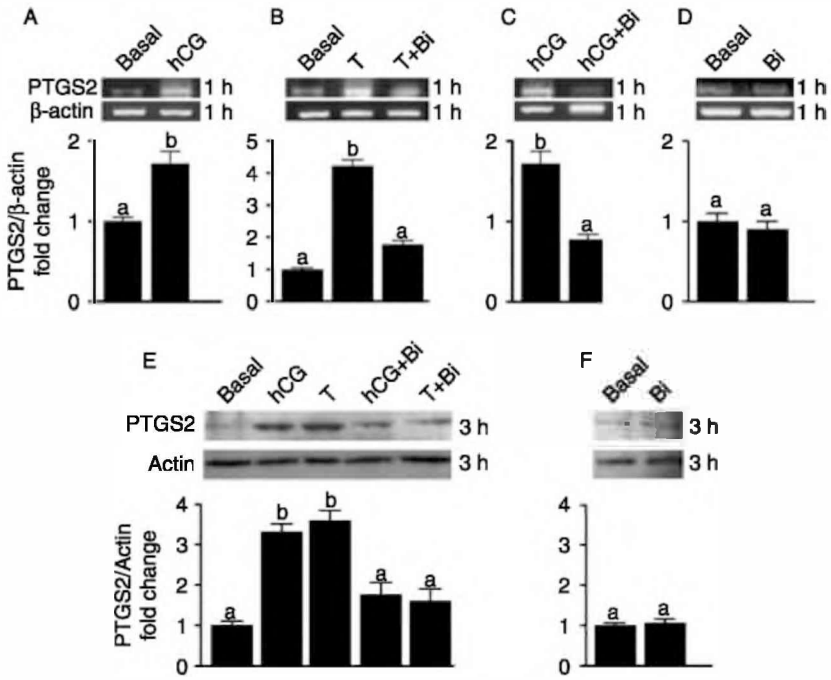

Figure 3 LH/hCG and testosterone (T) induction of mRNA and protein PTGS2 expression in Syrian hamster Leydig cells. Effect of bicalutamide (Bi). Leydig cells were incubated in Medium M199 for 1 (A–D) and 3 h (E and F) in the presence or absence of hCG (100 mIU/ml) (A, C and E) or testosterone (T, 1 μM) (B and E), either with or without bicalutamide (Bi, 10 μM) (B–F). *PTGS2* and β-actin mRNA levels were determined by RT-PCR. PTGS2 (72 kDa) and actin (42 kDa) protein levels were determined by western blot. These representative ethidium bromide-stained agarose gels and immunoblots show results obtained from one of three experiments performed on different cell preparations. For each experiment, cells were isolated from eight young adult hamsters kept under a long-day (LD) photoperiod (14 h light:10 h darkness). Bar plot graphs represent the mean ± S.E.M. 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. All groups were compared; different letters above the bars denote a statistically significant difference between the groups ($P<0.05$).

effect on MAPK3/1 phosphorylation and PTGS2 protein expression. Total MAPK3/1 were unaffected by testosterone, U0126 and/or Bi (Fig 7C and D).

When hamster Leydig cells were treated for 2 and 3 h with a physiological DHT concentration (0.1 μM) or a supraphysiological DHT dose (1 μM), MAPK3/1 phosphorylation and total MAPK3/1 remained unchanged (data not shown).

Identification of androgen receptors in hamster testes

The immunohistochemical analyses revealed the presence of androgen receptors in Leydig cells from young adult LD Syrian hamster testes (Fig. 8A). In addition, positive immunostaining for androgen receptors was detectable in myoid cells and Sertoli cells from young adult LD hamster testes (Fig. 8A). No reaction was observed in testis sections from young adult hamsters in which the androgen receptor antiserum was replaced by normal rabbit serum (Fig. 8B).

Some, but not all, androgen receptor-immunoreactive Leydig cells found in the hamster testis were also positively stained for PTGS2 (Fig. 8C–F).

Positive immunostaining for androgen receptors was also detectable in Leydig cells from prepubertal (18-day-old) and pubertal (46- and 60-day-old) LD hamsters (data not shown). In addition, androgen receptors were found in Leydig cells from young adult hamsters exposed to a SD photoperiod for 16 weeks (data not shown).

The existence of androgen receptors in isolated Leydig cells from LD young adult hamsters was confirmed by immunocytochemistry (Fig. 8G). When the androgen receptor antiserum was omitted, immunostaining was not found in isolated LD young adult hamster Leydig cells (Fig. 8H).

Discussion

This study provides novel evidence for a direct involvement of testosterone in the regulation of testicular PTGS2 expression and consequently of $PGF_{2\alpha}$ synthesis which, as we have previously demonstrated, inhibits LH-stimulated testosterone production in the reproductively active seasonal breeder Syrian hamster (Frungieri *et al.* 2006). Our results indicate that PTGS2 is mostly expressed in Leydig cells of pubertal and adult hamsters showing significant circulating levels of LH and total androgens (testosterone and DHT). Furthermore, a physiological dose of testosterone (1 μM), significantly

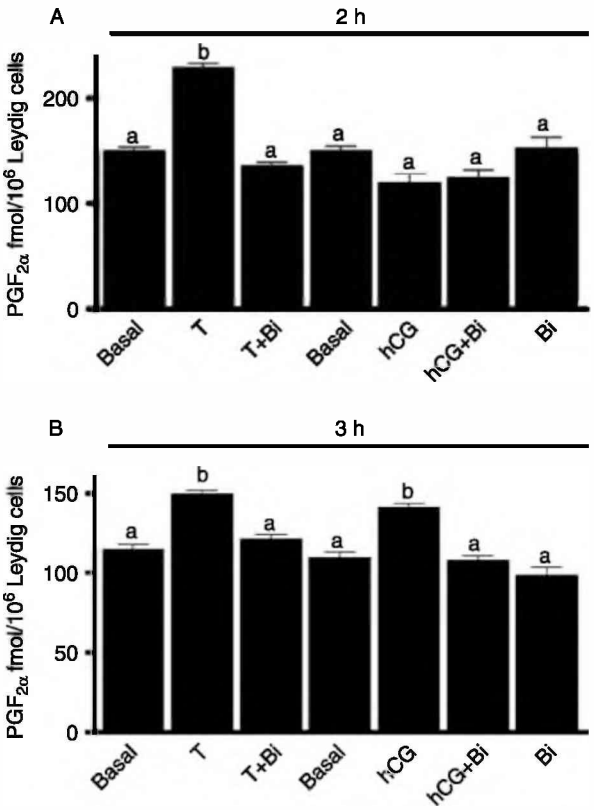

Figure 4 Testosterone and LH/hCG induction of $PGF_{2\alpha}$ production in Syrian hamster Leydig cells. Effect of bicalutamide (Bi). Leydig cells were incubated in the presence or absence of testosterone (T, 1 μM) or hCG (100 mIU/ml) for 2 (A) and 3 h (B), either with or without bicalutamide (Bi, 10 μM). $PGF_{2\alpha}$ levels in the incubation media were determined by immunoassay. Bar plot graphs represent the mean ±S.E.M. from one of two experiments performed in different cell preparations (six to eight replicates per experiment). For each experiment, cells were isolated from eight young adult hamsters kept under a long-day (LD) photoperiod (14 h light:10 h darkness). All groups were compared; different letters above the bars denote a statistically significant difference between the groups ($P<0.05$).

induced PTGS2 expression and $PGF_{2\alpha}$ production in hamster Leydig cells through androgen receptors and a non-classical mechanism that involves MAPK3/1 phosphorylation.

It is well established that PGs regulate physiological and pathological processes in reproduction, mainly in the ovary (Ahsan *et al.* 1997, Basini & Tamanini 2001, Tai *et al.* 2001, Schams & Berisha 2004). However, a possible role for PGs in testes is not yet fully understood.

Recently, we have screened testes from species ranging from mice to monkeys in order to find an animal model for further investigation of testicular PTGS2/PGs (Frungieri *et al.* 2006). By immunohistochemistry, we found only positive PTGS2 immunostaining in Leydig cells of the reproductively active seasonal breeder Syrian hamster (Frungieri *et al.* 2006). However, other authors have been able to detect PTGS2 expression in testes and Leydig cells from rats, mice and even humans using different experimental techniques such as RT-PCR, real-time RT-PCR and western blot (O'Neill & Ford-Hutchinson 1993, Neeraja *et al.* 2003, Wang *et al.* 2005, Balaji *et al.* 2007, Chen *et al.* 2007, Winnall *et al.* 2007). One plausible explanation for these discrepancies is that the levels of PTGS2 expression in normal testes of most species, including the men, may be too low to be detected by immunohistochemistry. By contrast, Leydig cells from reproductively active Syrian hamsters could have much higher levels of PTGS2 than the other species mentioned. Since our previous studies in hamster Leydig cells have also shown that PGD_2 and $PGF_{2\alpha}$ participate in the modulation of testosterone secretion (Frungieri *et al.* 2006, Schell *et al.* 2007), this seasonal breeder species, which undergoes a morphological and physiological testicular regression/recrudescence transition when exposed to a SD photoperiod, provides an excellent natural model to study the regulation of testicular PTGS2 expression and PG production in the control of reversible (in)fertility.

The first aim of the current study was to further investigate PTGS2 in Syrian hamster testes. We initially evaluated testicular expression of PTGS2 in Syrian hamsters during sexual development and the exposition of young adult animals to an inhibitory photoperiod (6 h light:18 h darkness) for 16 weeks in order to achieve the maximum testicular regression. By immunohistochemistry, we found that prepubertal and young adult

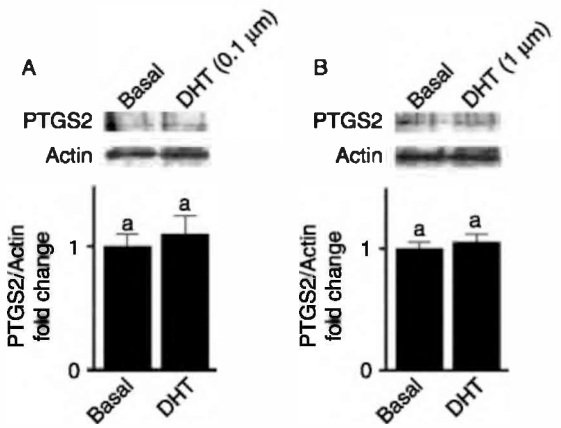

Figure 5 Role of dihydrotestosterone (DHT) in protein PTGS2 expression in hamster Leydig cells. Leydig cells were incubated in Medium M199 for 3 h in the presence or absence of 0.1 μM (A) and 1 μM (B) DHT. PTGS2 (72 kDa) and actin (42 kDa) protein levels were determined by western blot. These representative immunoblots show results obtained from one of three experiments performed on different cell preparations that showed comparable results. For each experiment, cells were isolated from eight young adult hamsters kept under a long-day (LD) photoperiod (14 h light:10 h darkness). Bar plot graphs represent the mean±S.E.M. 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. The same letter above the bars denotes that the difference between the groups is not statistically significant.

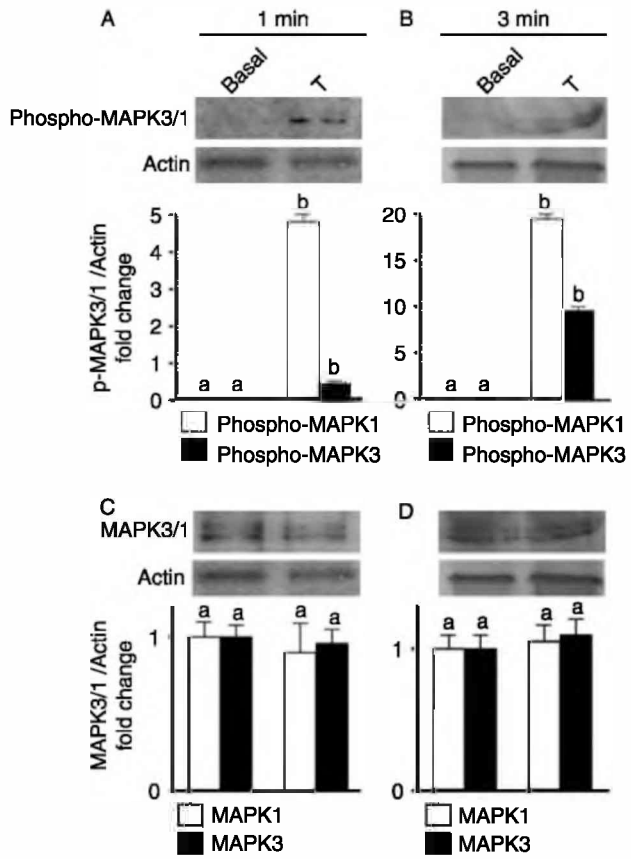

Figure 6 Short-term effect of testosterone (T) in MAPK3/1 phosphorylation in hamster Leydig cells. Leydig cells were incubated in Medium M199 for 1 (A and C) and 3 min (B and D) in the presence or absence of testosterone (T, 1 μM). Phospho-MAPK3/1 (44/42 kDa), MAPK3/1 (44/42 kDa) and actin (42 kDa) protein levels were determined by western blot. These representative immunoblots show results obtained from one of three experiments performed on different cell preparations. For each experiment, cells were isolated from eight young adult hamsters kept under a long-day (LD) photoperiod (14 h light:10 h darkness). Bar plot graphs represent the mean ± S.E.M. 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. When protein expression is not detected in basal conditions, results are expressed as 'fold change' relative to the background, which was assigned a value of 1. White and black bars represent MAPK1 and MAPK3 respectively. Different letters above the bars denote a statistically significant difference between the groups ($P<0.05$).

hamsters exposed to a SD photoperiod for 16 weeks with low serum concentrations of LH and total androgens did not express PTGS2 in testes. On the contrary, pubertal and adult reproductively active hamsters with increased circulating concentrations of LH and total androgens expressed PTGS2 in testicular interstitial cells. When we extended our studies using western blot analyses, 300 μg protein extracted from approximately two million cells were necessary to be loaded in order to detect PTGS2 expression in Leydig cells from prepubertal hamsters and young adult animals exposed to a SD photoperiod. Moreover, PTGS2 protein levels in Leydig cells from reproductively active adult hamsters were 12-fold higher than those detected in prepubertal and photoperiodically regressed adult hamster Leydig cells. These results suggest that the increment observed in LH and androgen levels at pubertal and adult ages might reflect the fact that these hormones could participate as modulators of testicular PTGS2 expression during sexual maturation and the photoperiodic-induced testicular active–inactive transition in hamsters.

Although immunohistochemical analyses showed the expression of PTGS2 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 PTGS2. Thus, for our RT-PCR and western blot studies, we purified Leydig cells from testes of reproductively active hamsters using a discontinuous Percoll density gradient. Cell preparations were 87–90% enriched with hamster Leydig cells. Less than 0.006% of the contaminating cells were macrophages whereas mast cells were not detected. It is important to mention that even though mast cells have been seen in the interstitial compartment and the tubular wall in human testes (Meineke *et al.* 2000), the same does not apply to the hamster testis. In fact, mast cells are exclusively located in the capsule in hamster testes (Frungieri *et al.* 1999), which is initially removed during the procedure used for Leydig cell purification.

Results obtained in purified hamster Leydig cells incubated in the presence or absence of LH/hCG seem to corroborate the hypothesis of a modulatory role of this hormone in the regulation of testicular PTGS2 expression. A maximum stimulatory dose of hCG (100 mIU/ml) significantly induced mRNA and protein levels of PTGS2 in Leydig cells isolated from active Syrian hamsters. In agreement with our findings in hamsters, Chen *et al.* (2007) have recently described that incubation of young and aged Leydig cells isolated from Brown Norway rats with LH or dibutyryl cAMP increases intracellular PTGS2 expression.

Previous reports suggest the existence of a link between testosterone production and PGs synthesis in testes (Syntin *et al.* 2001, Wang *et al.* 2003, 2005) pointing out the role of testicular PTGS2 in the regulation of steroidogenesis. In agreement with these studies, we have recently shown that Leydig cells purified from active seasonal breeder hamsters express PTGS2 and produce PGD_2 and $PGF_{2\alpha}$ that participate in the regulation of steroidogenesis (Frungieri *et al.* 2006, Schell *et al.* 2007). Taking into account this negative role played by PTGS2 and $PGF_{2\alpha}$ in testicular testosterone production, in the current study, we used Syrian hamster Leydig cells as an experimental model for elucidating the potential action of testosterone on testicular PTGS2 expression and $PGF_{2\alpha}$ production. Testosterone (1 μM) significantly increased PTGS2

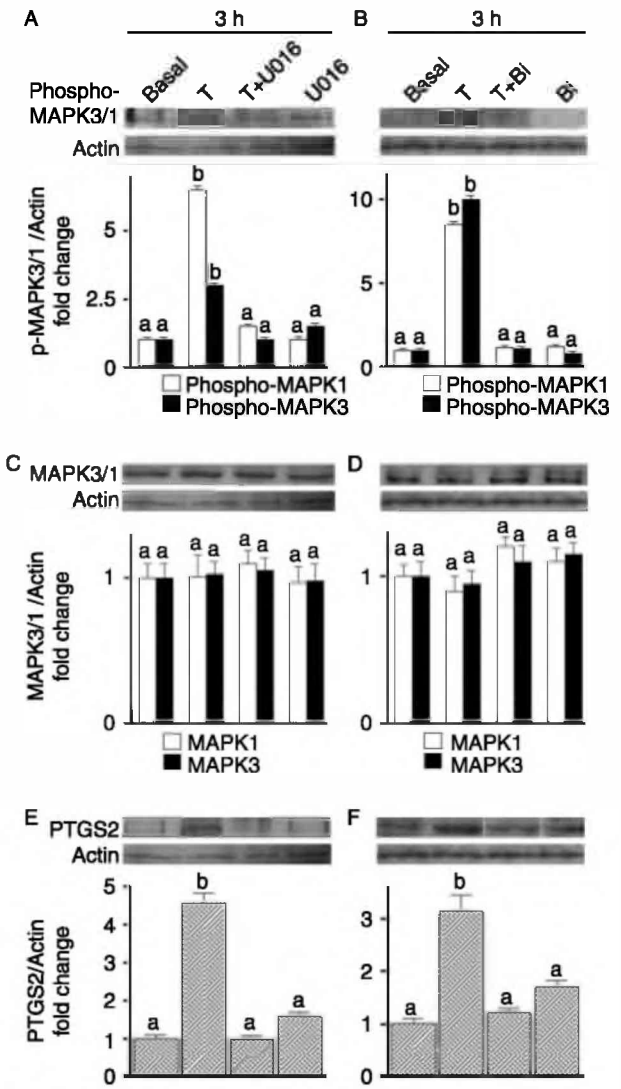

Figure 7 Long-term effect of testosterone (T) in MAPK3/1 phosphorylation and PTGS2 expression in hamster Leydig cells. Leydig cells were incubated in Medium M199 for 3 h in the presence or absence of testosterone (T, 1 μM), either with or without bicalutamide (Bi, 10 μM) (B, D and F) or U0126 (10 μM) (A, C and E). Phospho-MAPK3/1 (44/42 kDa), MAPK3/1 (44/42 kDa), PTGS2 (72 kDa) and actin (42 kDa) protein levels were determined by western blot. These representative immunoblots show results obtained from one of three experiments performed on different cell preparations. For each experiment, cells were isolated from eight young adult hamsters kept under a long-day (LD) photoperiod (14 h light:10 h darkness). Bar plot graphs represent the mean±S.E.M. 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. White and black bars represent MAPK1 and MAPK3 respectively. Different letters above the bars denote a statistically significant difference between the groups ($P<0.05$).

expression at mRNA and protein levels in Leydig cells isolated from reproductively active Syrian hamsters. Furthermore, testosterone significantly increased $PGF_{2\alpha}$ production. Nevertheless, stimulation of $PGF_{2\alpha}$ production is rather small taking into account the marked changes in PTGS2 levels by testosterone. It may arise from the activity of the other enzymes involved in this PGs biosynthetic pathway, as well as from $PGF_{2\alpha}$ conversion to other PGs or its inactive catabolites (Ellis & Jorgensen 1982, Wohlrab & Essbach 1984, Franchi *et al.* 1985, Nemetallah & Ellis 1985). The testicular weight and the specific testicular weight in these hamsters are 1.4–1.6 g and 1.0–1.2 g/cm^3 respectively, and the testicular testosterone content is 600–900 pmol/testis (Frungieri *et al.* 1996*a*). Thus, in the *in vitro* incubations of hamster Leydig cells for determination of PTGS2 expression and $PGF_{2\alpha}$ production, doses from 0.5 to 1 μM testosterone can be considered as physiological concentrations.

As expected, testosterone had no effect on PTGS2 expression and $PGF_{2\alpha}$ production when hamster Leydig cells were incubated in the presence of Bi, a pure non-steroidal antiandrogen that competitively inhibits the action of androgens by binding to androgen receptors in the target tissue (Furr *et al.* 1987).

For a better understanding of the mechanisms underlying the action of LH on testicular PTGS2 expression and $PGF_{2\alpha}$ production, we also used Bi. Both the stimulatory effect of hCG on PTGS2 mRNA and protein expressions and the positive regulatory action of hCG on $PGF_{2\alpha}$ production were reversed in the presence of Bi. These findings indicate that LH action results from its stimulatory role in testosterone synthesis and not from a direct mechanism on PTGS2/PGs.

Testosterone can also convert into DHT by the action of the 5α-reductase. Testosterone and DHT are the most potent androgens binding to the androgen receptor with K_d of 2.0×10^{-10} and 4.0×10^{-10} M respectively (Ge *et al.* 1999). Viger & Robaire (1995) have described that progenitor, immature and early pubertal rat Leydig cells possess high 5α-reductase activity and produce DHT from testosterone. By contrast, adult Leydig cells show low 5α-reductase expression and, as a consequence, low DHT production from testosterone. Testicular DHT production and DHT circulating levels in peripubertal, reproductively active and regressed adult hamsters have been previously established (Frungieri *et al.* 1999). In this study, a potential action of DHT on testicular PTGS2 expression has been addressed in young adult hamster Leydig cells. We found that DHT does not alter protein PTGS2 expression, therefore suggesting a direct action of testosterone in the regulation of PTGS2/$PGF_{2\alpha}$ exerted through androgen receptors. Since the testicular DHT content is 200–300 pmol/testis (Frungieri *et al.* 1996*a*), the 0.1 and 1 μM DHT concentrations used in the *in vitro* incubations of hamster Leydig cells for evaluation of PTGS2 expression can be considered a physiological and a supraphysiological dose respectively. Nevertheless, how the two major physiological androgens, testosterone and DHT, acting through the same androgen

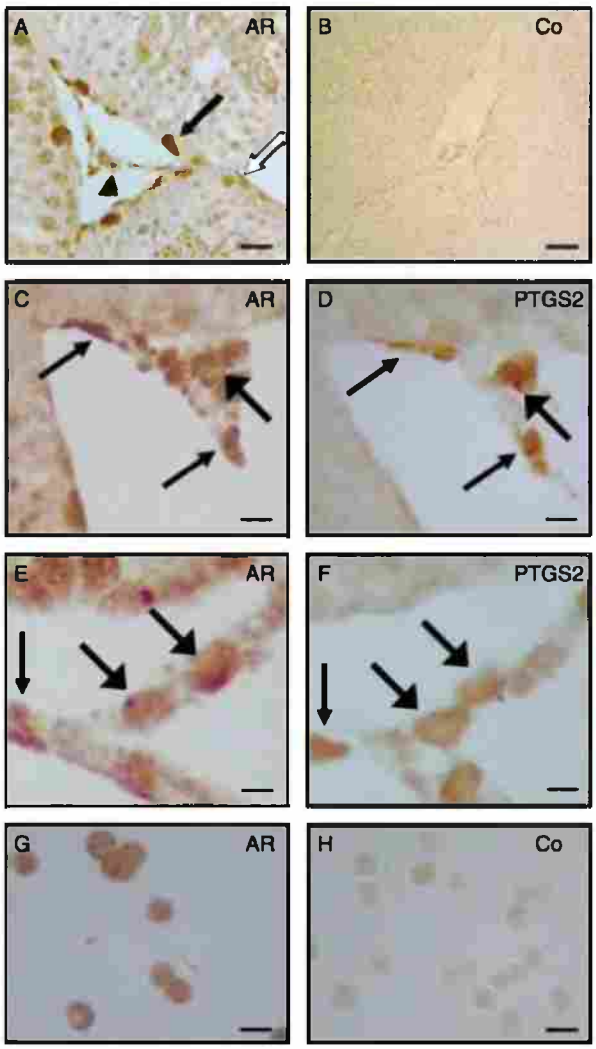

Figure 8 Immunolocalization of androgen receptors in Syrian hamster testes. Positive immunostaining for androgen receptor (AR) was found in Leydig cells (arrowhead), Sertoli cells (black arrow) and myoid cells (white arrow) in testes from young adult (90-day-old) Syrian hamsters kept under a long-day (LD) photoperiod (14 h light:10 h darkness) (A). Some, but not all, androgen receptors-immunoreactive Leydig cells found in the hamster testis were also positively stained for PTGS2 (black arrows) (C–F). C and D as well as E and F are consecutive testicular sections from a young adult hamster kept under a LD photoperiod. Positive staining for androgen receptors was also found in purified LD young adult hamster Leydig cells (G). No reaction was observed in sections from young adult LD hamster testes (B) and isolated LD young adult hamster Leydig cells (H) incubated only with normal nonimmune serum and the conjugated antibody (Co, controls). Bar, 40 μm (A and B), 10 μm (C, D, G and H) and 5 μm (E and F).

receptor protein can induce different responses remains unknown. There is no doubt that some effects of androgens are regulated by testosterone but not by DHT during development and aging, although the explanation remains obscure (Imperato-McGinley *et al.* 1974, 1979, Imperato-McGinley & Zhu 2002, Bimonte-Nelson *et al.* 2003). Hsiao *et al.* (2000) have recently hypothesized the existence of two kinds of androgen response elements, which might show different androgen receptor transactivation upon testosterone and DHT induction in mammalian cells. Alternatively, the effect of some testosterone metabolite, which cannot be produced from 5α-reduced androgens, could account for the different responses triggered by testosterone and DHT on PTGS2 expression. In this context, testosterone 7α-hydroxylation has been previously detected in rat testis, and 7α-hydroxytestosterone has been postulated as an inhibitor of 5α-reduced steroids production (Rosness *et al.* 1977, Mittler 1985, Sonderfan *et al.* 1989). Moreover, testosterone and/or DHT effects could take place through non-classical mechanisms. It has been recently reported the activation of the MAP kinase pathway and the phosphoinositide-3 (PI3) kinase/serine threonine kinase AKT signalling cascade by androgens in Sertoli, prostate and osteoblast cells (Fix *et al.* 2004, Kang *et al.* 2004, Chen *et al.* 2007, Cinar *et al.* 2007, Pinthus *et al.* 2007, Rahman & Christian 2007, Agoulnik *et al.* 2008). As a consequence, we have investigated the participation of those signalling pathways in the regulation of PTGS2 expression by testosterone and DHT in hamster Leydig cells. DHT did not activate MAPK3/1 or PI3K/AKT signalling pathways in hamster Leydig cells (results not shown). Although testosterone failed to activate PI3K/AKT (results not shown), this androgen induced a rapid phosphorylation of MAPK3/1 within short-term incubations (1–3 min), which was also detected after 2–3 h. These results are similar to those observed in studies performed in breast cancer, skeletal muscle cells, prostate stroma cells and Sertoli cells in which androgen activates MAPK3/1 too quickly to be explained by the classical androgen receptor pathway (Peterziel *et al.* 1999, Zhu *et al.* 1999, Estrada *et al.* 2003, Fix *et al.* 2004, Rahman & Christian 2007). We also found that Bi prevented the effects of testosterone on phospho-MAPK3/1 indicating that androgen receptors are required for testosterone-mediated MAPK activation. Furthermore, U0126, a very selective and highly potent inhibitor of the MAP kinase kinases 1 and 2 (MAP2K1/2), blocked the stimulatory action of testosterone treatments on PTGS2 expression. These results suggest that the MAPK pathway is involved in testosterone-mediated PTGS2 up-regulation. Thus, we might conclude that testosterone induction of PTGS2 expression in hamster Leydig cells seems to be exerted via androgen receptors and a non-classical mechanism that involves phosphorylation of MAPK3/1. Nevertheless, a concomitant effect of testosterone on PTGS2 taking place via a classical mechanism cannot be ruled out and should be further investigated.

Androgen receptors were identified by immunohistochemistry and immunocytochemistry in Leydig cells from young adult Syrian hamsters in agreement with previous reports in mice (Pelletier 2000, Zhou *et al.* 2002), rats (Pelletier 2000, Zhu *et al.* 2000), pigs (Christensen 1996) and humans (Van Roijen *et al.* 1995,

Pelletier 2000, Loukil *et al.* 2005). Some of those androgen receptor positive Leydig cells were also immunoreactive for PTGS2. Therefore, detection of androgen receptors in hamster Leydig cells allows us to speculate the occurrence of a stimulatory action of testosterone on PTGS2 expression taking place *in vivo* as well.

Previous reports from other authors (Syntin *et al.* 2001, Wang *et al.* 2003, 2005) and our group (Frungieri *et al.* 2006) support an inhibitory role played by PTGS2/$PGF_{2\alpha}$ in testicular STAR/17β-hydroxysteroid dehydrogenase (HSD17B) expression and testosterone production via $PGF_{2\alpha}$ (FP) receptors. Current data indicate a stimulatory action of testosterone on testicular PTGS2/$PGF_{2\alpha}$. Therefore, we may hypothesize about the existence of a regulatory loop in which testosterone induces PTGS2 expression and $PGF_{2\alpha}$ synthesis, but in which PTGS2/$PGF_{2\alpha}$ inhibits STAR and HSD17B expression and consequently testosterone production, thus setting a brake on testicular steroidogenesis. This regulatory loop might be of relevance in physiological conditions (i.e. the photoperiodic-induced testicular active–inactive transition in hamsters, sexual development and aging) and/or pathological states.

In summary, by elucidating details of how testicular testosterone locally influences PTGS2 expression, our work suggests that the androgen environment might be crucial for the regulation of testicular $PGF_{2\alpha}$ production at least in hamsters. We have also detected the expression of PTGS2 (Frungieri *et al.* 2002), FP receptors (Frungieri *et al.* 2006) and androgen receptors (data not shown) in human Leydig cells of infertile men. On the contrary, PTGS2 seems to be absent in normal human testes (Frungieri *et al.* 2002). Thus, we could speculate that testosterone/PTGS2/$PGF_{2\alpha}$ play a role under physiological conditions in hamsters although they might be of relevance in pathological situations in humans. Nevertheless, further investigations are required to determine whether our results can be extended to non-seasonal reproductive species including humans, in which factors other than testosterone may be responsible for the regulation of PTGS2 expression.

Materials and Methods

Animals

Male Syrian hamsters (*M. auratus*) were raised in our animal care unit (Charles River descendants, Animal Care Lab., IBYME, Buenos Aires) and kept from birth to adulthood in rooms at 23±2 °C under a LD photoperiod (14 h light:10 h darkness; lights on 0700–2100 h). Hamsters aged 18, 46, 60, 90 and 200 days exposed to LD photoperiod were used in this study. In addition, young adult hamsters aged 90 days were transferred to a SD photoperiod (6 h light:18 h darkness; lights on 0900–1500 h) for 16 weeks. It is important to mention that hamsters from our colony reach the maximum testicular regression after 16 weeks of SD photoperiod (see additional information in Frungieri *et al.* 1996*b*). Animals had free access to water and Purina formula chow. 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-CONICET), following the NIH guidelines. At the time of killing, trunk blood was collected. Serum was obtained by centrifugation and stored at −20 °C for further LH and total androgen (testosterone and DHT) determinations. Left testes were dissected, fixed for at least 48 h in Bouin or formaldehyde fluids followed by dehydration, and then embedded in paraffin wax for histological and immunohistochemical studies. Right testes were rapidly removed, dissected and used for Leydig cell purification. *In vitro* incubations of Leydig cells followed by determination of mRNA expression (by RT-PCR), protein expression (by immunoblotting) or $PGF_{2\alpha}$ levels in the incubation media (by immunoassay) were performed.

LH and total androgen assays

Serum levels of LH were measured by a heterologous RIA previously validated in hamsters (Frungieri *et al.* 1996*b*) using materials and protocols supplied by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK, Bethesda, MD, USA). For LH RIA, rLH-RP3 standard and rLH-S-10 antiserum were used. The sensitivity of the assay was 0.250 ng/ml. The intra-assay coefficient of variation (CV) was <4%. All serum samples were measured, in duplicate, in the same assay to avoid inter-assay variations.

Serum testosterone levels were measured by RIA after extraction with diethyl ether (Merck) and following the method validated in hamsters by Frungieri *et al.* (1996*b*, 1996*c*). An antibody to testosterone-7α-butyrate-BSA (Medicorp Inc., Montreal, Canada) that has 35% cross-reaction with DHT and 0.04% cross-reaction with androstane-3α,17β diol (3α-diol) was used. As a consequence, results are expressed in terms of total androgen (testosterone and DHT) serum levels. The minimum detectable concentration was 0.042 ng/ml, the intra-assay CV was <12% and the inter-assay CV was <15%.

Table 1 summarizes the serum levels of LH and total androgens from prepubertal (18-day-old), early pubertal (46-day-old), late pubertal (60-day-old), young adult (90-day-old) and adult (200-day-old) male Syrian hamsters kept under a LD photoperiod, as well as from young adult (90-day-old) hamsters kept under a LD photoperiod or transferred to a SD photoperiod for 16 weeks.

Histological, immunohistochemical and immunocytochemical analyses

Testes from prepubertal (18-day-old), early pubertal (46-day-old), late pubertal (60-day-old), young adult (90-day-old) and adult (200-day-old) LD hamsters, as well as testes from young adult hamsters exposed to a SD photoperiod for 16 weeks, were examined by histological and 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. Formaldehyde-fixed purified Leydig cells

Table 1 Serum levels of LH and total androgens (testosterone and DHT) in Syrian hamsters: influence of age and photoperiod.

	LH (ng/ml)	**Total androgens** (testosterone and DHT; ng/ml)
18-day-old	1.91 ± 0.23^{a}	0.66 ± 0.06^{a}
46-day-old	8.29 ± 0.76^{b}	5.12 ± 0.32^{b}
60-day-old	$4.32 \pm 0.85^{a,b}$	6.36 ± 0.35^{b}
90-day-old	$4.66 \pm 0.92^{a,b}$	5.23 ± 0.33^{b}
200-day-old	$3.23 \pm 1.02^{a,b}$	4.02 ± 0.51^{b}
LD photoperiod	4.69 ± 0.55^{a}	5.72 ± 0.21^{a}
16 weeks in SD photoperiod	0.46 ± 0.08^{b}	0.83 ± 0.17^{b}

The data represent the mean ± S.E.M. for 10–12 animals per group. All groups were compared; different letters denote a statistically significant difference between the groups ($P<0.05$). DHT, dihydrotestosterone; LD, long day; SD, short day.

from young adult hamsters exposed to LD were also 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). Then, sections and cells were permeabilized by a 5-min incubation with 0.5% saponin in PBS and non-specific proteins were blocked by subsequent incubation with protein block buffer (5% goat normal serum in PBS for PTGS2 immunodetection and 5% horse normal serum prepared in 1.5% milk in PBS for androgen receptor immunodetection) for 30 min. After several wash steps, the incubation with the antiserum (polyclonal rabbit anti-PTGS2 serum, 1:100, Oxford Biomedical Research, Oxford, MI, USA; polyclonal rabbit anti-androgen receptor serum, 1:200, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) diluted in incubation buffer (2% goat normal serum in PBS for PTGS2 immunodetection; 0.02 M $NaPO_4H_2{\cdot}H_2O$, 0.15 M ClNa, sodium azide 1% BSA pH 7.6 for androgen receptor immunodetection) was carried out overnight in a humidified chamber at 4 °C. On the second day, cells and testicular sections were washed and incubated with biotinylated secondary antiserum (goat anti-rabbit IgG; 1:500, Vector Lab., CA, USA) for 2 h at room temperature. Finally, immunoreaction was visualized with 0.01% H_2O_2 and 0.05% 3,3-diaminobenzidine solution (in 0.05 M Tris–HCl, pH 7.6) and an avidin–biotin–peroxidase system (Vector Lab).

Antigen retrieval, required for androgen receptor immunodetection, was performed either by microwave irradiation of hamster testes sections in citrate buffer 0.01 M (pH 6.0), or by incubation of formaldehyde-fixed purified hamster Leydig cells in 20 μg/ml proteinase K solution at 37 °C for 15 min.

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

Hamster Leydig cell purification and in vitro *incubations*

Syrian 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 0.15 mg/ml collagenase type I (Sigma Chemical Co). At the end of the incubation, collagenase activity was stopped by adding Medium 199, and the tubules were allowed to settle for 1 min. Supernatants were transferred to 25 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. Attached cells were more than 95% enriched with macrophages positive for Indian Ink, ED-1 antigen and ED-2 antigen.

Filtered cells were employed for Leydig cells 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 under a light microscope. Viability of Leydig cells preparation was 97.5–98.5%. In order to evaluate enrichment in Leydig cells, 3β-hydroxysteroid dehydrogenase (HSD3B) 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 mast cells were not detected. The remaining cell types had the morphology of either peritubular or endothelial cells. Petri dishes with 1.5 ml Medium 199 containing 2.5×10^5 (for RT-PCR) or 7.5×10^5 (for immunoblotting and $PGF_{2\alpha}$ production) cells were incubated at 37 °C under a humid atmosphere with 5% CO_2 and in the presence of the following chemicals: 100 mIU/ml hCG (Ayerst, Princeton, NJ, USA; specific activity, 59 UI/mg), 1 μM testosterone (Sigma) or 0.1 and 1 μM DHT (Sigma), either with or without 10 μM U0126 (Sigma) or 10 μM of *N*-[4-cyano-3-(trifluoromethyl)phenyl]-3-[(4-fluorophenyl) sulphonyl]-2-hydroxy-2-methyl-propanamide (Bi, Casodex, ICI 176 334, AstraZeneca Pharmaceuticals, Macclesfield, England). U0126 is a specific MAP2K1/2 inhibitor. Bi is a well-known pure anti-androgen. In this study, testosterone and DHT stock solutions were prepared in ethanol. These solutions were then further diluted in Medium 199. An appropriate volume of ethanol diluted in Medium 199 (0.03 μl ethanol/ml Medium 199 and 0.3 μl ethanol/ml Medium 199) was added to control experiments in order to account for possible effects of the ethanol. U0126 stock solution was dissolved in DMSO (ICN Biomedicals Inc., Aurora, OH, USA) and further diluted in Medium 199. An appropriate volume of DMSO diluted in Medium 199 (5 μl DMSO/ml Medium 199) was added to control experiments. Both hCG and Bi were dissolved in Medium 199, which was then used as vehicle for control incubations. After incubation, cells in media were transferred to tubes and centrifuged at 1200 ***g*** for 10 min. Cells were used either for RNA extraction followed by RT-PCR, or for protein extraction followed by immunoblotting. Media were frozen at −70 °C until $PGF_{2\alpha}$ concentrations were determined by immunoassay.

Immunoblotting

Approximately 7.5×10^5 hamster Leydig cells were homogenized in 20 mM Tris–HCl (pH 8), 137 mM NaCl buffer containing 10% glycerol, 1% lysis buffer (NP40, Sigma) and 1% of a pre-formed mixture of protease inhibitors (P8340, Sigma). Samples were heated at 95 °C for 5 min under reducing

conditions (10% mercaptoethanol). Leydig cells protein homogenates (100 μg) were loaded onto tricine-SDS-polyacrylamide gels (10%), electrophoretically separated and blotted onto nitrocellulose (Frungieri *et al.* 2002). Protein concentrations were measured by the method of Lowry *et al.* (1951). Blots were incubated with rabbit polyclonal anti-PTGS2 antiserum (1:250, Cayman Chemical, Ann Arbor, MI, USA), mouse monoclonal anti-actin antibody (1:5000, Calbiochem, La Jolla, CA, USA), mouse monoclonal anti-phospho-MAPK3/1 antibody (1:500, Cell Signalling Technology Inc., Beverly, MA, USA) and rabbit polyclonal anti-MAPK3/1 antiserum (1:500, Cell Signalling Technology Inc.), and subsequently with peroxidase-labelled secondary antibodies (1:2000 goat anti-mouse IgM, Calbiochem, for actin; 1:1000 sheep anti-mouse IgG, GE Healthcare, Wauwatosa, WI, USA, for phospho MAPK3/1 and goat anti-rabbit IgG, 1:2500, Sigma, for PTGS2 and MAPK3/1). Signals were detected with an ECL kit (Amersham Pharmacia Biotech AB).

RT-PCR analysis

RNA was extracted from 2.5×10^5 purified Leydig cells of young adult hamsters kept under the LD photoperiod conditions using the QIAGEN RNeasy mini kit (Qiagen Inc). Then, RT-reaction using dN6 random primers followed by PCR amplification was performed (Frungieri *et al.* 2002).

The oligonucleotide primers used to amplify a 193 bp region of the hamster β-actin gene by PCR were 5′-GGA TGC AGA AGG AGA TCA-3′ (sense primer) and 5′-CTA GAA GCA TTT GCG GTG-3′ (antisense primer). Since information about exon structure was available at GenBank, oligonucleotide primers were designed as homologous to regions of different exons.

The oligonucleotide primers used to amplify hamster *PTGS2* cDNAs of 292 bp were 5′-TGT ATG TAT GAG TGT GGG A-3′ (sense primer) and 5′-GGC TTC CCA GCT TTT GTA-3′ (antisense primer). These primers were previously tested for amplification of *PTGS2* cDNAs in hamster Leydig cells (Frungieri *et al.* 2006). Since information about complete Syrian hamster *PTGS2* mRNA is not available at GenBank, *PTGS2* primers were designed as homologous to regions of different exons of the human *PTGS2* sequence.

PCR conditions were 95 °C for 5 min, followed by cycles of 94 °C for 1 min, 55 °C (annealing temperature) for 1 min and 72 °C for 1 min, and a final incubation at 72 °C for 5 min. The number of cycles used (30–35 cycles) was previously validated in order to avoid saturation of the band intensities. PCR products were separated on 2% agarose gels, visualized with ethidium bromide, and bands of expected molecular sizes were gel eluted and subcloned in pGEM-T vector (Promega Corporation). The identity of the cDNA clones was confirmed by sequencing, using a fluorescence-based dideoxy-sequencing reaction and an automated sequence analysis on an ABI 373A DNA sequencer. The final *PTGS2* cDNA sequence obtained from the analysis of LD hamster Leydig cells (representing four independently derived identical sequences) was submitted to GenBank (accession number AY426532). This partial sequence showed 99.0% homology with human, 82.9% homology with rat and 85.6% homology with mouse *PTGS2* at nucleotide level, and represents between 6.5 and 7.5% of the predicted entire *PTGS2* mRNA sequences for human, rat and mouse.

$PGF_{2\alpha}$ immunoassay

Approximately, 7.5×10^5 hamster Leydig cells were used to determine $PGF_{2\alpha}$ levels in the incubation media. After 2- and 3-h incubations, media were acidified using HCl 2 N (pH 3.5), injected into a 200 mg C18 column, and 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. $PGF_{2\alpha}$ was assayed using a commercially available kit (Cayman Chemical) according to the method described by Frungieri *et al.* (2002). The minimum detectable immunoassay concentration was 0.4 fmol/tube. Intra- and inter-assay CVs were $<10\%$ and $<8\%$ respectively. $PGF_{2\alpha}$ levels were expressed as fmol per 10^6 Leydig cells.

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 ± S.E.M.

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

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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