Testosterone induction of prostaglandin-endoperoxide synthase 2 expression and prostaglandin F2α production in hamster Leydig cells

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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 PGF2α 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/PGF2α. In active hamster Leydig cells, LH/hCG and testosterone induced PTGS2 and PGF2α production, and their actions were abolished by the antiandrogen bicalutamide (B). 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 B 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/PGF2α in hamster Leydig cells via androgen receptors and a non-classical mechanism that involves MAPK3/1 activation. Since PGF2α 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 isozenzyme type 1 (PTGS1) is found in most cell types. By contrast, expression of the inducible isozenzyme 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 isozenzymes 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. PGD2, PGE2, PGF2α) 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
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-PGJ2 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 PGF2α 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
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
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 PGF2α synthesis which, as we have previously demonstrated, inhibits LH-stimulated testosterone production in the reproducibly 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
induced PTGS2 expression and PGF$_{2a}$ 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 (Frunieri et al. 2006). By immunohistochemistry, we found only positive PTGS2 immunostaining in Leydig cells of the reproductively active seasonal breeder Syrian hamster (Frunieri 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$_{2a}$ participate in the modulation of testosterone secretion (Frunieri 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 4](https://example.com/fig4.png)

**Figure 4** Testosterone and LH/hCG induction of PGF$_{2a}$ 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$_{2a}$ 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).

![Figure 5](https://example.com/fig5.png)

**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.
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 photoperiod-induced testicular active–inactive transition in hamsters.

Although immunohistochemical analyses showed the expression of PTGS2 in the cytoplasm of interstitial cells with the characteristic punctate 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 dibutylr 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 PGD2 and PGE2, 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 PGE2 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 PGE2 production. Testosterone (1 μM) significantly increased PTGS2

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 immunoblot 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 analysis, 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.
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 (Frungeri et al. 1996a). 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 antiandrosten 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 $2.0 \times 10^{-10}$ and $4.0 \times 10^{-10}$ 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 repressed adult hamsters have been previously established (Frungeri 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 (Frungeri et al. 1996a), 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
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, Sonderfian 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/potential 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, Pintus 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 Rijnen et al. 1995).

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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/PGF2α in testicular STAR/17β-hydroxysteroid dehydrogenase (HSD17B) expression and testosterone production via PGF2α (FP) receptors. Current data indicate a stimulatory action of testosterone on testicular PTGS2/PGF2α. Therefore, we may hypothesize about the existence of a regulatory loop in which testosterone induces PTGS2 expression and PGF2α synthesis, but in which PTGS2/PGF2α 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 PGF2α 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/PGF2α 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. 1996b). Animals had free access to water and Purina formula chow. Hamsters were killed by asphyxia with carbon dioxide (CO2) 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 PGF2α 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. 1996b) 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 antisera 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. (1996b, 1996c). An antibody to testosterone-7α-butyrate-BSA (Medicorp Inc., Montreal, Canada) that has 35% cross-reaction with DHT and 0.04% cross-reaction with androstan-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 SD 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.

<table>
<thead>
<tr>
<th>Age</th>
<th>LH (ng/ml)</th>
<th>Total androgens (testosterone and DHT ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18-day-old</td>
<td>1.91 ± 0.23b</td>
<td>0.66 ± 0.06b</td>
</tr>
<tr>
<td>46-day-old</td>
<td>8.29 ± 0.76b</td>
<td>5.12 ± 0.32b</td>
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<tr>
<td>60-day-old</td>
<td>4.32 ± 0.85b</td>
<td>6.36 ± 0.35b</td>
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<tr>
<td>90-day-old</td>
<td>4.66 ± 0.92b</td>
<td>5.23 ± 0.33b</td>
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<tr>
<td>200-day-old</td>
<td>3.23 ± 1.02b</td>
<td>4.02 ± 0.51b</td>
</tr>
<tr>
<td>LD photoperiod</td>
<td>4.69 ± 0.55a</td>
<td>5.72 ± 0.21a</td>
</tr>
<tr>
<td>16 weeks in SD photoperiod</td>
<td>0.46 ± 0.08b</td>
<td>0.83 ± 0.17b</td>
</tr>
</tbody>
</table>

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% H2O2 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 NaPO4, H2O2, 0.15 M CaNa, 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 antisera (goat anti-rabbit IgG; 1:500, Vector Lab., CA, USA) for 2 h at room temperature. Finally, immunoreaction was visualized with 0.01% H2O2 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 antisera 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 1 (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 cm2 sterile flasks, and placed in an incubator at 37 °C under a humid atmosphere with 5% CO2 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 Frangieri 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 × 105 (for RT-PCR) or 7.5 × 105 (for immunoblotting and PGR2a production) cells were incubated at 37 °C under a humid atmosphere with 5% CO2 and in the presence of the following chemicals: 100 μl/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 PGR2a concentrations were determined by immunoassay.
conditions (10% mercaptoethanol). Leydig cells protein homogenates (100 μg) were loaded onto tricine-SDS-polyacrylamide gels (10%), electrophoretically separated and blotted onto nitrocellulose (Fangrui 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 2.5×10⁵ 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 (Frugieri et al. 2002).

The oligonucleotide primers used to amplify a 193 bp region of the hamster β-actin gene were 5'-GGA TGC AGA AGG AGA TCA-3' (sense primer) and 5'-GGA TAA GAA GCA TTT GCG GT-3' (antisense primer). Hence 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'-TTT AGT 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 (Frugieri 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 AF426532). 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.

**PGF₂α immunoassay**

Approximately, 7.5×10⁵ hamster Leydig cells were used to determine PGF₂α 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. PGF₂α was assayed using a commercially available kit (Cayman Chemical) according to the method described by Frugieri et al. (2002). The minimum detectable immunoassay concentration was 0.4 fmol/tube. Intra- and inter-assay CVs were <10% and <8% respectively. PGF₂α levels were expressed as fmol per 10⁶ 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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