Endocrine, *vol. 24, no. 1, 39–45, June 2004* 0969–711X/04/24:39–45/$25.00

L-Prostaglandin D Synthase Expression and Regulation in Mouse Testis and Epididymis During Sexual Maturation and Testosterone Treatment after Castration

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Lipocalin-type prostaglandin D synthase (L-PGDS) is highly expressed in the adult testis and epididymis of many mammals. The present study was to investigate L-PGDS expression in mouse testis and epididymis during sexual maturation, and the effects of testosterone replacement on L-PGDS expression in epididymis by *in situ* hybridization and immunohistochemistry. Both L-PGDS mRNA and protein were highly expressed in the interstitial tissue of adult testis. L-PGDS mRNA was first detected on d 30 after birth and exhibited an abundant signal in adult caput and cauda epididymis. L-PGDS immunostaining was first observed on d 30 after birth. There was a strong level of L-PGDS immunostaining in adult epididymis. Castrated male mice were treated with either vehicle or testosterone propionate following 3 d postcastration. L-PGDS expression steadily declined in a time-dependent fashion in control groups. No L-PGDS mRNA expression or immunostaining was detected in the controls for 12 d. When the castrated mice were treated with testosterone propionate for 5 or 12 d, L-PGDS expression was significantly increased in the whole epididymis. These data suggest that L-PGDS expression in mouse epididymis gradually declined in parallel to the declining concentration of endogenous androgen after castration and increased with the treatment of exogenous testosterone, indicating that L-PGDS expression in mouse epididymis was modulated by androgen levels. However, differential expression in different areas of the epididymis may also be influenced by factors derived from the testis.

Key Words: L-PGDS; mouse; testis; epididymis.

Introduction

Prostaglandin D synthase (PGDS) catalyzes the isomerization of PGH_2 to PGD_2. Two distinct enzymes have been cloned as PGDS, lipocalin (brain)-type PGDS (L-PGDS) and hematopoietic PGDS (hPGDS) *(1)*. As a bifunctional protein, L-PGDS catalyzes the conversion of PGH_2 to PGD_2, and also binds and transports small lipophilic ligands such as retinoids, steroids, thyroid hormones, biliverdin, and bilirubin *(2)*. L-PGDS, formerly identified as β-trace, has been localized in the central nervous system and male genital organs of various mammals, and is secreted into cerebrospinal fluid, seminal plasma, and plasma, respectively *(3)*. Furthermore, hPGDS mRNA is highly expressed in female reproductive organs such as the oviduct and uterus *(4)*.

L-PGDS expression has been detected in adult testis and epididymis of rat, hamster, and cynomolgus monkey by *in situ* hybridization and immunohistochemistry *(5)*. However, in humans the concentration of L-PGDS is significantly lower in oligozoospermic men than in normozoospermic men *(6)*. In addition, L-PGDS protein was 3.5-fold greater in the seminal plasma of bulls with above average fertility when compared to bulls of average and below average fertility *(7)*. Tissue levels of L-PGDS in rat testis and epididymis during sexual maturation and castration was examined by RIA *(8)*. The expression and regulation of L-PGDS in rat testis and epididymis were recently reported *(9)*. PGDS mRNA expression was localized to seminiferous tubules of neonates but was only found in the testicular interstitium of the adult in mouse testis *(10)*. By using *in situ* hybridization and immunohistochemistry, the present study provided a more detailed investigation of L-PGDS expression and regulation in mouse testis and epididymis during sexual maturation and following castration with androgen replacement.

Results

L-PGDS Expression in Mouse Testis During Sexual Maturation

In the neonatal testis of d 1, a low level of L-PGDS mRNA expression was observed and was predominantly within the seminiferous tubules (Fig. 1A). L-PGDS mRNA expression was not detected in testis of d 10, 20, 25 after birth (Figs. 1B–D); however, at 30 d after birth, weak L-PGDS mRNA signals were first detected in the Leydig cells (Fig. 1E). By d 50 postpartum, a strong level of L-PGDS mRNA expression was present in the Leydig cells (Fig. 1F).

Received January 12, 2004; Revised March 30, 2004; Accepted April 30, 2004.

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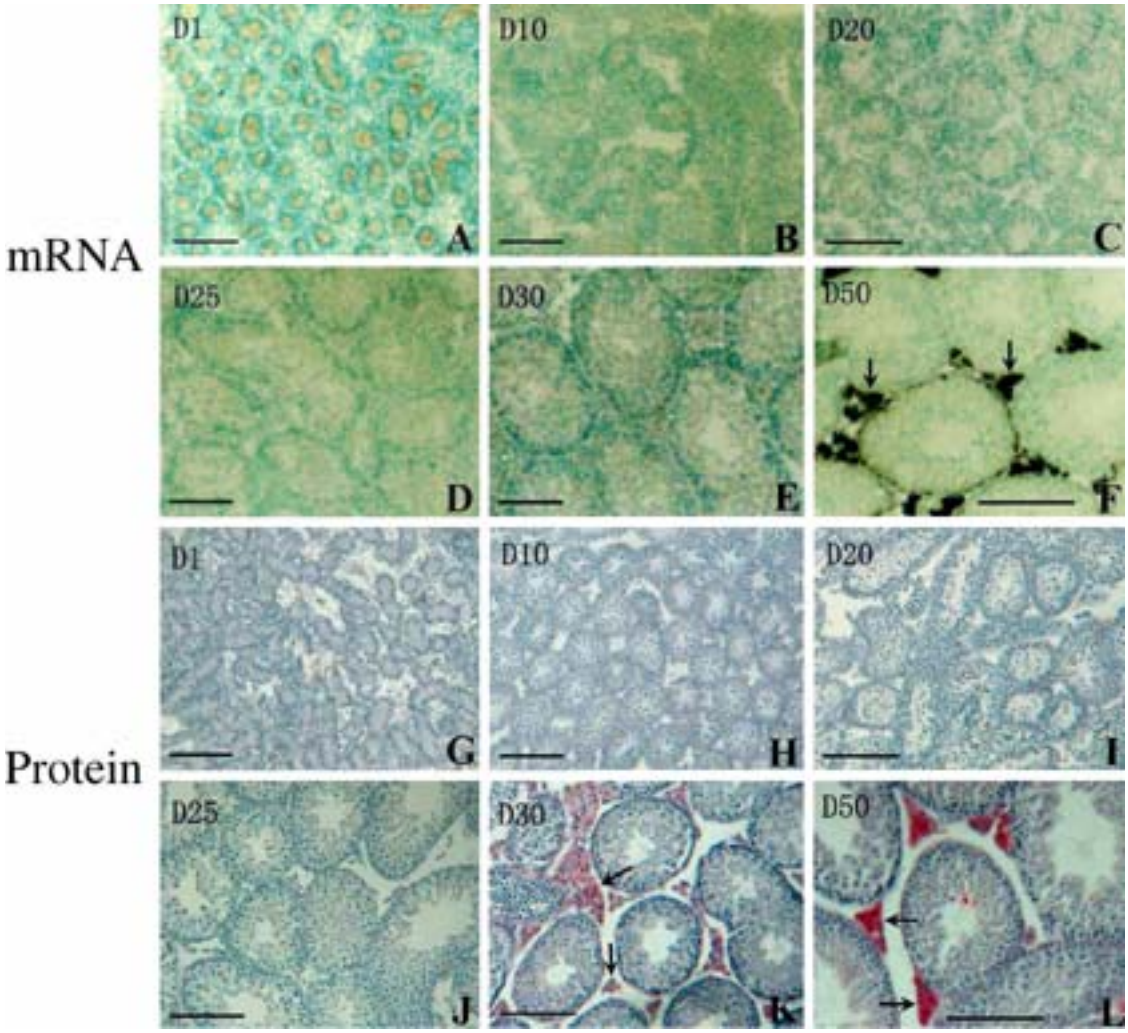

Fig. 1. *In situ* hybridization and immunostaining of L-PGDS in mouse testis on d 1 (**A**, **G**), d 10 (**B**, **H**), d 20 (**C**, **I**), d 25 (**D**, **J**), d 30 (**E**, **K**), and d 50 (**F**, **L**) after birth, respectively. Arrow: Leydig cells. Bar = 40 μm.

When the DIG-tagged sense L-PGDS probe was used as a control for hybridization background, no signal was detected in the testis of 50 d old mice (data not shown).

There was no detectable L-PGDS immunostaining of the protein in the testis until d 30 after birth (Figs. 1G–K) at which time a low level of L-PGDS immunostaining was observed in the interstitial tissue (Fig. 1K). By 50 d postpartum, strong L -PGDS immunostaining was localized to the Leydig cells (Fig. 1L). No detectable L-PGDS immunostaining was observed when normal rabbit IgG was used to replace rabbit anti-L-PGDS antibody for evaluation of nonspecific background (data not shown).

L-PGDS Expression in Caput Epididymis During Sexual Maturation

No L-PGDS mRNA expression was observed in caput epididymis on d 10 and 15 after birth (data not known). However, a basal level of L-PGDS expression was seen in the epididymal tubules on d 20 (data not known), gradually increased on d 25 and 30 (Figs. 2A,B), and reached a very strong expression by d 50 after birth (Fig. 2C).

L-PGDS protein as detected by immunostaining was not observed in caput epididymis from d 10 to 30 after birth during the sexual maturation (Figs. 2D,E). On d 50 after birth, a low level of L-PGDS immunostaining was seen in the epithelial cells of epididymal tubules. Interestingly, L-PGDS immunostaining was strongly detected in the lumen of some epididymal tubules (Fig. 2F).

L-PGDS Expression in Cauda Epididymis During Sexual Maturation

In cauda epididymis L-PGDS mRNA signal not detected on d 10 and 15 after birth (data not shown); however, by d 20 a basal level of L-PGDS expression was observed in the epididymal tubules (data not shown). On d 25, a clear signal for L-PGDS expression was seen in some epididymal

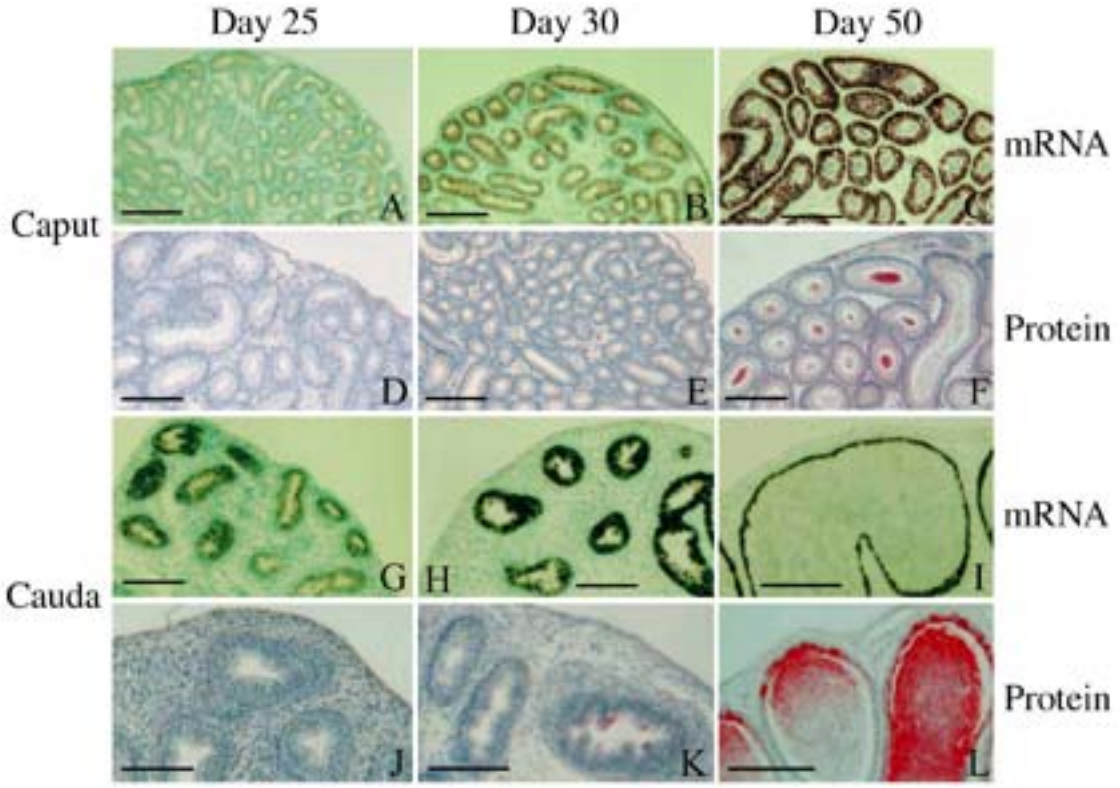

Fig. 2. *In situ* hybridization and immunostaining of L-PGDS in caput epididymis on d 25 (**A**, **D**), 30 (**B**, **E**), and 50 (**C**, **F**) after birth, respectively. *In situ* hybridization and immunostaining of L-PGDS in cauda epididymis on d 25 (**G**, **J**), 30 (**H**, **K**), and 50 (**I**, **L**) after birth, respectively. Bar = 40 μm.

tubules (Fig. 2G). However, on d 30 and 50, L-PGDS mRNA signals were strongly detected in all epididymal tubules (Figs. 2H,I).

The immunostaining of the L-PGDS protein was not detected in cauda epididymis on d 10, 15, 20, and 25 after birth (Fig. 3J). A basal level of L-PGDS immunostaining was observed in some of the epididymal tubules on d 30 (Fig. 3K). L-PGDS immunostaining was strongly detected in the epithelial cells and the lumen of cauda epididymal on d 50 after birth (Fig. 3L).

Effect of Castration and Hormone Supplementation on L-PGDS Expression in Caput Epididymis

Castrated mice were treated with either vehicle or testosterone propionate 3 d after surgery. In caput epididymis, a basal level of L-PGDS mRNA expression was observed following the treatment with vehicle for 1 and 5 d (data not shown). After castrated mice were treated with vehicle for 12 d, there was no detectable L-PGDS expression in caput epididymis (Fig. 3A). However, a low level of L-PGDS expression was seen in the epithelial cells after the mice were treated with testosterone propionate for 1 d (data not known). After the castrated mice were treated with testosterone propionate for 5 and 12 d, L-PGDS expression was strongly observed in the epithelial cells of caput epididymis (Fig. 3B).

L-PGDS immunostaining was not detectable in caput epididymis after the castrated mice were treated with vehicle for 1, 5, and 12 d, respectively (Fig. 3C). After these mice were treated with testosterone propionate for 1, 5, or 12 d, there was a low level of L-PGDS immunostaining in the epithelial cells of epididymal tubules (Fig. 3D).

Effect of Castration and Hormone Supplementation on L-PGDS Expression in Corpus Epididymis

In corpus epididymis, a low level of L-PGDS expression was observed in the epithelial cells after treated with vehicle for 1 and 5 d (data not shown). However, no L-PGDS mRNA expression was seen when the castrated mice were treated with vehicle for 12 d (Fig. 3E). After the castrated mice were treated with testosterone propionate for 1 d, only a low level of L-PGDS expression was observed, similar to that treated with vehicle for 1 d (data not shown). L-PGDS expression was stronger after treatment with testosterone propionate for 5 d compared to the control for 5 d (data not shown). After the castrated mice were treated with testosterone propionate for 12 d, L-PGDS expression was clearly detectable in the epithelial cells (Fig. 3F).

There was no detectable L-PGDS immunostaining in the corpus epididymis after the castrated mice were treated with vehicle for 1, 5, or 12 d (Fig. 3G). However, L-PGDS immunostaining was detected in the principal cells after the

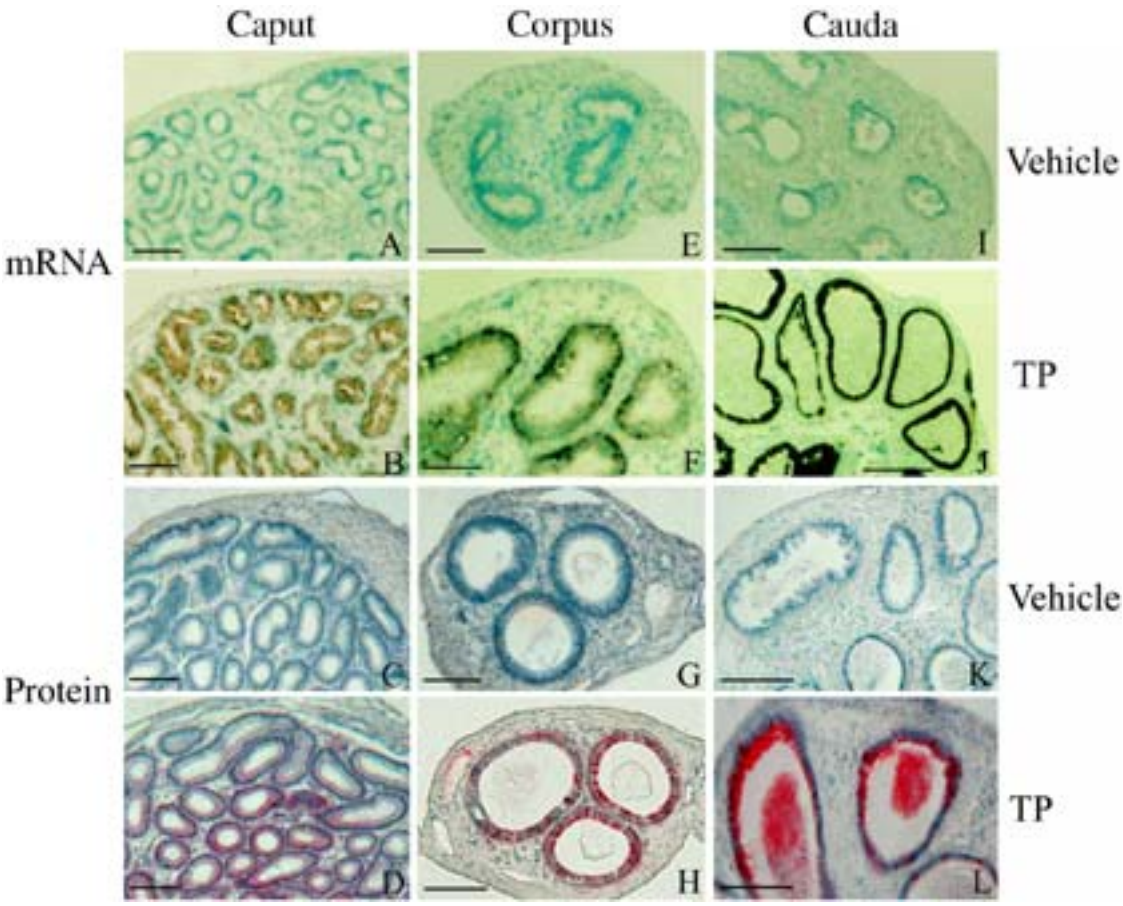

Fig. 3. *In situ* hybridization of L-PGDS mRNA in caput (**A**, **B**), corpus (**E**, **F**), and cauda (**I**, **J**) epididymis of castrated mice treated with vehicle and testosterone propionate (TP) for 12 d, respectively. L-PGDS immunostaining in caput (**C**, **D**), corpus (**G**, **H**), and cauda epididymis (**K**, **L**) of castrated male mice treated with vehicle or testosterone propionate (TP) for 12 d, respectively. Bar = 40 μm.

castrated mice were treated with testosterone propionate for 1, 5, or 12 d (Fig. 3H).

Effect of Castration and Hormone Supplementation on L-PGDS Expression in Cauda Epididymis

L-PGDS mRNA signals were strongly seen in the epithelial cells of epididymal tubules in the control group treated with vehicle for 1 d (data not shown). L-PGDS expression was declined in the 5-d control group (data not shown) and was not detectable in the control group treated with vehicle for 12 d (Fig. 3I). The treatment with testosterone propionate for 1 d had no obvious effect on L-PGDS expression compared to 1-d control (data not shown). L-PGDS expression was significantly stronger after the castrated mice were treated with testosterone propionate for 5 d compared to the control for 5 d (data not shown). There were no detectable signals in the control for 12 d (Fig. 3I). When the castrated mice were treated with testosterone propionate for 12 d, L-PGDS expression was strongly detected in the epididymal tubules (Fig. 3J).

A low level of L-PGDS immunostaining was observed in some of the epithelial cells of epididymal tubules in the control for 1 and 5 d (data not shown), but no immunostaining was seen in the control for 12 d (Fig. 3K). After the castrated mice were treated with testosterone propionate for 1, 5, or 12 d, L-PGDS immunostaining was strongly detected in the epithelial cells and the lumen (Fig. 3L).

Discussion

L-PGDS expression in mouse testis during sexual maturation was studied by *in situ* hybridization for mRNA expression and immunohistochemistry for detection of protein presence. On d 1 after birth, a low level of L-PGDS mRNA was seen within the seminiferous tubules, while no L-PGDS protein was seen. Neither L-PGDS mRNA nor L-PGDS immunostaining was detected on d 10, 20, and 25 after birth. However, both L-PGDS mRNA and L-PGDS protein were clearly evident in the interstitial tissue on d 50 after birth. In a previous study, PGDS mRNA appeared to be expressed

only in the seminiferous tubules of neonatal mouse testes and only in the interstitial tissue of adult testis as evaluated by *in situ* hybridization *(2)*. Hoffmann et al. *(11)* reported that L-PGDS mRNA expression was localized only in Leydig cells of mouse testis by *in situ* hybridization. However, L-PGDS immunostaining was localized within the Sertoli cells only at stages VI–VIII of the cycle of the seminiferous epithelium, with intense immunostaining observed throughout the interstitial tissue *(12)*. In humans, L-PGDS expression was localized in both seminiferous tubules and Leydig cells *(6)*. In sexually mature rats, L-PGDS mRNA was weakly expressed only in the testicular peritubular cells, whereas L-PGDS immunostaining was highly detected in the Leydig cells *(9)*. In our study, L-PGDS mRNA and immunostaining were localized in the interstitial tissue of adult testis; however, we observed no expression of L-PGDS mRNA or protein in the seminiferous tubules of adult mice. It was difficult to identify the cell type that the immunostaining localized too in the interstitium. However, based on the data from rat and other species, L-PGDS immunostaining should be located in Leydig cells.

In our study, L-PGDS expression between caput and cauda epididymis was slightly different. On d 25, 30, and 50 after birth, L-PGDS mRNA signals in the caput epididymis were weaker than that in the cauda epididymis. This expression pattern contrasted to our previous studies in rat and bull. In rat epididymis, the steady-state L-PGDS mRNA level was the highest in the caput, followed by the cauda and corpus by RT-PCR analysis. During maturation, L-PGDS concentration increased steadily in the epididymis and reached its highest level on d 60 after birth *(8)*. In Holstein bulls, prostaglandin D synthase expression was observed in the epithelial cells of the epididymides with greatest expression occurring in the caput epididymidis *(13)*. Additionally, there was a difference between L-PGDS mRNA signals and immunostaining in mouse epididymis in our study. Although L-PGDS mRNA expression was seen in the caput epididymis on d 25 and 30 after birth, no corresponding immunostaining was detected. Moreover, L-PGDS mRNA signals were strongly observed in the cauda epididymis on d 25 and 30 after birth, while a low level of L-PGDS immunostaining was seen only on d 30 after birth. These data support the idea of a posttranscriptional regulation for L-PGS expression. In the epididymis of 4.5-mo-old sheep, L-PGDS mRNA was present in all of the epididymis, but translation occurred in only the proximal caput *(14)*.

Both L-PGDS mRNA signals and L-PGDS protein immunostaining were strongly detected in adult mouse epididymis and to evaluate androgen requirements for L-PGDS expression in epididymis, male mice were treated with either vehicle or testosterone propionate 3 d after castration. L-PGDS expression in the control groups exhibited a significant decline in a time-dependent fashion. Although there were detectable levels of L-PGDS expression in the castrate controls at 1 or 5 d, L-PGDS mRNA signals and immunostaining were undetectable in the control for 12 d. After androgen replacement in the castrated mice for 5 or 12 d, L-PGDS expression significantly increased in the whole epididymis. These data suggest that L-PGDS expression in mouse epididymis gradually decreased as endogenous androgen levels declined following castration and that L-PGDS expression increased with androgen replacement. These data strongly suggest that L-PGDS expression is under the control of androgen. In the rat, both L-PGDS mRNA expression and protein immunostaining were significantly reduced in the caput, corpus, and cauda epididymis after castration. Testosterone propionate treatment induced a significant increase of L-PGDS expression in the epididymis of castrated rats *(9)*. In another study, orchiectomy also induced a drastic reduction of L-PGDS concentration in all three epididymal compartments of rat epididymis. Although dihydrotestosterone (DHT) treatment for 5 d induced a significant increase of L-PGDS concentration in caput epididymis after castration, there was a slight increase in corpus and cauda epididymis after DHT administration *(8)*. In the ram, castration and efferent duct ligation led to a decrease in PGDS mRNA synthesis and PGDS secretion. PGDS mRNA was not detected in the epididymis of stallion 1 mo after castration, but was restored by testosterone supplementation *(15)*. In our study, testosterone propionate treatment had differential effects on the L-PGDS expression in the caput, corpus, and cauda epididymis. L-PGDS expression in cauda epididymis following testosterone propionate treatment was greater than that in caput and corpus epididymis. Moreover, L-PGDS expression in caput epididymis following 5- or 12-d treatment with testosterone propionate was diminished when compared to that in adult caput epididymis. On the one hand, this suggests that the epididymal L-PGDS level is not entirely regulated by androgen and, on the other hand, factors from the gonad such as testis-derived growth factors *(16)* or spermatozoa-associated factors *(17)* are also likely to be involved in its regulation. It was reported that intratesticular administration of PGD_2 in the rat induced a significant decrease in testicular testosterone level *(18)*. PGD_2 may have a role in regulating testosterone level essential for sperm maturation. An alternate explanation is that this may reflect a consequence of regionalized expression pattern and function in each epididymal region (caput, corpus, and cauda). The acquisition of the sperm motility and zona pellucida binding capability mainly occur in the caput and corpus epididymis *(19,20)*, while the cauda epididymis is specialized in sperm storage *(21)*. Thus, the regionalized expression of L-PGDS in caput, corpus and cauda epididymis may have something to do with their differential functions for sperm transport and maturation.

In conclusion, L-PGDS was highly expressed in adult mouse testis and epididymis. Castration led to a gradual decrease of L-PGDS expression in the epididymis in a time-

dependent fashion. L-PGDS expression in the epididymis of castrated males was significantly increased by testosterone propionate treatment.

Materials and Methods

Animal Treatments

Mature mice (Kunming White, outbred) were caged in a controlled environment with a 14 h light/10 h dark cycle. All animal procedures were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University. To confirm reproducibility of results, at least three mice per group were used in each stage or treatment in this study.

Sexual Maturation

For the development and maturation study, the testis and epididymis were collected from male mice on d 1, 5, 10, 15, 20, 25, 30, and 50 after birth. In newborn mice, only caput and cauda epididymal tissues were collected for analysis because the epididymis was very small. One testis and one epididymis of each mouse were fixed in Bouin's solution for 24 h and embedded in paraffin for immunostaining. The contralateral testis and epididymis were frozen in liquid nitrogen and stored at −70°C for *in situ* hybridization.

Castration and Hormonal Replacement

Surgical procedures on mature male mice were performed using sterile technique with anesthesia. After male mice (weighing about 30 g) were bilaterally orchiectomized, the epididymides were returned into the scrotum. Three days after surgery, the mice were intramuscularly injected with testosterone propionate (0.1 mg/mouse/d, Sigma, St. Louis, MO, USA) or sesame oil (as a vehicle, Sigma) for 1, 5, and 12 d. Mature male mice were sham operated as the control for the castrate group. The caput, corpus, and cauda epididymis were collected and treated as above, respectively.

Hybridization Probe

The hybridization probe used in this study was originally cloned from total RNAs of mouse uterus by reverse transcription and amplification with forward primer 5'-CGGAATTCATGCTGTGGATGGGTTTG and reverse primer 5'-GCGGATCCAGTGACAGAGCAAGGGAG designed from mouse L-PGDS (75–704 bp, Genbank accession number AB006361). In these primers, protection bases (CG) and *EcoR*I sites(GAATTC, underlined) were added at the 5' end of the forward primer, and protection bases (CG) and *BamH*I sites (GGATCC, underlined) at the 5' end of the reverse primer. The PCR fragment (630 bp + 16 bp from the protection bases and *EcoR*I/*BamH*I sites) was recovered from the agarose gel and cloned into pGEM-3Zf (+) plasmid through *EcoR*I and *BamH*I sites, respectively. The cloned L-PGDS fragment was verified by sequencing. The plasmid containing the L-PGDS fragment was linearized with the appropriate enzymes and labeled. Digoxigenin (DIG)-labeled antisense and sense cRNA probes were transcribed in vitro using a DIG RNA labeling kit (Roche Diagnostics GmbH, Mannheim, Germany).

In Situ *Hybridization*

Mouse testis and/or epididymis were flash frozen in liquid nitrogen. Frozen sections (10 μm) were mounted on 3-aminopropyltriethoxy-silane (Sigma)-coated slides and fixed in 4% (w/v) paraformaldehyde solution in phosphate-buffered saline (PBS). The sections were washed in PBS twice, treated in 1% (v/v) Triton-100 for 20 min, and again washed three times in PBS. Following prehybridization washes in 50% (v/v) formamide and 5X SSC (1X SSC is 0.15 *M* sodium chloride, 0.015 *M* sodium citrate) at room temperature for 15 min, the sections were hybridized in the hybridization buffer [5X SSC, 50% (v/v) formamide, 0.02% (w/v) BSA, 250 μg/mL yeast tRNA, 10% (w/v) dextran sulfate, 1 μg/mL denatured DIG-labeled antisense or sense RNA probe for mouse L-PGDS] at 55°C for 16 h. After hybridization, the sections were washed for stringency in 50% (v/v) formamide/5X SSC at 55°C for 15 min, 50% (v/v) formamide/2X SSC at 55°C for 30 min, 50% (v/v) formamide/0.2X SSC at 55°C twice for 30 min each, and 0.2X SSC at room temperature for 5 min. After nonspecific binding was blocked in 1% (w/v) block mix (Roche Diagnostics GmbH) for 1 h, the sections were incubated in sheep anti-DIG antibody conjugated to alkaline phosphatase (1:5000, Roche Diagnostics GmbH) in 1% block mix (Roche Diagnostics GmbH) overnight at 4°C. The signal was visualized with 0.4 m*M* 5-bromo-4-chloro-3-indolyl phosphate and 0.4 m*M* nitroblue tetrazolium in the buffer containing 100 m*M* Tris-HCl, pH 9.5, 100 m*M* NaCl and 50 m*M* $MgCl_2$. Endogenous alkaline phosphatase activity was inhibited with 2 m*M* levamisole (Sigma). Sections were counterstained with 1% (w/v) methyl green in 0.12 *M* glacial acetic acid and 0.08 *M* sodium acetate for 30 min.

Immunohistochemistry

Sections (7 μm) were cut from paraffin embedded tissues, mounted onto 2% 3-aminopropyltriethoxysilane-coated slides, deparaffinized, and rehydrated. Nonspecific binding of sections was blocked by applying 10% normal horse serum in PBS for 1 h. The sections were incubated with rabbit anti-human L-PGDS (1:250, Cayman Chemical, Ann Arbor, MI) in 10% horse serum overnight at 4°C. After three washes in PBS for 5 min each, the sections were incubated with biotinylated goat anti-rabbit IgG followed by incubation with avidin–alkaline phosphatase complex and Vector Red according to the manufacturer's protocol (Vectastain ABC-AP kit, Vector Laboratories, Burlingame, CA). Endogenous alkaline phosphatase activity was inhibited with levamisole (Sigma). In some sections, rabbit anti-human L-PGDS was replaced with normal rabbit IgG as a negative control. For visualiza-

tion of nuclear morphology, the sections were counterstained with hematoxylin and mounted. The degree of staining was assessed subjectively by blinded examination of the slides by two investigators.

Acknowledgment

This work was supported by Chinese National Natural Science Foundation grants 30270163, 30200196, 30170110 and 30330060, and the Special Funds for Major State Basic Research Project (G1999055903).

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