Expression of the lipocalin-type prostaglandin D synthase gene in the reproductive tracts of Holstein bulls

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The aim of this study was to localize expression of the prostaglandin D synthase gene in the reproductive tracts of Holstein bulls using northern blotting and *in situ* hybridization. For northern blotting, a digoxigenin-labelled prostaglandin D synthase cDNA probe was used to probe blots containing RNA isolated from the testes, epididymides, vas deferens, ampullae, seminal vesicles, prostate and bulbourethral glands of bulls. The digoxigenin-labelled cDNA for the bovine homologue of prostaglandin D synthase hybridized to a single band (approximately 0.9 kb) to RNA samples from the caput, corpus and cauda epididymides, as well as RNA samples from the vas deferens and the ampulla. The probe also detected a single band in testis samples, although the transcript size was slightly larger (approximately 1.0 kb) than the transcript found in the other tissues. The highest expression of prostaglandin D synthase was observed in the testes and caput epididymides. Prostaglandin D synthase transcripts were not found in the seminal vesicles or the prostate or bulbourethral glands using northern blotting. For *in situ* hybridization, antisense and sense riboprobes were synthesized and used to hybridize to cryosections obtained from the reproductive tissues of bulls. *In situ* hybridization of bull testes showed that prostaglandin D synthase transcripts were present within the germ cells in the adluminal compartment of the seminiferous tubules containing round and elongated spermatids, indicating that expression varied with stage of development of the seminiferous tubules. Prostaglandin D synthase expression was observed in the epithelial cells of the epididymides with greatest expression occurring in the caput epididymidis. Some expression was also observed in the epithelial cells of the vas deferens and a few cells of some lobules in the prostate and bulbourethral glands. Expression of the prostaglandin D synthase gene was not detected in ampullae or seminal vesicles by *in situ* hybridization.

Introduction

Gerena *et al.* (1998) recently identified a 26 kDa protein associated with high fertility that is present in bull seminal plasma, as lipocalin-type prostaglandin D synthase. Prostaglandin D synthase is a member of the lipocalin superfamily (Nagata *et al.*, 1991; Urade *et al.*, 1995), which is a group of proteins that bind small hydrophobic molecules and specific cell surface receptors. Members of the lipocalin superfamily include retinol-binding protein, purpurin, β -lactoglobulin, α -1-microglobulin and C8 γ (Flower, 1994). Prostaglandin D synthase also catalyses the isomerization of PGH₂ to PGD₂, a major prostaglandin found in the brain of humans and rats (Urade *et al.*, 1985). PGD₂ appears to be involved in several functions *in vivo* including induction of sleep (Ueno *et al.*, 1993; Urade, 1996), temperature regulation

(Ueno *et al.*, 1982), suppression of LH release (Kinoshita *et al.*, 1982), anticonvulsion, nociception and modulation of odour response (Ito *et al.*, 1989).

Studies indicate that lipocalin-type prostaglandin D synthase, also known as beta-trace, may play a role in male reproductive function. Immunochemical and immunofluorescence studies have determined that beta-trace is present in human testes, epididymides, spermatic cords, ovaries and fallopian tubes, as well as several other tissues (Olsson and Nord, 1973). However, within the genital system, human epididymides contained the greatest amounts of beta-trace (Olsson and Nord, 1973). High concentrations (5 mg (100 ml)⁻¹) of beta-trace have been detected in human seminal fluid and reduced concentrations of beta-trace were found in semen from azoospermic men (Olsson, 1975). These data indicate that a reduction in the concentrations of lipocalin-type prostaglandin D synthase in semen may be associated with human infertility. More recently, Tokugawa *et al.* (1998) detected prostaglandin D synthase activity in human seminal plasma (0.05–1.83 nmol min⁻¹ per mg protein).

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Northern blotting revealed that prostaglandin D synthase mRNA was present in human testes, epididymides and prostate glands. Furthermore, immunohistochemical studies using anti-lipocalin type prostaglandin D synthase antibody detected prostaglandin D synthase in the Leydig cells of the testes, the epididymal epithelium and the secretory elements of the prostate gland (Tokugawa *et al.*, 1998).

Prostaglandin D synthase has also been detected in the reproductive tissues of other species. Prostaglandin D synthase activity was observed in the epididymides of rats (Ujihara *et al.*, 1988) and mice, and northern blotting demonstrated abundant expression of prostaglandin D synthase in the brain, testes, epididymides, ovaries and oviducts of rats (Eguchi *et al.*, 1996). Sorrentino *et al.* (1998) detected mRNA transcript in the testes and epididymides of rats using RT-PCR and demonstrated that mRNA transcripts within the testes were expressed by Sertoli cells and developing germ cells. The concentration of prostaglandin D synthase in the caput epididymides was six and 80 times greater than in the brain and testes, respectively, and indicated that prostaglandin D synthase gene expression was dependent on androgens, as well as other testicular factors (Sorrentino *et al.*, 1998).

Recent studies in our laboratory have focused on determining the origin of prostaglandin D synthase in the reproductive tracts of bulls. Prostaglandin D synthase was found in fluid from the rete testis and cauda epididymides of bulls, as well as in tissue extracts and histological sections from the testes, and caput, corpus and cauda epididymides (Gerena *et al.*, 1998; Rodríguez *et al.*, 1998).

The aim of the present study was to localize expression of the prostaglandin D synthase gene in the reproductive tracts of Holstein bulls to gain insight into possible fertility-related functions of prostaglandin D synthase in male reproductive tracts.

Materials and Methods

Collection of bull reproductive tissues

Reproductive tracts of four bulls previously maintained at the J. O. Almquist Research Center were excised within 30 min after the bulls were killed. The testes, epididymides (caput, corpus and cauda), vasa deferentia, ampullae, seminal vesicles, prostate and bulbourethral glands were isolated and plunged into liquid nitrogen. After transport to the laboratory, the tissues were stored at -80°C until used.

Amplification of prostaglandin D synthase cDNA

The pCR-Script vector containing the lipocalin-type prostaglandin D synthase cDNA was transformed into *Epicurian coli* XL1-blue competent cells (catalogue no 200249; Stratagene, La Jolla, CA) in accordance with the manufacturer's instructions. After the transformation, a bacterial colony was grown and the bacteria were harvested and lysed by the alkali method (Sambrook *et al.*, 1989). The plasmid was isolated and purified by precipitation with

polyethylene glycol (Sambrook *et al.*, 1989) and stored at -20°C until used for the preparation of probes for northern blotting and *in situ* hybridization.

Northern blotting

Total RNA was isolated from bull reproductive tissues using the Tri reagent (T-9424; Sigma Chemical Company, St Louis, MO) according to the manufacturer's instructions. RNA samples (10 μg) were loaded onto 1% (w/v) agarose gels containing 2.2 mol formaldehyde l^{-1} and electrophoresis was performed at 100 V (constant voltage) for 3–4 h. After gel electrophoresis, the RNA was transferred onto a nylon membrane (Nytran; Schleicher and Schuell, Keene, NH) by capillary action and the membrane was baked under vacuum for 2 h at 80°C .

The prostaglandin D synthase cDNA was released from the pCR-Script Amp SK vector by restriction enzyme digestion with EcoRI (Pharmacia, Piscataway, NJ) and SacI (Pharmacia). After digestion, the sample was run on a 0.8% (w/v) agarose gel and the prostaglandin D synthase cDNA was isolated and purified using the Gene Clean Kit (Catalogue no 1001-200; Bio 101 Inc, La Jolla, CA).

Northern blotting was performed using the digoxigenin (DIG) labelling and detection system (Boehringer Mannheim, Indianapolis, IN). Briefly, the prostaglandin D synthase cDNA was labelled by random priming using the DIG-high prime solution (Catalogue no 1585606; Boehringer Mannheim). The membrane was pre-hybridized in standard hybridization solution (5 $\times$ SSC (sodium chloride and sodium citrate), 0.1% (w/v) N-lauroylsarcosine, 0.02% (v/v) SDS and 1% (v/v) blocking reagent) for 30 min at 68°C and hybridized overnight at 68°C in 30 ml standard hybridization solution containing the DIG-labelled prostaglandin D synthase cDNA.

After hybridization, the membrane was washed for 2×15 min in $2 \times$ SSC and 0.1% (w/v) SDS at room temperature followed by 2×15 min washes in $0.1 \times$ SSC and 0.1% (w/v) SDS at 68°C . The membrane was rinsed in washing buffer (0.3% (v/v) Tween 20 in maleic acid buffer; maleic acid buffer: 0.1 mol maleic acid l^{-1} and 0.15 mol sodium chloride l^{-1} , pH 7.5), incubated for 30 min in blocking solution and incubated for 30 min in anti-DIG-AP conjugate solution (1:10 000 dilution; Catalogue no 1093274; Boehringer Mannheim). The membrane was washed for 2×15 min in washing buffer and equilibrated for 5 min in 30 ml detection buffer (prepared by diluting $10 \times$ blocking solution 1:10 in maleic acid buffer). The hybridized prostaglandin D synthase probe was visualized with the chemiluminescence substrate CSPD (disodium 3-(4-methoxy-spiro[1,2-dioxethane-3,2'-(5'-chloro)tricyclo[3.3.1^{3,7}]decan]-4-yl) phenyl phosphate) ready to use solution (Catalogue no 1755633; Boehringer Mannheim). The membrane was exposed to X-ray film (Fuji film; Fisher Scientific, Pittsburg, PA) at 37°C to enhance the luminescence reaction. After northern blotting the membrane was stripped and hybridized to a DIG-labelled 18S ribosomal RNA to check that the wells had been loaded evenly and that the RNA samples had been transferred completely to the nylon membrane.

In situ hybridization

Bull reproductive tissues were sectioned (10 µm thickness) using a cryostat and placed on poly-L-lysine (Sigma P-8920) coated slides. The slides were stored at -80°C until used for *in situ* hybridization.

The pCR-Script Amp SK vector containing the prostaglandin D synthase cDNA was linearized using either the restriction enzyme SacI (Pharmacia) or EcoRI (Pharmacia) to produce antisense and sense riboprobes, respectively. Linearized templates were treated with proteinase K, extracted in phenol-chloroform and precipitated with ethanol. Template DNA was resuspended in RNase-free water (approximately 1 µg µl⁻¹) and stored at -20°C until used for synthesis of riboprobes.

Riboprobes were synthesized using the T₃/T₇ Riboprobe *in vitro* transcription system (Catalogue no P1450; Promega, Madison, WI) according to the manufacturer's instructions. For the synthesis of prostaglandin D synthase antisense and sense riboprobes the EcoRI digest was transcribed with the T₃ polymerase and the SacI digest was transcribed with the T₇ polymerase. After synthesis of the riboprobes, the DNA templates were digested with RQ₁ DNase (Catalogue number M610A; Promega) and unincorporated nucleotides were removed using a NucTrap probe purification column (Catalogue no 400701; Stratagene) and push column beta shield device (Stratagene, Cat no. 400700) according to the manufacturer's instructions. The riboprobes were recovered by ammonium acetate precipitation and were resuspended in hybridization buffer at 7083 Beq µl⁻¹.

Slides with frozen sections were brought to room temperature on a slide warming plate, fixed for 30 min in 4% (w/v) paraformaldehyde in PBS (pH 7.0) and washed for 3 × 10 min in PBS. The sections were rinsed in ddH₂O and 0.1 mol triethanolamine (TEA) l⁻¹ for 1 min each before acetylation for 10 min in 0.1 mol TEA l⁻¹ containing 0.25% (v/v) acetic acid. The slides were rinsed in ddH₂O for 1 min and dehydrated in a 50, 70, 85, 95 and 2 × 100% ethanol series for 3 min each. The slides were dried on a slide warming plate. The riboprobes were denatured at 68°C for 10 min. Approximately 5–10 µl riboprobe and hybridization buffer were added to each section and a coverslip was placed over each section. Tissue sections were hybridized at 50°C for 3 h in a humidified chamber. Some sections were incubated with the labelled probe in the presence of 100 times excess of unlabelled probe to assess probe specificity. After hybridization, the slides were incubated in 4 × SSC and 0.02 mol dithiothreitol l⁻¹ for 15 min, and were rinsed in 4 × SSC for 10 min. The slides were treated with RNase A (20 µg RNase A ml⁻¹ in 5 × TE buffer) at 37°C for 30 min, and subjected to a high criterion wash for 1 h at 55°C, followed by an overnight wash in 0.5 × SSC and 0.02 mol β-mercaptoethanol l⁻¹ at room temperature. The following day, the sections were dehydrated, air-dried, taped to a piece of cardboard and exposed to film overnight at room temperature. After autoradiography, the slides were dipped in emulsion (Type NTB2; Kodak, Rochester, NY) and exposed in the dark for 2–5 days. The slides were developed with D-19 developer (Kodak) for 5 min, fixed (Kodak fixer) for 10 min and rinsed for at least 1 h under running

deionized water. Finally, the sections were counterstained with haematoxylin-eosin and mounted with Permount (Fisher Scientific). Slides were air-dried for 2 days before examination by bright field microscopy.

Results

The digoxigenin-labelled cDNA for the bovine homologue of prostaglandin D synthase hybridized to a single band (approximately 0.9 kb) in RNA samples from the caput, corpus and cauda epididymides (Fig. 1a). A single band was also observed in the testis samples, but the transcript size was slightly larger than the transcript size found in the other tissues (Fig. 1a). Highest expression of prostaglandin D synthase was observed in the testes and caput epididymides (Fig. 1a). Prostaglandin D synthase transcripts were also observed using northern blotting in the vas deferens and ampullae, but were not detected in the seminal vesicles, prostate or bulbourethral glands (Fig. 2a). After northern blotting with the DIG-labelled prostaglandin D synthase cDNA, the membrane was stripped and hybridized to a DIG-labelled 18S ribosomal RNA to check that the wells had been loaded evenly and that the RNA samples had been transferred completely to the nylon membrane (Figs 1b and 2b).

Prostaglandin D synthase transcripts were detected by *in situ* hybridization of bull testes within the germ cells in

Fig. 1. Expression of the prostaglandin D synthase gene in testis and cauda, corpus and caput epididymides of bulls visualized by northern blotting. (a) Northern blot probed with digoxigenin-labelled lipocalin-type prostaglandin D synthase cDNA. (b) Northern blot probed with digoxigenin-labelled ribosomal 18S RNA.

Fig. 2. Expression of the lipocalin-type prostaglandin D synthase gene in vas deferens and accessory sex gland tissues (ampulla, seminal vesicle, prostate and bulbourethral glands) of bulls visualized by northern blotting. (a) Northern blot probed with digoxigenin-labelled prostaglandin D synthase cDNA. (b) Northern blot probed with digoxigenin-labelled ribosomal 18S RNA.

the adluminal compartment of some seminiferous tubules (Fig. 3a,b,c). As prostaglandin D synthase transcripts were only detected in tubules containing elongated spermatids (Fig. 3a,b), it appears that prostaglandin D synthase gene expression varies with stage of spermatogenesis. Unfortunately, the cryosections used in the present study did not provide the morphological detail necessary for proper staging of the seminiferous tubules.

Within the epididymides, expression of prostaglandin D synthase was observed in the epithelial cells of the caput, corpus and cauda epididymides (Fig. 3d,e,f). In the caput epididymidis, considerable expression of prostaglandin D synthase was observed in most of the epithelial cells (Figs 2 and 3d). Some sections were incubated with labelled

antisense riboprobe in the presence of 100 times excess unlabelled antisense riboprobe to assess probe specificity. The signal disappeared when the labelled antisense riboprobe was incubated in the presence of excess unlabelled antisense riboprobe. Expression of prostaglandin D synthase was observed in only a few cells of the epithelium of the corpus (Fig. 3e) and cauda (Fig. 3f) epididymides. Some expression was also observed in the epithelial cells of the vas deferens (data not shown). In the prostate (Fig. 3g) and bulbourethral glands (Fig. 3h) expression of prostaglandin D synthase was restricted to a few cells of some lobules. Prostaglandin D synthase was not detected in the ampulla or seminal vesicles by *in situ* hybridization. Signal intensities obtained from *in situ* hybridizations did not differ among the four bulls examined. No specific hybridization was observed with the sense riboprobes.

Discussion

The DIG-labelled cDNA for the bovine homologue of prostaglandin D synthase hybridized to a single band (approximately 0.9 kb) in RNA samples from the caput, corpus and cauda epididymides, as well as RNA samples from the vas deferens and ampullae. This was the expected size for the prostaglandin D synthase transcript based on previous studies. Northern blotting of RNA from rat brain and epididymis showed a transcript size of approximately 850 bp (Urade *et al.*, 1989), and single positive transcripts of approximately 900 bp were also observed in northern blotting of human brain mRNA (Nagata *et al.*, 1991) and total RNA (White *et al.*, 1992). In the present study, the single band observed in the testis samples had a transcript size slightly larger (approximately 1.0 kb) than that found in the other tissues. As studies conducted to date indicate that a single prostaglandin D synthase gene exists in the haploid genome (White *et al.*, 1992), the larger transcript size detected in the testis may be the result of alternative splicing patterns or different polyadenylation lengths of a transcript synthesized from the single prostaglandin D synthase gene. This is the first study in which a different sized prostaglandin D synthase transcript is reported.

Prostaglandin D synthase transcripts were localized in bull testes by *in situ* hybridization in the germ cells within the adluminal compartment of the seminiferous epithelium. Although the use of cryosections did not provide the morphological detail necessary for proper staging of spermatogenesis in the seminiferous tubules, expression of prostaglandin D synthase was observed primarily in tubules containing elongated spermatids lining the lumen of the seminiferous epithelium. In bulls, the cycle of the seminiferous epithelium has been divided into eight (Amann

Fig. 3. Cellular localization of lipocalin-type prostaglandin D synthase transcripts in the reproductive tissues of bulls by *in situ* hybridization. Reproductive tissues of bulls were probed with either a ^{35}S -radiolabelled antisense (a, b, d, e, f, g and h) or negative control sense riboprobe (c). Positive signals are observed as silver grains. (a, b) Silver grains were distributed over the germ cells within the adluminal compartment of the seminiferous tubules. (c) No specific hybridization was observed with the sense riboprobe. (d) In the epididymis, silver grains were distributed heavily over the caput epithelium, whereas the distribution of silver grains in the epithelium of the (e) corpus and (f) cauda epididymides was spotty. The distribution of silver grains in the epithelium of the vas deferens (not shown), (g) prostate gland and (h) bulbourethral glands was limited to a few cells only. Scale bars represent 100 μm .

Fig. 3. For legend see opposite page.

and Schanbacher, 1983; Johnson, 1994) or 12 stages (Berndson and Desjardins, 1974). When the eight-stage classification scheme was used, prostaglandin D synthase expression was limited to stages VII and VIII of the seminiferous epithelium. These stages are characterized by the presence of type A and type B spermatogonia, pachytene primary spermatocytes, round spermatids, and elongated spermatids interfacing with the lumen of the seminiferous epithelium (Johnson, 1994). No specific hybridization was observed in the interstitial compartment.

Different patterns of expression of prostaglandin D synthase were observed within the three regions of the epididymis. In the caput epididymidis, expression of prostaglandin D synthase was observed throughout the epithelium of the tubule. In contrast, expression of prostaglandin D synthase in the corpus and cauda epididymides was restricted to specific regions of the epithelium. These results support those obtained by immunohistochemical localization of prostaglandin D synthase in bull testicular and epididymal tissues. When anti-prostaglandin D synthase polyclonal antibody was used, prostaglandin D synthase protein was localized within the seminiferous tubules and the epithelium of the caput, corpus and cauda epididymides, with the most intense staining observed in the epithelium of the caput epididymidis (Rodríguez *et al.*, 1998).

Garrett *et al.* (1991) reported regional localization of other epididymal proteins. There is evidence which indicates that region-specific patterns of protein localization within the epididymides are the result of region-specific gene expression for these proteins (Garrett *et al.*, 1991; Akmal *et al.*, 1996). Protein and mRNA expression of the retinoic acid receptor- α were highest in the caput epididymides, low in the corpus epididymides, but high in the cauda epididymides of rats (Akmal *et al.*, 1996). Other rat epididymal proteins that show region-specific gene expression include protein B/C, protein D/E, sulphated glycoproteins 1 and 2, cellular retinol binding protein and proenkephalin (Garrett *et al.*, 1991).

Expression of the prostaglandin D synthase gene was also observed in the epithelium of the vas deferens and within the prostate and bulbourethral glands, although silver grains were not detected in ampullae or seminal vesicles using the antisense riboprobe. This is believed to be the first study in which expression of the prostaglandin D synthase gene in the vas deferens, seminal vesicles and bulbourethral glands has been investigated.

The results obtained from *in situ* hybridization generally support those obtained by northern blotting. The testes and the caput epididymides are the primary tissues in which prostaglandin D synthase is expressed. Both techniques also detected the prostaglandin D synthase transcript in the vas deferens, as well as the corpus and cauda epididymides. However, transcripts were detected in ampullae using northern blotting, but transcripts were not detected in ampullae by *in situ* hybridization. It is possible that expression of prostaglandin D synthase mRNA was low throughout the different cell types in the ampullae. Although not readily detectable by *in situ* hybridization, the concentration of prostaglandin D synthase transcripts after

RNA isolation may have enabled the detection of such transcripts by northern blotting (McFadden, 1995). In contrast, prostaglandin D synthase transcripts were detected in the prostate and bulbourethral glands by *in situ* hybridization but not by northern blotting. In this case the prostaglandin D synthase mRNAs were highly localized to particular cells within these tissues. Although readily detectable by *in situ* hybridization, the dilution effect of non-expressing cells after RNA isolation may have made it difficult to detect the prostaglandin D synthase transcripts by northern blotting (McFadden, 1995).

The function of prostaglandin D synthase in the reproductive tracts of bulls remains speculative. It is possible that lipocalin-type prostaglandin D synthase behaves as a bifunctional protein acting as both a lipocalin and a prostaglandin D₂-producing enzyme.

Prostaglandin D synthase catalyses the isomerization of PGH₂ to PGD₂ in the presence of various sulphhydryl compounds. Bull seminal plasma, fluid from the cauda epididymidis and fluid from the rete testis also have prostaglandin D synthase activity (Gerena *et al.*, 1998), but it is not known whether PGD₂ receptors exist in the reproductive tracts of bulls.

As a lipocalin, prostaglandin D synthase may be involved in the transport of bioactive lipophilic substances, which is analogous to the function of other lipocalins, such as retinol-binding protein and beta lactoglobulin. Prostaglandin D synthase binds all *trans*- and 9-*cis*-retinoic acids with the same affinity as other retinoid transporters (Tanaka *et al.*, 1997). Retinoid derivatives of vitamin A play an important role in the male reproductive system including induction of epithelial differentiation, testicular function and maintenance of spermatogenesis (Skinner, 1991). Prolonged vitamin A deficiency results in germ cell depletion and cessation of spermatogenesis at the preleptotene spermatocyte stage (Morales and Griswold 1987). In the epididymides, prostaglandin D synthase may supply retinoic acid for the maintenance of epithelial tissue and normal epididymal function, including sperm maturation (Newcomer, 1995). In rats, the highest expression of cellular retinol binding protein was observed in the initial segment and the segments of the caput epididymidis (Garrett *et al.*, 1991), which parallels the prostaglandin D synthase expression observed in the present study and in another study of protein localization in the epididymides of bulls (Gerena *et al.*, 2000).

It is also possible that prostaglandin D synthase may be involved in the transfer of other lipophilic substances, such as androgens. Production of testosterone by the Leydig cells can raise the intratesticular concentrations of testosterone to > 300 ng, or 100–300 times the concentration found in peripheral blood plasma (Amann, 1983), which appears to be necessary for maintenance of spermatogenesis. Lipocalin-type prostaglandin D synthase may aid in the transport and concentration of androgens near germ cells at specific stages of development.

Likewise, prostaglandin D synthase in fluid from the rete testis and cauda epididymidis (Gerena *et al.*, 1998) may be involved in the transport of androgens from the testis to the efferent ducts and the caput epididymidis, and contribute to

the marked differences in steroid profiles of fluids entering and leaving the epididymides of bulls (Ganjam and Amann, 1976).

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