BIOLOGY OF REPRODUCTION 58, 600–607 (1998)

Lipocalin-Type Prostaglandin D Synthase in Human Male Reproductive Organs and Seminal Plasma[1]

Yoshihiro Tokugawa,[2,3] Ichiro Kunishige,[3] Yasue Kubota,[3] Koichiro Shimoya,[3] Toshikatsu Nobunaga,[3] Tadashi Kimura,[3] Fumitaka Saji,[3] Yuji Murata,[3] Naomi Eguchi,[4] Hiroshi Oda,[5] Yoshihiro Urade,[6] and Osamu Hayaishi[6]

Department of Obstetrics and Gynecology,[3] Osaka University Medical School, Osaka, Japan
Precursory Research for Embryonic Science and Technology,[4] Research Development Corp. of Japan, Osaka, Japan
Central Research Institute of Maruha Corp.,[5] Ibaraki, Japan
Department of Molecular Behavioral Biology,[6] Osaka Bioscience Institute, Osaka, Japan

ABSTRACT

Prostaglandin D synthase (PGDS) activity was detected in human seminal plasma (0.05–1.83 nmol/min per milligram protein). The enzyme was purified from human seminal plasma by immunoaffinity chromatography and found to be 27 kDa in size and *N*-glycosylated, similar to PGDS in the cerebrospinal fluid. The *N*-terminal amino acid sequence of 16 residues of the seminal enzyme, APEAQVSVQPNFQQDK, was identical to that of the cerebrospinal fluid PGDS. Although PGDS activity and the content determined by the immunoassay each highly varied in the seminal plasma, the concentration was significantly ($p < 0.001$) lower in the oligozoospermic group (2.47 ± 0.51 μg/ml) than in the normozoospermic group (9.75 ± 1.49 μg/ml). Prostaglandin (PG) D_2 was detected in the seminal plasma (5.00 ± 0.65 ng/ml) with a positive correlation to the PGDS concentration ($p < 0.05$). PGD_2 was converted to the J series of PGs in the seminal plasma with a half-life of 6.5 h. Northern blot analysis revealed that mRNA for PGDS was expressed in the testis, prostate, and epididymis. Through immunohistochemistry, PGDS was localized in Leydig cells of the testis and in epithelial cells of the prostate and ductus epididymidis.

INTRODUCTION

Seminal plasma contains a large variety of chemical components: enzymes, hormones, and inorganic components [1–3]. Prostaglandins (PGs) are among the seminal plasma constituents. Although intense research efforts have been focused on PGs in the male reproductive tract, the relationships between seminal PGs and human fertility are still unknown. Many researchers have studied PGE_2 and $PGF_{2\alpha}$ in the seminal plasma, whereas only a few studies have been reported on PGD_2. PGD_2 is actively produced in various organs [4] and has marked effects on a number of biological processes, including platelet aggregation [5], and relaxation of vascular and nonvascular smooth muscle [6, 7].

Three distinct enzymes have been characterized as prostaglandin D synthase (PGDS): 1) glutathione (GSH)-independent PGDS, which is a brain-type PGDS [8, 9]; 2) GSH-requiring PGDS, a spleen-type PGDS [10, 11]; and 3) GSH S-transferase [12]. GSH-independent PGDS is localized in the rat brain, spinal cord [4], cochlea [13], retina [14], and epididymis [4], whereas GSH-requiring PGDS is widely distributed in peripheral tissues of rats including spleen, thymus, bone marrow, and digestive tract [4]. Although several isozymes of GSH S-transferase contribute to the production of PGD_2 in homogenates of the liver and testis [12], their physiological contribution to this production remains unclear.

The cDNAs and genes for the rat and human GSH-independent PGDS enzymes have been isolated [15–18]. Through a homology search in databases of protein primary structure, the enzyme was demonstrated to be a member of the lipocalin superfamily, a group of proteins comprising a variety of secretory proteins that bind and transport small lipophilic molecules [19]. GSH-independent PGDS is the only enzyme among the proteins encoded by this gene family and is termed lipocalin-type PGDS. The lipocalin-type PGDS is also secreted into the extracellular space, similarly to other lipocalins; i.e., the major protein constituent of human cerebrospinal fluid (CSF), classically termed β-trace, is identical to lipocalin-type PGDS [20–22]. β-Trace was originally discovered by immunoelectrophoresis as a protein specific to human CSF [23]. The β-trace-like immunoreactive protein was later detected in various other human body fluids, e.g., the serum, urine, and ascites, and also in the seminal plasma [24–26]. However, the previous studies were semiquantitative owing to the lack of knowledge on the enzymic and protein properties of lipocalin-type PGDS.

In this study, we purified and characterized the lipocalin-type PGDS in human seminal plasma and determined the seminal plasma concentrations of the enzyme and PGD_2. We also determined the metabolism of PGD_2 in the seminal plasma and the tissue distribution and cellular localization of the enzyme in human male genital organs.

MATERIALS AND METHODS

Samples

Semen samples were collected by masturbation after 4 days of abstinence from 51 males of infertile couples who had come to Osaka University Medical School for treatment of infertility. Semen samples were analyzed for the following parameters: semen volume, sperm concentration, total sperm count, motility, and morphology. Liquefied

Accepted October 2, 1997.
Received August 11, 1997.
[1]This work was supported in part by grants-in-aid from the Ministry of Education, Science, and Culture of Japan (09671677 to C.A., 09671679 to K.O., 09470359 to F.S., 07671781 to Y.S., 08671888 to K.S., 08671889 to Y.T., 07558108 and 07457033 to Y.U., and 06508003 to O.H.) and by grants from the Mitsubishi Foundation, Sankyo Foundation of Life Science, the Naito Foundation, Naito Medical Foundation, Ono Medical Research Foundation, Suntory Institute for Bioorganic Research Foundation, Japan Foundation for Applied Enzymology, and The Cell Science Research Foundation (to Y.U.).
[2]Correspondence: Yoshihiro Tokugawa, Department of Obstetrics and Gynecology, Osaka University Medical School, 2–2 Yamadaoka, Suita, Osaka, 565, Japan. FAX: 81–6–879–3359; e-mail: tokugawa@gyne.med.osaka-u.ac.jp

samples were centrifuged at 1000 × *g* for 10 min, and the supernatants were recentrifuged at 10 000 × *g* for 15 min to remove any cellular elements and debris. After centrifugation, the clear seminal plasma was collected and stored at −80°C until used. Human testis, epididymis, and prostate specimens were obtained by autopsy from individuals without known diseases of these organs. Informed consent was obtained from all patients in this study.

Enzyme Assays

The PGDS activity was measured by incubation of human seminal plasma at 25°C for 1 min with [1-^{14}C]PGH_2 (final concentration, 40 μM) in 50 μl of 0.1 M Tris-HCl (pH 8.0) in the presence of 1 mM dithiothreitol (DTT) as reported previously [9]. The reaction was stopped by addition of 5 vol of diethyl ether:methanol:1 M citric acid (30:4:1). The substrate and products were extracted to be recovered in the ether layer and applied to a silica gel plate (Whatman, Clifton, NJ). Thin-layer chromatography (TLC) was conducted at −20°C with a solvent system of diethyl ether:methanol:acetic acid (90:2:1). The radioactivity on the plate was visualized and counted with BAS-2000 (Fuji film, Tokyo, Japan). The dose-dependent conversion of PGH_2 to PGD_2 was then calculated. [1-^{14}C]PGH_2 was prepared from [1-^{14}C]arachidonic acid (2.20 GBq/mmol; DuPont NEN, Boston, MA) [9]. Protein concentration was determined by the method of Lowry et al. [27] as modified by Bensadoun and Weinstein [28].

Sandwich ELISA

The PGDS content was determined by the sandwich ELISA assay with two mouse monoclonal antibodies (mAbs), 1B7 and 7F5, against human lipocalin-type PGDS. These mAbs were prepared as previously described [29]. The enzyme purified from human CSF [22] was used as the standard. The PGD_2 concentration was measured by the PGD_2 MOX EIA kit (Cayman Chemical, Ann Arbor, MI) after derivatization of PGD_2 to PGD_2 methoxime.

Degradation of PGD_2 in Seminal Plasma

[1-^{14}C]PGD_2 was prepared from [1-^{14}C]PGH_2 by incubation with recombinant PGDS [30]. Seminal plasma (45 μl) was incubated with 30 μM of [1-^{14}C]PGD_2 in a total volume of 50 μl of 0.1 M Tris-HCl (pH 8.0) at 37°C for 1, 2, and 12 h. After the incubation, the mixture was acidified with 0.1 N HCl and extracted with diethyl ether. The extract was evaporated to dryness and then applied on a silica gel plate. TLC was conducted with a solvent system of benzene:ethyl-acetate:acetic acid (50:50:2). A half-life ($t_{1/2}$) of PGD_2 was calculated after the radioactivity on the plate was counted with BAS-2000. Authentic PGD_2, PGJ_2, [Δ^{12}]PGJ_2, and 15-deoxy-[$\Delta^{12,14}$]PGJ_2 (Cayman Chemical) were applied as markers and visualized by exposure to iodine vapor.

Western Blot Analysis

Seminal plasma (5 μl) was electrophoresed on a 14% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (0.45 μm; Schleicher and Schuell, Dassel, Germany). The membrane was incubated with 5% dried milk protein followed by anti-human lipocalin-type PGDS mAb 1B7 or 7F5 (1 μg/ml). The PGDS immunoreactivity was visualized with an ECL Western blotting analysis system (Amersham, Aylesbury, UK) operated according to the manufacturer's instructions.

Purification of Human Seminal Plasma PGDS

The mAb 1B7-conjugated immunoaffinity resin was prepared with an Affi-Gel Hz immunoaffinity kit (Bio-Rad Laboratories, Hercules, CA). Seminal plasma (1 ml) was diluted 7-fold in PBS and applied to the immunoaffinity column (1-ml bed). The column was washed sequentially with 4 ml of PBS containing 2 M NaCl, 10 ml of PBS containing 0.1% Triton X, and 5 ml of PBS. After the column had been washed, PGDS bound to the resin was eluted with 4 ml of 0.1 M sodium citrate (pH 3.0). The eluate was concentrated with a Centricon 3 (Amicon, Beverly, MA) and dialyzed against 50 mM Tris-HCl, pH 8.0.

Liberation of N-Glycans from PGDS

The purified PGDS (5 μg of protein) was boiled for 5 min in 100 μl of 0.5 M Tris-HCl (pH 8.0), 0.5% SDS, and 50 mM β-mercaptoethanol. Nonidet P-40 (final concentration of 1%) and recombinant *N*-Glycanase [peptide-N^4(*N*-acetyl-β-glycosaminyl) asparagine amidase] (1.2 units; Genezyme, Cambridge, MA) were added to the solution. After incubation at 37°C for 12 h, the reaction mixture was used for Western blot analysis.

Determination of Amino-Terminal Sequence of the Purified PGDS

The *N*-terminal amino acid sequence was determined with a 477A protein sequencer and an on-line 120A PTH analyzer (Applied Biosystems, Foster City, CA).

RNA Extraction and Northern Blotting

Total RNA was extracted from the prostate, epididymis, and testis by the acid guanidium-phenol-chloroform method [31]. Total RNA (10 μg) was electrophoresed on a 1% agarose-2.2 M formaldehyde gel and blotted onto a nylon membrane (Biodyne A, Pall, NY) in 20-strength SSC (single-strength SSC is 0.15 M sodium chloride and 0.015 M sodium citrate). The membrane was fixed and hybridized in QuickHyb hybridization solution (Stratagene, La Jolla, CA) with the cDNAs for human lipocalin-type PGDS (accession no. M61900 [16]) and β-actin, which had been labeled with [^{32}P]dCTP (DuPont NEN) by the random-primer method. The membrane was washed with 0.2-strength SSC containing 0.1% SDS and exposed to Kodak XAR film (Eastman Kodak, Rochester, NY).

Immunohistochemistry

The tissues obtained from autopsy were embedded in Tissue-Tek (Miles, Elkhart, IN) and rapidly frozen on dry ice. The frozen tissues were sectioned in a cryostat (10 μm thick) and fixed with acetone for 10 min. The sections were then treated for 30 min with methanol containing 0.3% hydrogen peroxide to eliminate the intrinsic peroxidase activity. Paraffin sections (10 μm thick) were also used in several experiments. Immunohistological staining was performed with a Vectastain ABC (avidin-biotin-peroxidase) kit (Vector Laboratories Inc., Burlingame, CA). The mAb 1B7 (10 μg/ml) was used as the primary antibody. The primary and second antibodies were incubated for 30 min, each at room temperature. A mixture of equal volumes of 0.02% hydrogen peroxide and 0.1% diaminobenzidine so-

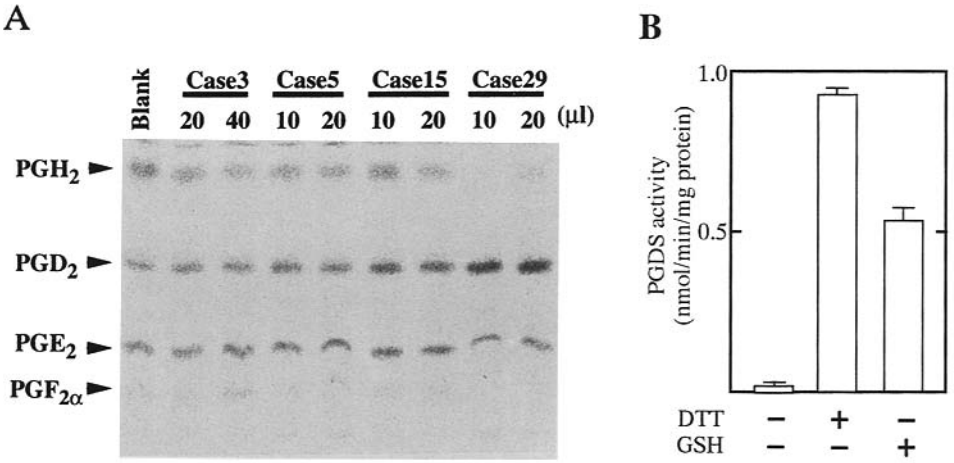

FIG. 1. **A**) PGDS activity in human seminal plasma. The positions of PGH_2, PGD_2, PGE_2, and $PGF_{2\alpha}$ are indicated on the left. PGH_2 gradually degraded to a mixture of PGD_2 and PGE_2 with a molar ratio of 1 to 2 even in the absence of the enzyme (blank), whereas it was selectively converted to PGD_2 in the presence of seminal plasma in a concentration-dependent manner (cases 3, 5, and 15). In case 29, PGH_2 was almost completely converted to PGD_2. It should be noted that the amounts of PGE_2 and $PGF_{2\alpha}$ formed in the presence of the plasma were almost the same as those produced by autodegradation of PGH_2. The PGDS activity was calculated to be 0.05, 0.08, 0.28, and 1.70 nmol/min per milligram protein in cases 3, 5, 15, and 29, respectively. **B**) Requirement of sulfhydryl compounds for the reaction catalyzed by seminal plasma PGDS. Pooled seminal plasma was dialyzed to remove endogenous GSH and incubated with PGH_2 in the presence or absence of 1 mM DTT or GSH. Values represent the mean ± SEM (n = 3).

lution was used as the color substrate solution. The IgG fraction obtained from nonimmunized mice was used for control staining.

Statistical Analysis

All data are presented as means ± SEM in the text and tables, with n representing the number of samples. Between-group comparisons were performed by Student's t-test when data were normally distributed, or by the Mann-Whitney test for nonparametric data. The correlation was analyzed by simple linear regression. All calculations were performed with a StatView program (Abacus Concepts, Berkeley, CA). The null hypothesis was rejected at $p < 0.05$.

RESULTS

Identification of Lipocalin-Type PGDS in Human Seminal Plasma

Human seminal plasma exhibited PGDS activity and converted PGH_2 to PGD_2 in a concentration-dependent manner. However, the activity varied highly among the samples and ranged from 0.05 to 1.83 nmol/min per milligram protein (Fig. 1A). The enzyme in the seminal plasma required sulfhydryl compounds such as DTT for the activity, but did not absolutely require GSH for the reaction (Fig. 1B). The catalytic properties were the same as those of lipocalin-type PGDS and differed from those of GSH-requiring PGDS and GSH-S-transferases (data not shown). In an immunoblotting test with mAbs 1B7 and 7F5, both of which are highly specific for human lipocalin-type PGDS [29], a 27-kDa band was observed in the seminal plasma at the same position as that of the human CSF enzyme, although the intensity deviated highly among the seminal plasma samples (Fig. 2).

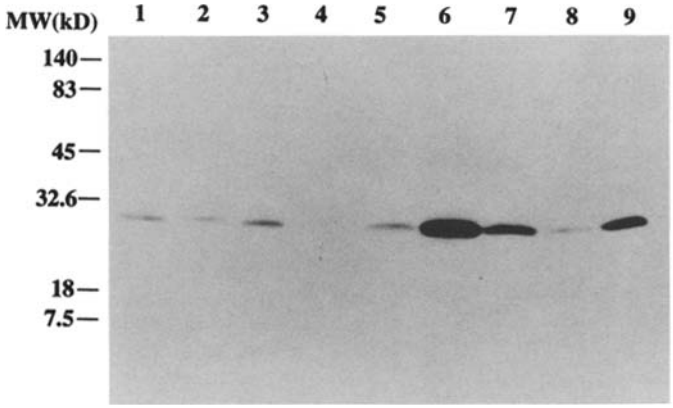

FIG. 2. Western blotting of lipocalin-type PGDS in seminal plasma. The signal of lipocalin-type PGDS of 27 kDa was detected in all seminal plasma. The molecular weight of marker proteins ($\times 10^{-3}$) is shown on the left. The seminal plasma concentration of lipocalin-type PGDS of these samples was determined by sandwich ELISA to be 4.4, 3.0, 4.8, 2.5, 8.0, 30.0, 10.5, 3.8, and 9.5 μg/ml for samples 1 to 9, respectively. All samples were normozoospermic (25 to 130 × 10^6/ml).

We then purified PGDS from the pooled human seminal plasma using mAb 1B7-conjugated immunoaffinity column chromatography; the PGDS activity was completely trapped on the column and recovered into the eluate by elution with acidic solution at a recovery of > 80% (Table 1). The acidic eluate contained the purified enzyme, which gave a single band at a position of M_r 27 000 by silver staining after SDS-PAGE (Fig. 3). The specific activity of the purified enzyme (1892 nmol/min per milligram protein) was comparable to that of the enzyme in human CSF (1000 nmol/min per milligram protein) [22]. The molecular mass of the purified enzyme shifted to about 21 000 after incubation with *N*-Glycanase (Fig. 3), indicating that the seminal plasma enzyme was *N*-glycosylated, similar to the CSF enzyme. Furthermore, the *N*-terminal sequence of seminal plasma PGDS was determined to be APEAQVSVQPNFQQDK, which is exactly the same as

TABLE 1. Purification of lipocalin-type PGDS from human seminal plasma by mAb 1B7-conjugated immunoaffinity column chromatography.

Step	Total activity (nmol/min)	Total protein (mg)	Specific activity (nmol/min per milligram protein)	Yield (%)	Purification (fold)
Pooled seminal plasma	16.4	38.9	0.422	100	1
Purified PGDS	13.2	0.007	1892	80.5	4483

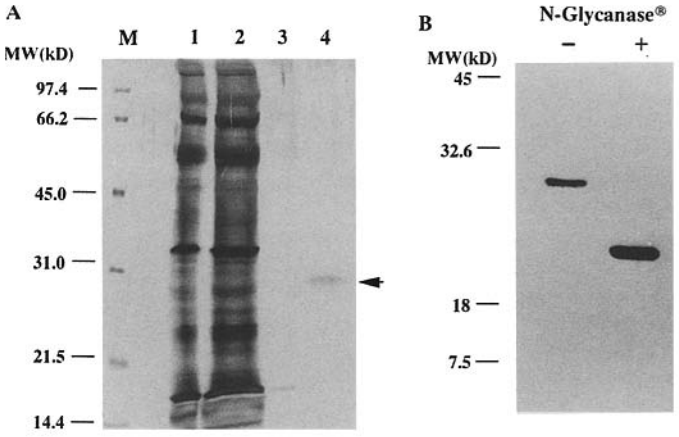

FIG. 3. **A**) Purification of lipocalin-type PGDS from human seminal plasma by mAb 1B7-conjugated immunoaffinity column chromatography. The flow-through fraction (lane 1), wash fractions obtained with PBS containing 2 M NaCl (lane 2) and 0.1% Triton X (lane 3), and the acidic eluate (lane 4) were electrophoresed on a 14% SDS-polyacrylamide gel and stained with silver. A single band at 27 kDa (arrow) was observed in the acidic eluate. **B**) Change in the size of lipocalin-type PGDS purified from seminal plasma by digestion with *N*-Glycanase. The lipocalin-type PGDS purified from human seminal plasma was incubated at 37°C for 12 h in the absence (lane 1) and the presence (lane 2) of *N*-Glycanase.

that sequence of the enzyme in the CSF [20–22]. These results, all taken together, indicate that the enzyme in human seminal plasma is lipocalin-type PGDS.

The seminal plasma concentration of the lipocalin-type PGDS was determined by the quantitative sandwich ELISA carried out with mAbs 1B7 and 7F5. Figure 4 shows a typical standard curve prepared with lipocalin-type PGDS purified from the CSF, as well as the dilution curves of the seminal plasma samples. In all cases, dilution curves of the samples were parallel to the standard curve. By the ELISA assay, the seminal plasma concentration of the enzyme was determined to be 0.3–42 μg/ml. When the correlations of the seminal plasma concentration of the enzyme with a variety of spermiogram parameters were analyzed, the concentration was significantly ($p < 0.001$) lower in the oligozoospermic group, defined as having a sperm count of less than 20 × 10^6/ml (2.47 ± 0.51 μg/ml, n = 10), than in the normozoospermic group (9.75 ± 1.49 μg/ml, n =

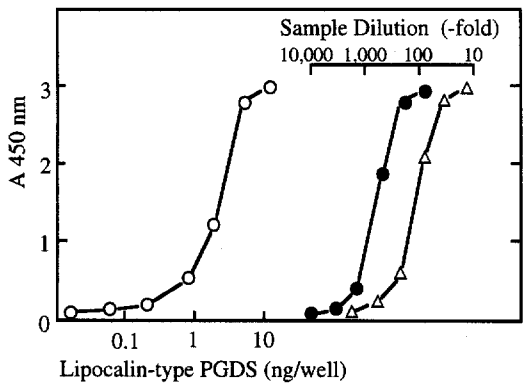

FIG. 4. Sandwich ELISA for lipocalin-type PGDS with mAb 1B7 and mAb 7F5. Symbols represent datum points of a standard curve for lipocalin-type PGDS purified from human CSF (open circles) and those of the dilution curves of seminal plasma obtained from two different persons (solid circles and triangles). One sample (solid circles) reflected normozoospermia (55 × 10^6/ml); the other (triangles), oligozoospermia (15 × 10^6/ml).

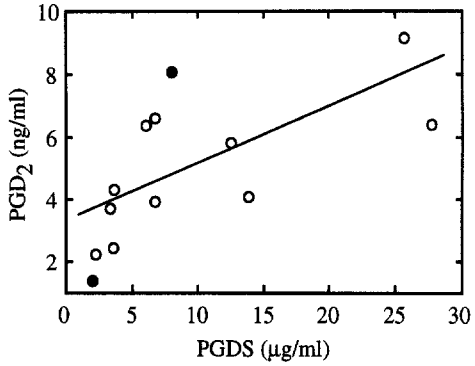

FIG. 5. Correlation between lipocalin-type PGDS and PGD_2 concentrations in the seminal plasma. The PGDS concentration is plotted on the x-axis and PGD_2 on the y-axis. PGDS and PGD_2 concentrations were determined by ELISA as described in the text. A positive correlation between the concentration of PGDS and PGD2 in the seminal plasma was observed (y = 0.183 x + 3.297, $r = 0.661$, $p < 0.05$, n = 13). Eleven samples (open circles) and two samples (solid circles) were collected from normozoospermic and oligozoospermic men, respectively.

41). However, no statistically significant correlation was found between the lipocalin-type PGDS and other spermiogram parameters, such as ejaculation volume of semen and percentage sperm motility.

Existence and Degradation of PGD_2 in Human Seminal Plasma

We then measured PGD_2 concentration in human seminal plasma and found that this fluid contained PGD_2 (5.00 ± 0.65 ng/ml, n = 13). Furthermore, there was a positive correlation between the concentration of lipocalin-type PGDS and PGD_2 in the seminal plasma ($r = 0.661$, $p < 0.05$, $n = 13$) (Fig. 5). However, the PGD_2 concentration was significantly low. We therefore examined the stability of PGD_2 in human seminal plasma. When PGD_2 was incubated at 37°C with seminal plasma, PGD_2 was degraded to a mixture of the J series of PGs, such as PGJ_2, $[\Delta^{12}]PGJ_2$, and 15-deoxy-$[\Delta^{12,14}]PGJ_2$, in a time-dependent manner (Fig. 6). The $t_{1/2}$ value of PGD_2 in seminal plasma was calculated to be 6.5 h, which was longer than the value in blood plasma (4.1 h, data not shown) yet much shorter than

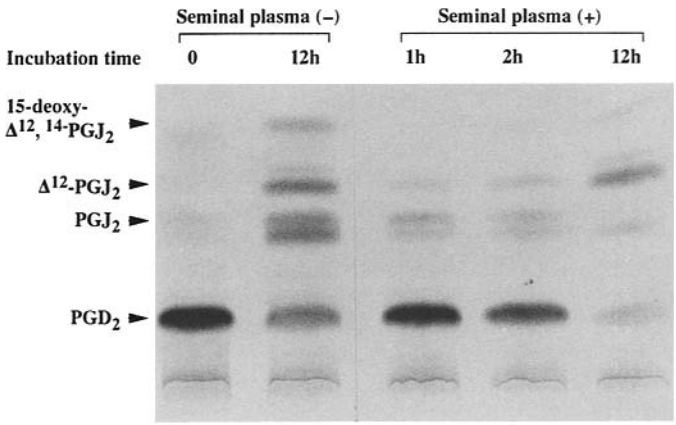

FIG. 6. Conversion of PGD_2 into the J series of PGs in human seminal plasma was determined by TLC as described in *Materials and Methods*. The positions of PGD_2, PGJ_2, Δ^{12}-PGJ_2, and 15-deoxy-$\Delta^{12,14}$-PGJ_2 are indicated on the left. Before the incubation, there were few autodegradation products. After 12-h incubation with or without the seminal plasma, 80% and 60% of PGD_2 was converted to the J series of PGs, respectively.

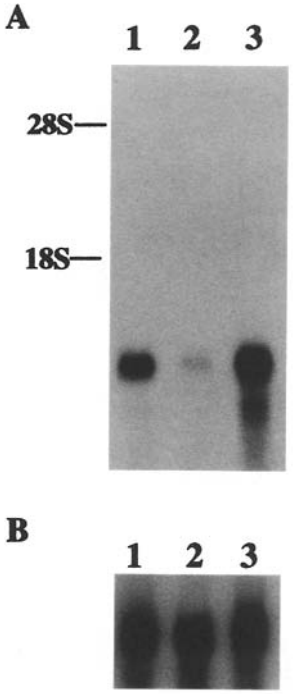

FIG. 7. Northern blot analysis of lipocalin-type PGDS mRNA in human male genital organs. Total RNAs (10 μg) from the prostate (lane 1), epididymis (lane 2), and testis (lane 3) were electrophoresed and hybridized with ^{32}P-labeled cDNAs for human lipocalin-type PGDS (**A**) and for β-actin (**B**).

that in a saline solution (9.7 h). Before the incubation, there were minor amounts of autolysis products. After 12 h of incubation, more than 80% of PGD_2 in seminal plasma was converted to the J series of PGs, mainly to $[\Delta^{12}]PGJ_2$. These results indicate that the low level of PGD_2 in seminal plasma was probably due to its conversion to the J series of PGs in this fluid.

Tissue Distribution and Cellular Localization of Lipocalin-Type PGDS in Male Reproductive Organs

To investigate the organs that synthesize the lipocalin-type PGDS and secrete it into the seminal plasma, we determined the distribution profile of mRNA for the enzyme among several male genital organs. Northern blot analysis with the cDNA for the enzyme revealed that the mRNA was highly expressed in the testis and prostate gland and weakly in the epididymis, in all of which a single transcript was observed at a position of 0.9 kilobases (Fig. 7).

The cellular localization of lipocalin-type PGDS in those genital organs was examined by immunoperoxidase staining with mAb 1B7 (Fig. 8). In the testis, positive staining was observed on Leydig cells, which were located in the intertubular spaces among the seminiferous tubules. In the epididymis, a structure that secretes a part of the seminal plasma, the ductus epididymidis was positively stained. The epithelial cells of the secretory elements of the prostate gland were also positively stained. No staining was observed in those cells when nonimmunized mouse IgG was used as the primary antibody.

DISCUSSION

In this study, by measurement of enzyme activity (Fig. 1), by Western blot analysis (Fig. 2), and by ELISA (Fig. 4), we demonstrate that lipocalin-type PGDS is present in human seminal plasma. The seminal fluid and CSF PGDS are the same [20–22] as judged by the molecular weight (27 000), *N*-glycosylation (Fig. 3), *N*-terminal amino acid sequence, and immunoreactivities against antibodies mAb 1B7 and 7F5 (Fig. 4).

Seminal plasma has long been known to be the richest source of PGs since their discovery by von Euler, and the predominant components of seminal plasma PG are PGE_2 (5.02–94.47 μg/ml), $PGF_{2\alpha}$ (0.75–2.93 μg/ml), and their 19-hydroxy derivatives, 19-hydroxy PGE_2 (77.28–498.60 μg/ml) and 19-hydroxy $PGF_{2\alpha}$ (2.5–12.46 μg/ml) [32]. Kuno et al. [33] reported that human semen was almost devoid of PGD_2 (< 0.2 μg/ml). Here, we determined the seminal plasma concentration of PGD_2 (5.00 ± 0.65 ng/ml, n = 13) and found a positive correlation between the concentrations of lipocalin-type PGDS and PGD_2 in the fluid (Fig. 5). These data suggest that lipocalin-type PGDS contributes to the production of PGD_2 in the seminal plasma.

However, PGD_2 is not a major prostanoid in seminal plasma. The concentration is 1000-fold less than those of PGE_2 and $PGF_{2\alpha}$. PGD_2 readily undergoes dehydration in vitro [34, 35] as well as in vivo [36], being converted to PGs of the J series, such as PGJ_2, $[\Delta^{12}]PGJ_2$, and 15-deoxy-$[\Delta^{12,14}]PGJ_2$, the last of which was recently identified as an endogenous ligand for peroxisome proliferator-activated receptor-γ and shown to promote adipocyte differentiation [37, 38]. We also demonstrate that PGD_2 is quickly converted to those dehydrated products in human seminal plasma with $t_{1/2}$ of 6.5 h (Fig. 6). These findings suggest that the low level of PGD_2 in human seminal plasma could be due to the rapid metabolism of PGD_2 in this fluid.

PGD_2 functions as a neuromodulator of several central actions such as sleep-wake cycles, body temperature, LH release, and odor responses [39]. It also acts as a negative-feedback regulator during platelet aggregation [5], produces relaxation of vascular and nonvascular smooth muscle [6, 7], and plays a role during immunologic reactions [40–42]. Rectal infusion of PGD_2 into rats resulted in a reduced response of T lymphocytes to phytohemagglutinin in vitro [33]. Antisperm antibodies are often found in the circulation as well as locally within the female reproductive tract and are implicated in female infertility [43]. Although the effect of vaginal infusion of PGD_2 has not been investigated, PGD_2 may act locally as an immunosuppressor in the vagina to reduce the production of antisperm antibodies.

The presence of PGD-sensitive receptors in nonpregnant human myometrium was indicated by Sanger et al. [44], who demonstrated the potent relaxant action of the PGD-receptor agonist BW 245C. The mRNA of the mouse PGD receptor is expressed in the uterus as well as in the lung and gastrointestinal tract [45]. Furthermore, PGD_2 reportedly raises the cAMP level in CHO-J cells, which stably express PGD receptor, with a 50% effective concentration (EC_{50}) of 6.8 nM [45]. PGD_2 concentration in the seminal plasma (5.0 ng/ml = 14.2 nM) determined in this study is higher than the EC_{50} of PGD_2, being sufficient to activate the receptor. Kunz et al. [46] reported that rapid sperm transport through the female genital tract was passive and was provided by uterine peristalsis. Since the uterus is a target organ of PGD_2, PGD_2 in the seminal plasma may play an important role in this uterine peristalsis in cooperation with PGE_2 and $PGF_{2\alpha}$, which are potent uterine contractants.

We show in this study that PGD_2 is readily converted to the J series of PGs in seminal plasma (Fig. 6). Since the J series of PGs have been reported to show various biological activities, such as suppression of tumor growth, antiviral

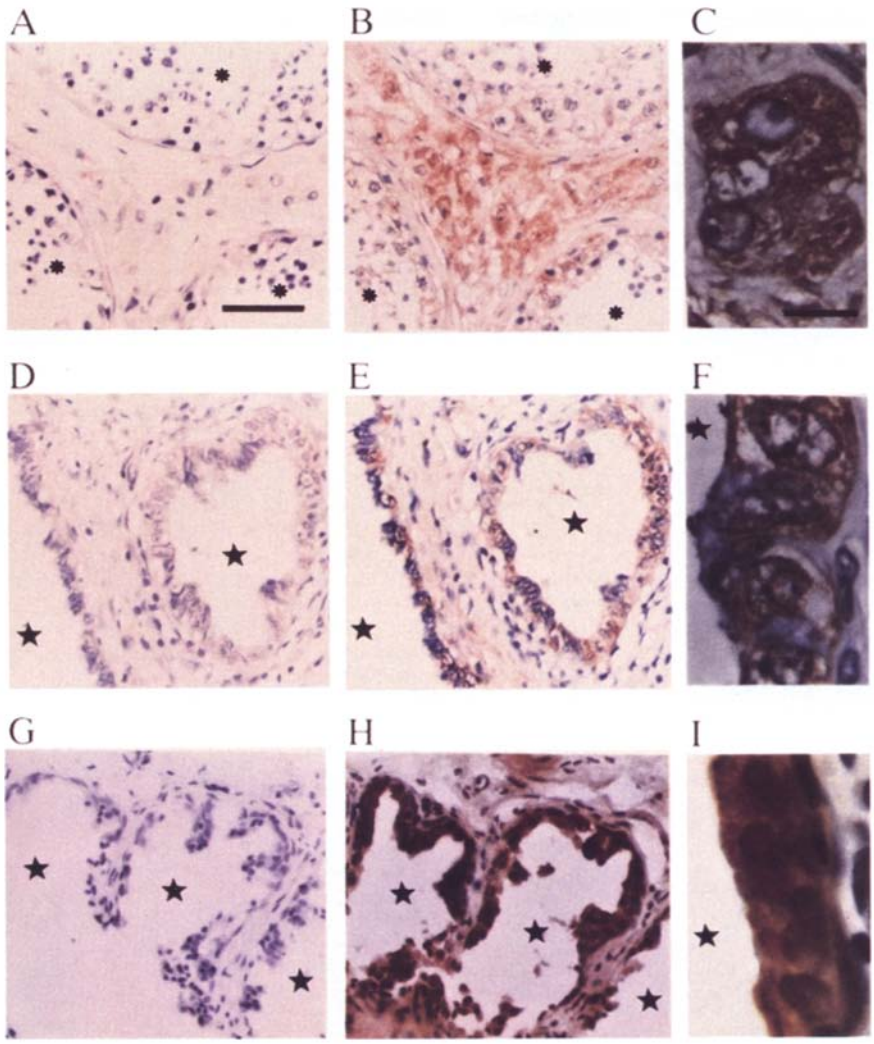

FIG. 8. Immunohistochemistry of human male genital organs with anti-lipocalin-type PGDS antibody. Fresh-frozen sections of the testis (**A–C**) and the epididymis (**D–F**) as well as paraffin sections of the prostate (**G–I**) were immunostained with mAb 1B7 (**B, C, E, F, H, I**) or nonimmunized mouse IgG (**A, D, G**). Leydig cells, which were located in the intertubular spaces among the seminiferous tubules (*), were positively stained (**B, C**). Positive staining was also observed on cells of the ductus epididymidis, and the epithelial cells of the secretory elements of the prostate gland were positively stained (**E, F, H, I**). Stars denote lumens (**D–I**). **C**, **F**, and **I** represent the higher-magnification views. The sections were counterstained with cresyl violet. Bars = 50 μm (**A, B, D, E, G, H**) and 10 μm (**C, F, I**).

activity, and stimulation of osteogenesis [47], these PGs also may function in the reproductive organs. It should be noted that these J series of PGs may be enriched in the seminal plasma, although no quantitative analysis for these PGs has been reported. If a high level of the J series of PGs are found in seminal fluid, then the potential importance of PGDS activity in the male reproductive organs may be more relevant.

The seminal plasma concentration of lipocalin-type PGDS measured in this study by our sandwich ELISA (0.3–42 μg/ml) is lower than the values (6–180 μg/ml) reported previously [25]. This discrepancy is probably due to the weak specificity of the polyclonal antisera used for the earlier assay. Both mAb 1B7 and 7F5 used for our ELISA are highly specific for lipocalin-type PGDS among the various proteins in the seminal plasma, as evidenced by the finding that they showed a single band in Western blot with the seminal plasma at the same position as that of lipocalin-type PGDS in the human CSF (data not shown). Therefore, we consider our results to be more accurate than the previous ones.

The seminal plasma concentration of lipocalin-type PGDS was lower in oligozoospermic than in normozoospermic samples, but no significant correlation was detected between the concentration and other spermiogram parameters. These findings are consistent with those in a previous report by Olsson [25], in which higher concentrations of the enzyme were measured (as discussed above). The enzyme may therefore be associated with fertility in humans. This possibility is supported by a recent report on bull seminal plasma. Killian et al. [48] found four fertility-associated proteins in bull seminal plasma: two of the proteins predominated in higher-fertility bulls, and the other two in the lower-fertility animals. A 26-kDa protein observed in the seminal plasma of the higher-fertility bulls was recently identified, by cDNA cloning, as the bovine homologue of lipocalin-type PGDS [49]; i.e., seminal plasma lipocalin-type PGDS is a fertility-associated protein in bulls.

For fertilization, a specific seminal plasma composition is required at ejaculation and during sperm transport through the female genital tract. Low levels of certain soluble seminal components are associated with subfertility or infertility. For example, the amount of PGE_2 in the semen of infertile men is approximately one half to one third of that found in fertile men [50]. Also, oligozoospermic men show decreased levels of albumin in their seminal plasma [51], and a deficiency of seminal plasma glycoprotein is associated with asthenospermia [52]. Although the concentrations of PGs other than PGD_2 and of individual proteins in seminal fluid were not measured in this study, it would be worthwhile to determine whether a relationship exists between the lipocalin-type PGDS and those components with respect to fertilization.

Lipocalin-type PGDS is a member of the lipocalin superfamily [16, 53], and it shares the feature of binding and

transport of lipophilic molecules with other superfamily members, such as retinol-binding protein and β-lactoglobulin. The enzyme is secreted into various body fluids including the seminal fluid as shown here, and binds retinoids at a molar ratio of 1:1 with affinities (K_d = 70–80 nM) comparable to those of other lipocalins [54]. Therefore, this enzyme is considered to be a bifunctional protein, acting as both a PGD_2-producing enzyme and a retinoid transporter [55]. It has been established that retinoids (vitamin A derivatives) are crucial for male fertility. In vitamin A-deficient male rats, spermatogenesis becomes arrested, and degeneration and loss of germ cells take place [56]. Administration of retinol acetate causes a massive and synchronized reinitiation of spermatogenesis in vitamin A-deficient rats [57]. Furthermore, retinoic acid receptor-γ-deficient mice exhibit male sterility due to squamous metaplasia of the seminal vesicles and prostate [58]. Two cytoplasmic proteins are involved in retinoic acid signal transduction, named cellular retinoic acid-binding protein (CRABP) and cellular retinoic acid-binding protein II (CRABP-II). Rat testis expresses CRABP and CRABP-II. CRABP-II is expressed in Leydig cells in rat testis [59]. CRABP and CRABP-II knockout mice remain essentially normal [60–62]. These reports suggest that other retinoid-binding proteins exist in the testis. We found that Leydig cells expressed lipocalin-type PGDS (Fig. 8). Taken together, these results indicate that lipocalin-type PGDS may play an important role as a retinoid-binding protein in Leydig cells to maintain spermatogenesis. Therefore, it is also important to evaluate the relationship between the lipocalin-type PGDS and lipophilic substances in the seminal plasma, such as PGs and retinoids, in relation to fertilization. Immunoaffinity purification of the enzyme from the seminal plasma (Fig. 3) is useful for identification of the predicted endogenous ligand for the enzyme in the seminal plasma. The purified lipocalin-type PGDS will also be useful for further experiments to examine whether it has any function in capacitation or in the acrosome reaction of spermatozoa.

In humans, the immunoreactivity of β-trace protein, which is identical to lipocalin-type PGDS, is detected immunologically in the brain, testis, and epididymis [63]. However, the tissue distribution and cellular localization of β-trace protein in humans have not been defined in detail. In this study, we demonstrate by Northern blotting and immunohistochemistry (Figs. 7 and 8) that the mRNA of lipocalin-type PGDS is expressed in the human testis, epididymis, and prostate and that the immunoreactivity is localized in the epithelial cells of the prostate gland and ductus epididymidis and in Leydig cells in the testis. The epithelial cells of the prostate gland and ductus epididymidis secrete many substances into the seminal plasma. The PGDS concentration in seminal fluid is higher than the reported concentration in serum (0.27 ± 0.01 μg/ml) [29]. On the other hand, Leydig cells are present in the intertubular tissues of the testis and are not considered to contribute directly to the secretion of lipocalin-type PGDS into the seminal plasma. These results suggest that lipocalin-type PGDS in human seminal plasma originates from tubular epithelial cells of the prostate and epididymis.

REFERENCES

1. Mann T, Lutwak-Mann C. Biochemistry of seminal plasma and male accessory fluids: application to andrological problems. In: Mann T, Lutwak-Mann C (eds.), Male Reproductive Function and Semen. New York: Springer; 1981: 269–336.
2. Polakoski KL, Kopta M. Seminal plasma. In: Zaneveld LJD, Chatterton RT (eds.), Biochemistry of Mammalian Reproduction. New York: J. Wiley; 1982: 89–117.
3. Rosecrans RR, Jeyendran RS, Perez PM, Kennedy WP. Comparison of biochemical parameters of human blood serum and seminal plasma. Andrologia 1987; 19:625–628.
4. Ujihara M, Urade Y, Eguchi N, Hayashi H, Ikai K, Hayaishi O. Prostaglandin D_2 formation and characterization of its synthetases in various tissues of adult rats. Arch Biochem Biophys 1988; 260:521–531.
5. Giles H, Leff P. The biology and pharmacology of PGD_2. Prostaglandins 1988; 35:277–300.
6. Braun M, Schror K. Prostaglandin D_2 relaxes bovine coronary arteries by endothelium-dependent nitric oxide-mediated cGMP formation. Circ Res 1992; 71:1305–1313.
7. Narumiya S, Toda N. Different responsiveness of prostaglandin D_2-sensitive systems to prostaglandin D_2 and its analogues. Br J Pharmacol 1985; 85:367–375.
8. Shimizu T, Yamamoto S, Hayaishi O. Purification and properties of prostaglandin D synthetase from rat brain. J Biol Chem 1979; 254: 5222–5228.
9. Urade Y, Fujimoto N, Hayaishi O. Purification and characterization of rat brain prostaglandin D synthetase. J Biol Chem 1985; 260:12410–12415.
10. Christ-Hazelhof E, Nugteren DH. Purification and characterization of prostaglandin endoperoxide D-isomerase, a cytoplasmic, glutathione-requiring enzyme. Biochim Biophys Acta 1979; 572:43–51.
11. Urade Y, Fujimoto N, Ujihara M, Hayaishi O. Biochemical and immunological characterization of rat spleen prostaglandin D synthetase. J Biol Chem 1987; 262:3820–3825.
12. Ujihara M, Tsuchida S, Satoh K, Sato K, Urade Y. Biochemical and immunological demonstration of prostaglandin D_2, E_2, and $F_{2\alpha}$ formation from prostaglandin H_2 by various rat glutathione S-transferase isozymes. Arch Biochem Biophys 1988; 264:428–437.
13. Tachibana M, Fex J, Urade Y, Hayaishi O. Brain-type prostaglandin D synthetase occurs in the rat cochlea. Proc Natl Acad Sci USA 1987; 84:7677–7680.
14. Goh Y, Urade Y, Fujimoto N, Hayaishi O. Content and formation of prostaglandins and distribution of prostaglandin-related enzyme activities in the rat ocular system. Biochim Biophys Acta 1987; 921:302–311.
15. Urade Y, Nagata A, Suzuki Y, Fujii Y, Hayaishi O. Primary structure of rat brain prostaglandin D synthetase deduced from cDNA sequence. J Biol Chem 1989; 264:1041–1045.
16. Nagata A, Suzuki Y, Igarashi M, Eguchi N, Toh H, Urade Y, Hayaishi O. Human brain prostaglandin D synthase has been evolutionarily differentiated from lipophilic-ligand carrier proteins. Proc Natl Acad Sci USA 1991; 88:4020–4024.
17. Igarashi M, Nagata A, Toh H, Urade Y, Hayaishi O. Structural organization of the gene for prostaglandin D synthase in the rat brain. Proc Natl Acad Sci USA 1992; 89:5376–5380.
18. White DM, Mikol DD, Espinosa R, Weimer B, Le Beau MM, Stefansson K. Structure and chromosomal localization of the human gene for a brain form of prostaglandin D_2 synthase. J Biol Chem 1992; 267:23202–23208.
19. Pervaiz S, Brew K. Homology and structure-function correlations between alpha 1-acid glycoprotein and serum retinol-binding protein and its relatives. FASEB J 1987; 1:209–214.
20. Kuruvilla AP, Hochwald GM, Ghiso J, Castano EM, Pizzolato M, Frangione B. Isolation and amino terminal sequence of beta-trace, a novel protein from human cerebrospinal fluid. Brain Res 1991; 565: 337–340.
21. Hoffmann A, Conradt HS, Gross G, Nimtz M, Lottspeich F, Wurster U. Purification and chemical characterization of beta-trace protein from human cerebrospinal fluid: its identification as prostaglandin D synthase. J Neurochem 1993; 61:451–456.
22. Watanabe K, Urade Y, Mader M, Murphy C, Hayaishi O. Identification of beta-trace as prostaglandin D synthase. Biochem Biophys Res Commun 1994; 203:1110–1116.
23. Clausen J. Proteins in normal cerebrospinal fluid not found in serum. Proc Soc Exp Biol Med 1961; 107:170–172.
24. Olsson JE, Link H, Nosslin B. Metabolic studies on ^{125}I-labelled beta-trace protein, with special reference to synthesis within the central nervous system. J Neurochem 1973; 21:1153–1159.
25. Olsson JE. Correlation between the concentration of beta-trace protein and the number of spermatozoa in human semen. J Reprod Fertil 1975; 42:149–151.
26. Hochwald GM, Thorbecke GJ. Use of an antiserum against cerebro-

spinal fluid in demonstration of trace proteins in biological fluids. Proc Soc Exp Biol Med 1962; 109:91–95.
27. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951; 193:265–275.
28. Bensadoun A, Weinstein D. Assay of proteins in the presence of interfering materials. Anal Biochem 1976; 70:241–250.
29. Oda H, Eguchi N, Urade Y, Hayaishi O. Quantitative sandwich enzyme-linked immunosorbent assay for human secretory prostaglandin D synthase (β-Trace). Proc Jpn Acad 1996; 72:108–111.
30. Urade Y, Tanaka T, Eguchi N, Kikuchi M, Kimura H, Toh H, Hayaishi O. Structural and functional significance of cysteine residues of glutathione-independent prostaglandin D synthase. Identification of Cys^{65} as an essential thiol. J Biol Chem 1995; 270:1422–1428.
31. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; 162:156–159.
32. Tusell JM, Gelpi E. Prostaglandins E and F, and 19-hydroxylated E and F (series I and II) in semen of fertile men. Gas and liquid chromatographic separation with selected ion detection. J Chromatogr 1980; 181:295–310.
33. Kuno S, Ueno R, Hayaishi O. Prostaglandin E_2 administered via anus causes immunosuppression in male but not female rats: a possible pathogenesis of acquired immune deficiency syndrome in homosexual males. Proc Natl Acad Sci USA 1986; 83:2682–2683.
34. Fitzpatrick FA, Wynalda MA. Albumin-catalyzed metabolism of prostaglandin D_2. Identification of products formed in vitro. J Biol Chem 1983; 258:11713–11718.
35. Kikawa Y, Narumiya S, Fukushima M, Wakatsuka H, Hayaishi O. 9-Deoxy-Δ^9,Δ^{12}-13,14-dihydroprostaglandin D_2, a metabolite of prostaglandin D_2 formed in human plasma. Proc Natl Acad Sci USA 1984; 81:1317–1321.
36. Hirata Y, Hayashi H, Ito S, Kikawa Y, Ishibashi M, Sudo M, Miyazaki H, Fukushima M, Narumiya S, Hayaishi O. Occurrence of 9-deoxy-Δ^9,Δ^{12}-13,14-dihydroprostaglandin D_2 in human urine. J Biol Chem 1988; 263:16619–16625.
37. Kliewer SA, Lenhard JM, Willson TM, Patel I, Morris DC, Lehmann JM. A prostaglandin J_2 metabolite binds peroxisome proliferator-activated receptor γ and promotes adipocyte differentiation. Cell 1995; 83:813–819.
38. Forman BM, Tontonoz P, Chen J, Brun RP, Spiegelman BM, Evans RM. 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 is a ligand for the adipocyte determination factor PPARγ. Cell 1995; 83:803–812.
39. Hayaishi O. Molecular mechanisms of sleep-wake regulation: roles of prostaglandins D_2 and E_2. FASEB J 1991; 5:2575–2581.
40. Lewis RA, Soter NA, Diamond PT, Austen KF, Oates JA, Roberts L2. Prostaglandin D_2 generation after activation of rat and human mast cells with anti-IgE. J Immunol 1982; 129:1627–1631.
41. Urade Y, Ujihara M, Horiguchi Y, Ikai K, Hayaishi O. The major source of endogenous prostaglandin D_2 production is likely antigen-presenting cells. Localization of glutathione-requiring prostaglandin D synthetase in histiocytes, dendritic, and Kupffer cells in various rat tissues. J Immunol 1989; 143:2982–2989.
42. Wheeldon A, Vardey CJ. Characterization of the inhibitory prostanoid receptors on human neutrophils. Br J Pharmacol 1993; 108:1051–1054.
43. Jones HW, Wentz AC, Burnett LS. Novak's Textbook of Gynecology. Baltimore: Williams & Wilkins; 1988: 286–287.
44. Sanger GJ, Jackson A, Bennett A. A prostaglandin analogue which potently relaxes human uterus but not gut muscle. Eur J Pharmacol 1982; 81:141–143.
45. Hirata M, Kakizuka A, Aizawa M, Ushikubi F, Narumiya S. Molecular characterization of a mouse prostaglandin D receptor and functional expression of the cloned gene. Proc Natl Acad Sci USA 1994; 91: 11192–11196.
46. Kunz G, Beil D, Deininger H, Wildt L, Leyendecker G. The dynamics of rapid sperm transport through the female genital tract: evidence from vaginal sonography of uterine peristalsis and hysterosalpingoscintigraphy. Hum Reprod 1996; 11:627–632.
47. Fukushima M. Biological activities and mechanisms of action of PGJ_2 and related compounds: an update. Prostaglandins Leukotrienes Essent Fatty Acids 1992; 47:1–12.
48. Killian GJ, Chapman DA, Rogowski LA. Fertility-associated proteins in Holstein bull seminal plasma. Biol Reprod 1993; 49:1202–1207.
49. Gerena RL, Irikura D, Urade Y, Eguchi N, Chapman DA, Killian GJ. Identification of a fertility-associated protein in bull seminal plasma as lipocalin-type prostaglandin D synthase. Biol Reprod 1998; (in press).
50. Lewy RI, Bills TK, Dalton J, Smith JB, Silver MJ. 19-Hydroxy-prostaglandin E and infertility in human males. Prostaglandins Leukotrienes Med 1979; 2:367–372.
51. Autiero M, Sansone G, Abrescia P. Relative ratios of lactoferrin, albumin, and acid phosphatase seminal levels as sperm quality markers in fertile and infertile men. J Androl 1991; 12:191–200.
52. Johnson WL, Hunter AG, Jeyendran RS, Perez PM. Association of asthenozoospermia with a deficiency of a seminal glycoprotein. Int J Fertil 1985; 30:57–60.
53. Urade Y, Watanabe K, Hayaishi O. Prostaglandin D, E, and F synthases. J Lipid Mediators Cell Signalling 1995; 12:257–273.
54. Tanaka T, Urade Y, Kimura H, Eguchi N, Nishikawa A, Hayaishi O. Lipocalin-type prostaglandin D synthase (beta-trace) is a newly recognized type of retinoid transporter. J Biol Chem 1997; 272:15789–15795.
55. Urade Y. Dual function of beta-trace. Prostaglandins 1996; 51:286.
56. Mitranond V, Sobhon P, Tosukhowong P, Chindaduangrat W. Cytological changes in the testes of vitamin A-deficient rats. I. Quantitation of germinal cells in the seminiferous tubules. Acta Anat 1979; 103: 159–168.
57. van Pelt AMM, de Rooij DG. The origin of the synchronization of the seminiferous epithelium in vitamin A deficient rats after vitamin A replacement. Biol Reprod 1990; 42:677–682.
58. Lohnes D, Kastner P, Dierich A, Mark M, LeMeur M, Chambon P. Function of retinoic acid receptor γ in the mouse. Cell 1993; 73:643–658.
59. Zheng WL, Bucco RA, Schmitt MC, Wardlaw SA, Ong DE. Localization of cellular retinoic acid-binding protein (CRABP) II and CRABP in developing rat testis. Endocrinology 1996; 137:5028–5035.
60. Gorry P, Lufkin T, Dierich A, Rochette EC, Decimo D, Dolle P, Mark M, Durand B, Chambon P. The cellular retinoic acid binding protein I is dispensable. Proc Natl Acad Sci USA 1994; 91:9032–9036.
61. Fawcett D, Pasceri P, Fraser R, Colbert M, Rossant J, Giguere V. Postaxial polydactyly in forelimbs of CRABP-II mutant mice. Development 1995; 121:671–679.
62. Lampron C, Rochette EC, Gorry P, Dolle P, Mark M, Lufkin T, LeMeur M, Chambon P. Mice deficient in cellular retinoic acid binding protein II (CRABPII) or in both CRABPI and CRABPII are essentially normal. Development 1995; 121:539–548.
63. Olsson JE, Nord L. Immunochemical and immunofluorescence studies of beta-trace protein in different species and organs, with special reference to the central nervous system. J Neurochem 1973; 21:625–633.