Cloning of equine prostaglandin dehydrogenase and its gonadotropin-dependent regulation in theca and mural granulosa cells of equine preovulatory follicles during the ovulatory process

Khampoune Sayasith, Nadine Bouchard, Monique Doré¹ and Jean Sirois

Centre de recherche en reproduction animale and Département de biomédecine vétérinaire, Faculté de médecine vétérinaire, Université de Montréal, 3200 Sicotte, Saint-Hyacinthe, Québec, Canada J2S 7C6 and ¹Département de pathologie et microbiologie, Faculté de médecine vétérinaire, Université de Montréal, Saint-Hyacinthe, Québec, Canada J2S 7C6

Correspondence should be addressed to K Sayasith; Email: k.sayasith@umontreal.ca

Abstract

The mammalian ovulatory process is accompanied by a gonadotropin-dependent increase in follicular levels of prostaglandin E₂ (PGE₂) and PGF₂ α , which are metabolized by 15-hydroxy prostaglandin dehydrogenase (PGDH). Little is known about ovarian PGDH regulation in non-primate species. The objectives of this study were to characterize the structure of equine PGDH and its regulation in follicles during human chorionic gonadotropin (hCG)-induced ovulation. The full-length equine PGDH was obtained by RT-PCR, 5'- and 3'-rapid amplification of cDNA ends (RACE). Its open reading frame encodes a 266-amino acid protein that is 72–95% homologous to other species. Semi-quantitative RT-PCR/Southern blot were used to study PGDH regulation in follicles isolated 0–39 h post-hCG. Results showed that PGDH mRNA expression was low in follicles obtained at 0 h, increased at 12 and 24 h ($P < 0.05$), and decreased at 36-h post-hCG. This induction of expression was biphasic, with elevated abundance of transcripts at 12 and 33 h post-hCG ($P < 0.05$) in mural granulosa and theca cells. Immunohistochemistry and immunoblotting confirmed regulated expression of PGDH protein in both cell types of preovulatory follicles after hCG. High levels of PGDH mRNA were observed in corpus luteum and other non-ovarian tissues tested, except kidney, muscle, brain, and heart. Thus, this study is the first to report the gonadotropin-dependent regulation of PGDH during ovulation in a non-primate species. PGDH induction was biphasic in theca and mural granulosa cells differing from primates in which this induction was monophasic and limited to granulosa cells, suggesting species-specific differences in follicular control of PGDH expression during ovulation.

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Introduction

Prostaglandins (PGs) are potent mediators of several biological processes, including ovulation and inflammation. Their synthesis from plasma membrane arachidonic acid is dependent on the expression of prostaglandin G/H synthase (PGHS; also known as cyclooxygenase or COX) and terminal synthases (Sirois *et al.* 2004), and their regulation can occur at different levels including biosynthesis, receptor, transport, and metabolism. The metabolism of PGs is mediated by nicotinamide adenine dinucleotide (NAD)⁺-dependent 15-hydroxy prostaglandin dehydrogenase (PGDH; also as type-I 15-PGDH), which belongs to the family of short chain dehydrogenase/reductase (SDR) and contains a region of 20 amino acids that is highly conserved within

this family of enzymes (Krook *et al.* 1990). PGDH catalyzes the first step of PG inactivation by cytoplasmic oxidation of the 15-hydroxyl group leading into the generation of 15 keto-metabolites (Ensor & Tai 1995, Okita & Okita 1996). This reaction occurs after the uptake of PGs across the plasma membrane by a PG transporter (Nomura *et al.* 2005). PGDH catalyzes not only the oxidation of PGs under physiological conditions, but also the reduction of 15-keto-metabolites under a lower pH (Yamazaki & Sasaki 1975). PGDH is ubiquitously expressed in mammalian tissues and can be found in vascular beds, including placenta, lung and kidney, as well as in peripheral tissues, suggesting that local metabolism can also contribute to peripheral regulation of PG activity (Bergholte & Okita 1986, Mak *et al.* 1990, Tombach *et al.* 1990).

PGDH cDNA has been cloned from several species and organs, including human placenta, mouse lungs, rat intestine, and bovine and porcine uterus (Ensor *et al.* 1990, Matsuo *et al.* 1996, Bracken *et al.* 1997, Zhang *et al.* 1997, Parent *et al.* 2006). Human PGDH encodes a protein of 266 amino acids with an apparent molecular weight of 29 kDa (Ensor *et al.* 1990). Its bioactive form is thought to be a dimer, although some believe that it can function as in a monomer (Hohl *et al.* 1993, Tai *et al.* 2006). Sequence alignment of PGDH from various species indicates that the primary structure of the enzyme is highly homologous, containing a conserved SDR domain, with a C-terminal domain and the region from residue 205 to 224 being more divergent (Tai *et al.* 2006). PGDH is known to inactivate PGs and, in the lungs, rapid PGDH inactivation renders primary PGs at low levels in the plasma (Erwich & Keirse 1992). In placenta, PGs play a key role in the initiation of parturition in which the expression of PGDH is decreased and concentrations of PGs are conversely increased at term (Sangha *et al.* 1994, Winchester *et al.* 2002). A decrease of PGDH expression is also observed in several cancer tissues and tumor cell lines, including gastrointestinal cancers and colon, lung, and breast carcinomas (Backlund *et al.* 2005), whereas functional inactivation of PGDH by genetic deletion increases PGE2 levels in tissues, and blocks production of the urinary PGE2 metabolite (Coggins *et al.* 2002, Backlund *et al.* 2005). As PGE2 is associated with resistance to programmed cell death and stimulation of cell migration, cell proliferation and angiogenesis (Sheng *et al.* 1998, Rozic *et al.* 2001), a loss of PGDH activity suggests its physiological role as tumor suppressor in cancer tissues. As noted above, little is known about the role of PGDH in the ovulatory process.

Ovulation is an luteinizing hormone (LH)-induced process that involves a series of biochemical and biophysical events, ultimately leading to rupture of the preovulatory follicle and release of the oocyte. This process is accompanied with a selective induction of PGHS-2 in granulosa cells and a marked increase in intrafollicular levels of PGE2 and PGF2 α (Espey 1980, Watson & Sertich 1990, Sirois & Doré 1997, Watson & Hinrichs 1998). Studies on the regulation of PGDH during ovulation are limited to one recent report from the preovulatory follicle of a primate (Duffy *et al.* 2005). However, studies on the expression and regulation of PGDH in mares are of interest because preovulatory follicular development and ovulation in this animal species show several distinctive characteristics compared with other species, such as a large diameter of the ovulatory follicle (40–45 mm), a relatively long ovulatory process (39–42 h), and the follicular rupture at the ovulatory fossa (Stabenfeldt *et al.* 1975, Duchamp *et al.* 1987, Ginther 1992, Sirois & Doré 1997, Kerban *et al.* 1999, Boerboom *et al.* 2000). Moreover, the preovulatory rise in gonadotropins in mares causes an

extensive expansion of the entire mural granulosa cell layer resulting in abundant accumulation of extracellular matrix (Kerban *et al.* 1999) that differs from that occurred in rodents in which the expansion process appears primarily limited to the cumulus oocyte complex. The unique and remarkable cellular features observed in equine follicles suggest that the regulation of PGDH may differ in this species. Therefore, the specific objectives of the study were to characterize the primary structure of equine PGDH and to investigate the gonadotropin-dependent regulation of PGDH in equine follicles during the ovulatory process.

Materials and Methods

Cloning of the equine PGDH cDNA

The full-length equine PGDH cDNA was isolated by a combination of RT-PCR, 5'- and 3'-RACE. RT-PCR was first performed using sense and anti-sense primers (primers 1 and 2) designed from a highly conserved region identified after sequence alignments of human (GenBank Accession number, NM_000860) and mouse (GenBank Accession number, NM_008278) PGDH homologues, 100 ng pooled equine ovarian RNA, and a OneStep RT-PCR System (Qiagen) as directed by the manufacturer (Fig. 1Aa). Pooled ovarian RNA consists of equal amounts of RNA prepared from a preovulatory follicle isolated before hCG, another follicle isolated 36 h after hCG, and a corpus luteum obtained on day 8 of the cycle (day 0, day of ovulation), as described previously (Boerboom *et al.* 2000). RT-PCR consisted of one cycle of 50 °C for 30 min and 95 °C for 15 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 60 s, and 72 °C for 1 min. The RT-PCR product was subcloned into pGEM-T Easy vector (Promega) and sequenced (Service de Séquençage de l'Université de Laval, Québec, Canada). A large equine PGDH cDNA fragment was obtained.

To characterize the 3'-end of equine PGDH cDNA, a 3'-RACE was performed as described previously (Boerboom *et al.* 2000) using 5 μ g total RNA isolated from a preovulatory follicle obtained 36 h post-hCG. Briefly, reverse transcription was performed using a poly-dT oligonucleotide with anchor sequences at its 5'-end (primer 3) followed by a first PCR done with sense primer 4 and anti-sense 5, and a second nested PCR with sense primer 6 and anti-sense primer 7 (Fig. 1Ab). Primers 4 and 6 were designed from the 3'-end of a large equine PGDH cDNA fragment.

To obtain the missing 5'-end of equine PGDH cDNA, the 5'-RACE system version 2.0 (Invitrogen Life Technologies) was used as directed by the manufacturer. Reverse transcription was performed as directed using anti-sense primer 8 and 3 μ g RNA from a 36-h post-hCG preovulatory follicle. The first 5'-RACE/PCR was performed with sense abridged anchor primer 9 (Invitrogen

Figure 1 Cloning strategy for isolating the equine PGDH cDNA. (A) The open reading frame (ORF) of equine PGDH is represented as an open box whereas the 3'-UTR are shown as solid lines; numbers in parenthesis indicate the size in base pairs of equine PGDH ORF and 3'-UTR. The equine PGDH cDNA was characterized by a combination of (a) RT-PCR, (b) 3'-RACE, and (c) 5'-RACE as described in Materials and Methods, and (d) the full-length equine PGDH coding sequence was isolated by RT-PCR; arrows and numbers refer to the orientation, relative position and identity of oligonucleotide primers used in each cloning procedure. (B) List of primers used in section A for equine PGDH cloning. The abridged anchor primer (9) and abridged universal amplification primer (11) are components of the 5'-RACE system (Invitrogen Life Technologies).

Life Technologies) and anti-sense primer 10, whereas, the second 5'-RACE/PCR employed the sense abridged universal amplification primer 11 (Invitrogen Life Technologies) and anti-sense primer 12 (Fig. 1Ac). The primers 10 and 12 were designed from the 5'-end of large equine PGDH cDNA fragment. PCRs for both 3'- and 5'-RACE consisted of 35 cycles of 94 °C for 30 s, 58 °C for 60 s, and 72 °C for 1 min. Final PCR products were subcloned into pGEM-T Easy vector, and sequenced. The complete equine PGDH coding region was isolated by RT-PCR using 100 ng RNA from a 36-h post-hCG preovulatory follicle, and anti-sense primer 13 and sense primer 14 (Fig. 1Ad), subcloned into pcDNA 3.1 (+) expression vector (Invitrogen Life Technologies) and sequenced.

Equine tissues and RNA extraction

Equine preovulatory follicles and corpora lutea were isolated at specific stages of the estrous cycle from Standardbred and Thoroughbred mares as previously described (Kerban *et al.* 1999). Briefly, when

preovulatory follicles reached 35 mm in diameter during estrus, the ovulatory process was induced by injection of hCG (2500 IU, i.v.) and ovariectomies were performed through colpotomy using an ovariome at 0, 12, 24, 30, 33, 36, or 39 h post-hCG ($n=4-6$ mares/time point). Corpora lutea ($n=3$ mares) were isolated on day 8 of the estrous cycle. Preovulatory follicles and corpora lutea were dissected from the surrounding ovarian tissues with a scalpel. Follicles were dissected into three cellular preparations referred to as the follicular wall (theca interna with attached granulosa cells), and isolated mural granulosa cells and thecal layers, as described (Sirois *et al.* 1991). The relative purity of each cellular preparation is estimated to exceed 95% based on the selective expression of P450 17 α -hydroxylase-C17-20 lyase (CYP17A1) and P450 aromatase (CYP19A1) mRNAs by theca interna and granulosa cells respectively (Boerboom *et al.* 1999). Testicular tissues were obtained from the Hôpital de grandes animaux, Faculté de médecine vétérinaire (Université de Montréal) following a routine castration, whereas, other non-ovarian tissues were collected at a local slaughterhouse. All animal

procedures were approved by the institutional animal use and care committee. Total RNA was isolated from tissues with TRIzol reagent (Invitrogen Canada, Inc.) according to the manufacturer's instructions using a Kinematica PT 1200C Polytron Homogenizer (Fisher Scientific, Montréal, Canada).

Semi-quantitative RT-PCR and Southern blot analysis

The semi-quantitative analysis of PGDH and rpL7a mRNA levels in equine tissues was performed using a OneStep RT-PCR System as directed by the manufacturer (Qiagen) and sense primer 1 and anti-sense primer 2 (Fig. 1B) specific for equine PGDH (generating a DNA fragment of 445 bp), and sense (5'-ACA GGA CAT CCA GCC CAA ACG-3') and anti-sense (5'-GCT CCT TTG TCT TCC GAG TTG-3') primers specific for equine rpL7a (generating a DNA fragment of 516 bp). The equine rpL7a primers were designed from a published sequence (GenBank Accession number, AF508309), and its transcript expression has been shown to be relatively constant during the equine ovulatory process (Brown *et al.* 2004, Sayasith *et al.* 2005). Each reaction was conducted using 100 ng total RNA, and cycling conditions were one cycle of 50 °C for 30 min and 95 °C for 15 min, followed by 24 PCR cycles of 94 °C for 30 s, 60 °C for 1 min and 72 °C for 2 min for PGDH, or 18 PCR cycles of 94 °C for 30 s, 58 °C for 1 min and 72 °C for 2 min for rpL7a. The number of PCR cycles used was determined by running a RT-PCR in 50 µl. A volume of 5 µl was pipetted after each three PCR cycles, starting from the 12th amplification cycle. PCR products were electrophoresed on 2% TAE-agarose gels, transferred to biotrans nylon membranes (ICN Pharmaceuticals, Montréal, Québec, Canada) and hybridized with corresponding radiolabeled PGDH and rpL7a cDNA fragments using QuikHyb hybridization solution (Stratagene, LaJolla, CA, USA). Membranes were exposed to a phosphor screen. Signals were quantified on a Storm imaging system using the ImageQuant software version 1.1 (Molecular Dynamics, Amersham Biosciences) and used to establish a standard curve of amplification in which the number of PCR cycles falling in linear range of amplification corresponding to optimal reactions (exponential amplification) was determined.

Cell cultures, transient transfection, cell extracts and western blot analysis

An equine granulosa cell line produced in our laboratory (unpublished results) was cultured in Dulbecco's modified Eagle's medium (DMEM)-F12 (Invitrogen Life Technologies) containing L-glutamine, non-essential amino acids, 10% fetal bovine serum, and penicillin (100 units/ml)–streptomycin (100 µg/ml). Cultures were maintained at 37 °C in a humidified atmosphere of 5%

CO₂, and transiently transfected with 4 µg/petris of PGDH expression vector using 30 µg LipofectAMINE PLUS reagent in 4 ml DMEM-F12, in accordance with the manufacturer's protocol. Three hours after transfection, cells were rinsed, incubated in culture media for 24 h and harvested. Protein extracts were prepared as previously described (Filion *et al.* 2001, Sayasith *et al.* 2004). Briefly, tissues and cells were homogenized and sonicated on ice in a buffer containing 20 mM Tris (pH 8.0), 50 mM EDTA, 0.1 mM diethyldithiocarbamic acid, and 1.0% Tween (TED buffer). Sonicates were centrifuged at 16 000 g for 15 min at 4 °C, and the recovered supernatants were stored at –80 °C. Protein concentration was quantified by the method of Bradford (Bradford 1976, Bio-Rad Protein Assay). Samples (50–100 µg/well) were resolved by one-dimensional SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes. Membranes were incubated with the anti-equine PGDH antibody (1:1000 dilution) or anti-human PGDH antibody (1:200; Cayman Chemical, Gardner, MI, USA). The anti-equine PGDH antibody was commercially produced (New England Peptide, Inc. MA, USA) using an equine PGDH peptide fragment CKAALDEQFEPRKTL to immunize rabbits. Immunoreactive proteins were visualized on Kodak X-OMAT AR film (Eastman Kodak Co., Rochester, NY, USA) after incubation with the horseradish peroxidase-linked donkey anti-rabbit secondary antibody (1:6000) and the enhanced chemiluminescence system (ECL Plus) following the manufacturer's protocol (Amersham Pharmacia Biotech).

Immunohistochemical localization of PGDH in equine follicles

Immunohistochemical staining was performed using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA), as previously described (Sirois & Doré 1997). Briefly, formalin-fixed tissues were paraffin-embedded, and 3 µm thick sections were prepared and then the paraffin was removed using a series of alcohol concentrations. Endogenous peroxidase was quenched by incubating the slides in 0.3% hydrogen peroxide in methanol for 30 min. After rinsing in PBS for 15 min, sections were incubated with diluted normal goat serum for 20 min at room temperature. The anti-equine PGDH antibody was diluted in PBS (1:2500) and applied, and sections were incubated overnight at 4 °C. Control sections were incubated with PBS. After rinsing in PBS for 10 min, a biotinylated goat anti-rabbit antibody (1:222; Vector Laboratories) was applied and sections were incubated for 45 min at room temperature. After washing in PBS for 10 min, sections were incubated with the avidin DH-biotinylated horseradish peroxidase H reagents for 45 min at room temperature, washed with PBS for 10 min, and incubated with diaminobenzidine tetrahydrochloride (Sigma) as the chromogen substrate.

Sections were counterstained with Gill's haematoxylin stain and mounted.

Statistical analysis

One-way ANOVA was used to test the effect of time after hCG on levels of PGDH mRNA in samples of theca and granulosa cells. PGDH mRNA levels were normalized with the control gene *rpL7a* prior to analysis. When ANOVA indicated significant differences ($P < 0.05$), the Dunnett's test was used for comparisons of individual means. Statistical analyses were performed using JMP software (SAS Institute, Inc., Cary, NC, USA).

Results

Characterization of the equine PGDH cDNA

To clone equine PGDH cDNA, an equine PGDH cDNA fragment of 445-bp was first isolated by RT-PCR using primers designed by sequence alignments of human and mouse PGDH homologues (Fig. 1Aa). Sense and anti-sense primers designed from the latter cDNA fragment were used in 3'- and 5'-RACE reactions to characterize the 3'-end and to obtain the missing 5'-end of equine PGDH cDNA (fragments of 543 and 91 bp respectively; Fig. 1Ab and Ac). Results showed that the equine PGDH cDNA was composed of an open reading frame (ORF) of 801 bp (including the stop codon), and a 3'-UTR of 278 bp (Fig. 2). The nucleotide sequence was submitted to GenBank with Accession number DQ385611.

The predicted complete coding region of equine PGDH was isolated by RT-PCR, subcloned and confirmed by sequencing. This region encodes a 266 amino acid protein, which is similar in length to human (GenBank Accession number, NM_000860), mouse (GenBank Accession number, NM_008278), fish (GenBank Accession number, XM_689239) and canine (GenBank Accession number, XM_543199), but one amino acid longer than pig (GenBank Accession number, Y07953) and chicken (GenBank Accession number, XM_420526) PGDH homologues (Fig. 2). Comparative analyses indicated that the equine PGDH amino acid sequence is relatively similar to its homologues, ranging from 72 to 95% identity (Fig. 2). Moreover, structural domains putatively involved in PGDH functions are conserved in the equine protein, including a SDR domain containing active sites for the enzyme, a signal peptide, an asparagine (N)-linked glycosylation consensus sequence and amino acids required for NAD^+ -binding (Fig. 2 (Tai *et al.* 2006)).

Tissue distribution of equine PGDH mRNA and its regulation in preovulatory follicles

The distribution of PGDH mRNA in various equine tissues was studied by RT-PCR/Southern blot. Results

revealed that levels of PGDH transcripts were low in kidney, muscle and brain, moderate in heart, and high in the preovulatory follicle isolated 12 h after hCG and in other non-ovarian tissues tested (Fig. 3A).

The regulation of PGDH mRNA during the ovulatory process was investigated by semi-quantitative RT-PCR/Southern blot using total RNA extracted from follicle walls (theca layers with attached granulosa cells) of equine preovulatory follicles isolated between 0 and 36 h after hCG treatment, and corpora lutea obtained on day 8 of cycle. Results showed that the expression of PGDH mRNA was low before hCG treatment (0 h), highly increased at 12 and 24 h, and decreased at 36-h post-hCG (Fig. 3C). Levels of the PGDH transcript were relatively high in corpora lutea (Fig. 3C). When results from several follicles ($n=4-6$ distinct follicles (each from different animal) per time point) and corpus luteum ($n=3$) were expressed as ratios of PGDH to *rpL7a*, a significant increase of PGDH mRNA at 12 and 24 h in preovulatory follicles and corpora lutea was observed as compared with 0 h post-hCG ($P < 0.05$; Fig. 3D).

To verify whether equine PGDH mRNA expression is present in granulosa cells and/or theca cells, isolated preparations of both cells were prepared from equine preovulatory follicles and analyzed by RT-PCR/Southern blot. In granulosa cells, results revealed that levels of PGDH mRNA were low at 0 h, increased markedly at 12 h, decreased at 24 and 30 h, increased again at 33 h, and decreased between 36- and 39-h post-hCG (Fig. 4A). When results from several follicles ($n=4-5$) were expressed as ratios of PGDH to *rpL7a*, increased levels of PGDH mRNA were observed after hCG, when compared with 0 h post-hCG ($P < 0.05$; Fig. 4B). In theca interna layer, results revealed that levels of PGDH mRNA were low at 0 h, increased at 12 h, decreased at 24 h, increased again between 30 and 33 h, and decreased from 36 to 39 h post-hCG (Fig. 4C). When results from multiple follicles ($n=4-5$) were expressed as ratios of PGDH to *rpL7a*, a marked increase in PGDH mRNA levels was detected at 12 and 33 h post-hCG, as compared with 0 h post-hCG ($P < 0.05$; Fig. 4D).

Regulation of PGDH protein in preovulatory follicles

To determine whether the induction of the PGDH transcript was associated to its protein production in preovulatory follicles, immunoblotting was performed. The specificity of the anti-equine PGDH antibody against PGDH protein was determined using extracts prepared from granulosa cells overexpressing equine PGDH. Results showed that no protein band was revealed by equine antibody in a mock transfection of granulosa cell (Fig. 5A, lane 1), whereas a single protein band was recognized by the antibody when transfected cell extracts were assayed (Fig. 5A, lane 2). This protein band appears in the range of the calculated molecular mass of the full-length PGDH protein (29 kDa) in the

	V	
equ	MHVNGKVALV <u>T</u> GAAQGI <u>I</u> GRAFAEELLHKGAKVALDCNLEAGVKCKAALD	50
hum	 L W Q A .	50
mou	 K A . LH W	50
pig	 G W	50
can	 S . A W	50
fis	 S . A W	50
chi	 A VQA . G L . R . P . . . QQS	50
equ	EQFEPRKTFLFIQC ⁺ DVANQEQLRD <u>TFR</u> KVVDFHGRLDILVN <u>N</u> AGVNNEKNW	100
hum	 Q D . Q	100
mou	 Q V D . K	100
pig	 Q D T Y	100
can	 Q D . G	100
fis	 Q D . G	100
chi	 QR . V TDT . . KGA . K . IE V D .	100
	* 	
equ	EKTLQINLVSVISGYLGLDYMSKQNGEGGGIIIN <u>MS</u> SLAGLIPVAQQPV	150
hum	 M	150
mou	. Q G M	150
pig	 V M	150
can	 H M	150
fis	 M	150
chi	. S . I T R E . . R . G . . . D . V . . . I M . A . F . . .	150
	* * 	
equ	YCASKHGIIIGFTRSAAMAANLMNSGVRLNAICPGFVNTPILESIEKEENM	200
hum	 V L A	200
mou	. S V D V	200
pig	 RK M S . Q	200
can		200
fis		200
chi	. . T . . V I . L . ME . Y . . . T Q . D . . .	200
equ	GQYIEYKDHIKDMMKFYGILDPSMIANGLITLIEDDALNGAIMKITTSKG	250
hum	 I . Y PL M . V	250
mou	. . . G . . Q . . A V . H . A D P . . SA . E .	250
pig	. P . . TG C E D T . N .	250
can	 S	250
fis	 S	250
chi	. . YS N . Q . . VM . . I . E . . I . . T . EV . . A . Q .	250
equ	IHFQNYDTIPFHTKTQ	266 (100%)
hum	. . . D . . T	266 (92%)
mou	. . . D . IS . LLV . AP	266 (86%)
pig	. . . D . . A . SYA . D -	265 (89%)
can	. . . D . E . T . . A . .	266 (95%)
fis	. . . D . E . T . . A . .	266 (95%)
chi	. . . E . SEO . Y . S . R -	265 (72%)

Figure 2 Deduced amino acid sequence of equine PGDH and its comparison with other mammalian homologues. The amino acid sequence of equine (equ) PGDH is aligned with the human (hum), mouse (mou), pig, canine (can), fish (fis), and chicken (chi) homologues. Identical residues are noted with a printed period, hyphens indicate gaps in protein sequences created to optimize alignment, numbers on the right are for the last amino acid residue on that line, and the percentage in parentheses refers to the percentage of identity (%) in amino acid residues when comparisons are made with equine PGDH. The putative domain of short-chain dehydrogenase/reductase family is boxed, with the active sites identified by an asterisk; the signal peptide and predicted cleavage site are overlined and marked by an empty triangle, respectively; the asparagine (N)-linked glycosylation consensus sequence is underlined; sites for protein kinase C phosphorylation are double-overlined; and amino acid residues forming NAD⁺-binding site are double-underlined.

absence of post-translational modifications. In parallel, anti-human PGDH antibody (Cayman Chemical, Ann Arbor, MI, USA) known to cross-react with bovine, guinea pig, and baboon PGDH was tested against equine

protein extracts to compare with the equine antibody specificity. Similar results were observed when compared with anti-equine PGDH antibody (Fig. 5B). Analyses performed with equine extracts obtained from

Figure 3 Expression of PGDH mRNA in equine tissues and its gonadotropin-dependent regulation in equine preovulatory follicles. Relative expression of equine PGDH (A) and rpL7a (B; control gene) were analyzed by a semi-quantitative RT-PCR/Southern blot using total RNA (100 ng) extracted from various equine tissues, as described in Materials and Methods. Numbers on the right indicate the size of the PCR fragment in bp. (C–E) RNA extracts were prepared from the wall (theca with attached granulosa cells) of equine preovulatory follicles at 0, 12, 24, and 36 h after administration of an ovulatory dose of hCG, and corpora lutea (CL) isolated on day 8 of the cycle. Samples (100 ng) were analyzed by a semi-quantitative RT-PCR/Southern blot for the expression of PGDH and rpL7a transcripts, as described in Materials and Methods. Representative results of PGDH (C) and rpL7a (D) mRNA levels are presented from one follicle/time point. (E) The PGDH signal was normalized with the control gene rpL7a, and results are presented as PGDH to rpL7a ratios. Mean $\pm$ S.E.M.; $n=4$ –6 distinct follicles, or $n=3$ CL (i.e. mares) per time point. Bars marked with an asterisk are significantly different from 0 h post-hCG ($P<0.05$).

preovulatory follicles isolated 0–36 h after hCG revealed that multiple protein bands were recognized by the equine antibody (Fig. 5C, lanes 2–5) situation, also observed in other species (Schlegel *et al.* 1974, Ensor & Tai 1995, Giannoulis *et al.* 2002): the lower band appears in the range of 29 kDa (a) corresponding to the monomer of full-length PGDH protein; the major band around 60 kDa (b) most likely represent dimeric form of the PGDH protein; and the bands between monomeric and dimeric forms most likely correspond to PGDH proteins with different extents of glycosylation. A similar protein band pattern was seen with equine testis extracts (Fig. 5D) in keeping with a high abundance of PGDH mRNA detected in this tissue (Fig. 3A). Interestingly, all protein bands followed the similar trend of changes: levels of protein bands were low at 0 h (Fig. 5C, lane 2) but increased markedly after hCG, with high levels detected at 24 h post-hCG (Fig. 5C, lanes 3–5); monomeric form of the protein was absent before hCG (0 h) but highly increased from 12 to 24 h post-hCG and decreased thereafter. Of note, similar results were also obtained when anti-human PGDH antibody was used (data not shown).

To investigate the intracellular localization of the PGDH protein, immunohistochemistry was performed on sections of equine preovulatory follicles isolated before and after hCG treatment using the anti-equine PGDH antibody. Results from one representative follicle showed that the intensity of a staining was very low in mural granulosa and theca layers of preovulatory follicles obtained before hCG (0 h; Fig. 6A), but high at 12, 24, 33, and 36 h post-hCG (Fig. 6B–E) in both cell types as compared with 0 h. The increase of a staining was accompanied by a progressive loosening (i.e. expansion) of the granulosa cell layer induced by hCG during which PGDH was localized to the cytoplasm of granulosa and theca cells. This increase was correlated with that of PGDH protein intensity from preovulatory follicular extracts obtained after hCG as demonstrated by immunoblotting (Fig. 5C). As a control of staining, the primary antibody was replaced with PBS and no staining was observed in follicles isolated after hCG (Fig. 6F).

Discussion

Prostaglandins are known to play important role in the ovulatory process during which levels of PGE2 and PGF2 α are highly increased just prior to ovulation (Espey 1980, Watson & Sertich 1990, Sirois & Doré 1997, Watson & Hinrichs 1998). PGDH is an important enzyme involved in PGE2 and PGF2 α catabolism. There is only one report on the regulation of PGDH in ovarian physiology of a primate (Duffy *et al.* 2005). However, the information obtained from animal species does not always reflect the spectrum of potential expression among species. For example, in mares, follicular development and ovulation show several distinct characteristics that are not observed elsewhere: the

Figure 4 Cellular localization of PGDH mRNA in equine preovulatory follicles. Total RNA was separately extracted from mural granulosa and theca cells isolated from equine preovulatory follicles between 0 and 39 h post-hCG. Semi-quantitative RT-PCR/Southern blot was performed with these samples (100 ng) to analyze for PGDH and rpL7a content, as described in Materials and Methods. Representative results of PGDH and rpL7a mRNA levels are presented from one granulosa (A) and one theca (C) sample per time point. (B and D) The PGDH signal was normalized to the control gene rpL7a, and results are presented as ratios of PGDH to rpL7a (mean $\pm$ S.E.M.; $n=4-5$ samples (i.e. mares) per time point). Bars marked with an asterisk are significantly different from 0 h post-hCG ($P<0.05$).

gonadotropin rise in preovulatory follicles induces a unique and remarkable modification of the granulosa cell layer in follicles prior to ovulation, characterized by extensive synthesis and assembly of a viscoelastic extracellular matrix (ECM) within the granulosa cell layer (Kerban *et al.* 1999) and the spatial and temporal regulation of several genes involved in ovulation and steroidogenesis in preovulatory follicles differs from those observed in the ovary of other mammals (Sirois *et al.* 1991, Boerboom *et al.* 1999, Kerban *et al.* 1999, 2000). This difference in the pattern of gene regulation is further supported by the present study. Indeed, in the primate ovary, PGDH induction occurs in a monophasic manner with a robust induction of PGDH detected at 12 h post-hCG and a marked decrease from then to ovulation (ovulation occurs around 40–42 h post-hCG in this species) (Duffy *et al.* 2005). The later induction is restricted only to granulosa cells (Duffy *et al.* 2005). In contrast, our findings indicate that the expression of gonadotropin-induced PGDH in the mare was biphasic with strong induction observed at 12 and 33 h after hCG, in both granulosa and theca cells, two main cellular compartments of preovulatory follicles. These differences underline the importance of use of different animal species to determine the physiological importance of genes.

As no previous study on PGDH regulation has been performed in mares, this study is the first to report that the ovulatory process induced by hCG is accompanied by high levels of PGDH expression and its regulation in equine preovulatory follicles. PGDH is the primary enzyme to metabolize PGE₂ as well as PGF₂ α , converting the latter into their 15-keto-derivative metabolites, biologically inactive forms (Tai *et al.* 2001). Thus,

the expression of PGDH as well as its regulation can be considered as an important regulator of biological fluid PG concentrations that complements their biosynthesis. PGE₂ and PGF₂ α synthesis is dependent on the PGHS-2 expression, a rate-limiting enzyme for PG synthesis, and terminal synthases, PG E synthase (PGES) and PG F synthase (PGFS) respectively (Sirois *et al.* 2004), and their biological action is known to mediate through specific receptors, EP1 to EP4 for PGE₂ and FP for PGF₂ α (Sirois *et al.* 2004). The obligatory role of PGHS-2 and the PGE receptor EP2 in ovulation has been well established in rodents. Indeed, mice deficient in PGHS-2 or receptor EP2 are infertile and display abnormal ovulation (Lim *et al.* 1997, Davis *et al.* 1999, Hizaki *et al.* 1999, Tilley *et al.* 1999), which can be restored by an administration of exogenous PGE₂ (Davis *et al.* 1999). In several species, follicular fluid PGE₂ and PGF₂ α levels rise rapidly after gonadotropin-dependent expression of PGHS-2 in preovulatory follicles (Wong & Richards 1992, Sirois 1994, Sirois & Doré 1997). In equine preovulatory follicles, PGHS-2 mRNA expression first became apparent at 30 h in granulosa cells, reached maximal levels at 33-h post-hCG, and declined thereafter; levels of the PGHS-2 protein were low or undetected from 0 to 30 h, but markedly increased from 33 to 39 h post-hCG, and high levels of follicular fluid PGE₂ and PGF₂ α were observed only between 36 and 39 h post-hCG just before ovulation (Sirois & Doré 1997, Boerboom & Sirois 1998). Of note, there is a marked increase of gonadotropin-stimulated PGHS-2 expression that precedes maximal follicular fluid PG levels, suggesting that higher levels of PGs in follicular fluid may be due to the combination of increased PG

Figure 5 Regulation of PGDH protein in preovulatory follicles. (A and B) Protein extracts were prepared from granulosa cells overexpressing equine PGDH (lane 2), and analyzed by one-dimensional SDS-PAGE and immunoblotting using specific polyclonal antibody raised against a fragment of the equine PGDH protein (A) or anti-human PGDH polyclonal antibody (B). An 'a' letter on the right indicates the migration of PGDH protein; lane 1 is for untransfected cells. (C) Immunoblotting of protein extracts prepared from preovulatory follicles isolated 0–36 h post-hCG (lanes 2–5), as described in Materials and Methods. Results from protein extracts (50 µg/well) of one representative follicle/time point are shown ($n=4$ samples (i.e. mares) per time point). Markers on the left indicate the migration of molecular weight standards. Protein bands indicate multiple forms of PGDH: a lower band is for PGDH monomer (a); a major band is for PGDH dimer (b); and bands between PGDH monomeric and dimeric forms are for PGDH proteins with different extent of glycosylation. Lane 1 is for a positive control using protein extracts obtained from granulosa cells overexpressing equine PGDH.

synthesis by PGHS-2 and to decreased PG catabolism by PGDH, since a decrease of PGDH protein expression is observed at this timing interval. In contrast, low levels of PGE2 and PGF2 α in equine follicular fluid detected from 0 to 33 h post-hCG may be due to low rate of their synthesis, since gonadotropin-dependent expression of PGHS-2 is very low at this period of time (Sirois & Doré

1997). However, marked levels of PGDH protein were observed during this time interval, suggesting a possible synthesis of PGDH, since small amount of PGDH mRNA was detected, or a possible induction of PGDH transcripts and their translation simultaneously by hCG at this period of time. Our findings indicate that both granulosa and theca cells express PGDH, suggesting that conversion of PGs by PGHD may occur in both follicular cell types. In all cases, quantification of PGs and their metabolites in follicular fluid during the preovulatory interval will be required to demonstrate the activity of PGDH to confirm its putative involvement in ovulation.

Gonadotropin-induced expression of phospholipase A2 (cPLA2), the enzyme that mobilizes plasma membrane arachidonic acids for PGHS-2, PGHS-2, and PGES, terminal enzyme for PGE2 synthesis, is well documented in granulosa cells of preovulatory follicles (Sirois 1994, Sirois & Doré 1997, Filion *et al.* 2001, Diouf *et al.* 2006). Our recent studies also have shown that aldo-keto reductase 1C23 (AKR1C23), a multi-functional enzyme believed to possess PGFS-like activity for PGF2 α production, is induced by hCG in equine preovulatory follicles (Brown *et al.* 2006). Findings from the present study indicate that PGDH is co-localized at identical follicular cells with cPLA2, PGHS-2, PGES, and AKR1C23. Since metabolism of PGs occurs only after their uptake by PG transporter, further studies on the expression and regulation of the PG transporter in follicular cells would be interesting to establish the correlation between the co-expression of biosynthetic, transport, and metabolic proteins in the same preovulatory follicles, and to shed light on the coordinated roles of these proteins in the ovulatory process.

Studies on molecular control of PGDH expression *in vivo* are limited because most of known PGDH inhibitors either inhibit PGHS-2, consequently inhibit PGE2 synthesis. Numerous reports have shown that PGDH expression is hormonally regulated in several tissues including ovary, lung, uterus, and reproductive tract (Abel & Kelly 1983, Tsai & Einzig 1989, Giannoulis *et al.* 2002, Winchester *et al.* 2002). Our data indicate that the expression of PGDH transcripts was low in the kidney, muscle, brain, and heart, but highly detected in corpus luteum and other equine tissues tested. Moreover, the present study identified high/ovulatory levels of gonadotropins as a primary physiological regulator of PGDH in equine preovulatory follicles. Sequence analysis displayed the presence of a cAMP response element and a progesterone receptor-binding site in the human and rodent PGDH promoter region (Matsuo *et al.* 1997, Greenland *et al.* 2000). Thus, hCG stimulation through interaction with the LH/CG receptor located on follicular cells acting through the classic protein kinase A pathway may regulate PGDH mRNA transcription. The induction of PGDH mRNA in equine preovulatory follicles was biphasic. This suggests the involvement of multiple intracellular factors and

Figure 6 Immunohistochemical localization of PGDH protein in equine preovulatory follicles. Immunohistochemistry was performed on formalin-fixed sections of preovulatory follicles isolated before and after hCG treatment, as described in Materials and Methods. Results from one representative follicle/time point are shown ($n=4$ samples (i.e. mares) per time point). These results indicate that PGDH staining in follicles obtained before hCG (0 h; A) was low in granulosa and theca cells, markedly increased in both cell types in preovulatory follicles isolated 12, 24, and 33 h post-hCG (B, C and D respectively), and slightly decreased at 36 h post-hCG (E). (F) Control staining from the follicular tissue presented in D (33 h post-hCG) was negative when the primary antibody was replaced with PBS. The limits of the granulosa cell (GC) layer are indicated (A and B). The region above the granulosa cell layer is for the antrum, whereas the region immediately beneath corresponds to thecal layers, including the limits of theca interna (TI) (A and B). Magnification, $\times 400$ with a scale bar equal to 0.05 mm.

events in the modulation of PGDH, including P4 and its receptor PR. P4 and PR have been shown to stimulate the activity PGDH promoter in the expression of PGDH in myometrial cells (Greenland *et al.* 2000). Interestingly, P4 and PR are known to be induced by gonadotropins in preovulatory follicles and are required for the follicle rupture and successful ovulation (Watson & Hinrichs 1998, Robker *et al.* 2000, Boerboom *et al.* 2003). Thus, the isolation and characterization of the equine PGDH promoter and studies using P4 inhibitors and PR antagonists would be interesting that should help us to get insight possible involvement of P4 and PR in the regulation of PGDH expression during the ovulatory process of the mares.

In summary, this study reports for the first time the primary structure of equine PGDH and its regulated expression induced by hCG in equine follicles during the ovulatory process *in vivo*. In contrast to a primate, in which the induction of PGDH was monophasic and limited only to granulosa cells, the latter induction in equine ovarian follicles was biphasic and occurred in both follicular granulosa and theca cells, suggesting species-specific differences in follicular control of

PGDH expression during the ovulatory process. The expression of PGDH protein was elevated in preovulatory follicles, but began to decrease at the time when levels of PGHS-2 protein in granulosa cells and of PGE2 and PGF2 α in follicular fluid were concomitantly increased just prior to ovulation. This suggests that PGDH is involved in the control of PG concentrations required for the ovulatory process. The molecular mechanisms behind the expression of PGDH during this important physiological process in mares and also in other species remain to be clearly determined.

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