**Human chorionic gonadotropin-dependent induction of an equine aldo-keto reductase (AKR1C23) with 20α-hydroxysteroid dehydrogenase activity during follicular luteinization in vivo**

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**Abstract**

Aldo–keto reductases (AKRs) are multifunctional enzymes capable of acting on a wide variety of substrates, including sex steroids. AKRs having 20α-hydroxysteroid dehydrogenase (20α-HSD) activity can reduce progesterone to 20α-hydroxy-4-pregnen-3-one (20α-DHP), a metabolite with lower affinity for the progesterone receptor. The objective of this study was to investigate the regulation of equine AKR1C23 during human chorionic gonadotropin (hCG)-induced ovulation/luteinization. The equine AKR1C23 cDNA was cloned and shown to encode a 322 amino acid protein that is conserved (71–81% identity) when compared with mammalian orthologs. RT-PCR/Southern blotting analyses were performed to study the regulation of AKR1C23 transcripts in equine preovulatory follicles isolated between 0 and 39 h after hCG treatment (ovulation occurring 39–42 h post-hCG). Results showed the presence of low AKR1C23 expression before hCG treatment, but a marked increase was observed in follicles obtained 12 h after hCG (P < 0.05). Analyses of isolated preparations of granulosa and theca interna cells identified low mRNA expression in both cell types prior to hCG treatment, with granulosa cells clearly being the predominant site of follicular AKR1C23 mRNA induction. A specific polyclonal antibody was raised against a fragment of the equine protein and immunoblotting analyses showed an increase in AKR1C23 protein in granulosa cell extracts when comparing follicles isolated at 36 h post-hCG vs those collected prior to treatment, in keeping with mRNA results. Immunohistochemical data confirmed the induction of the enzyme in follicular cells after hCG treatment. The enzyme was tested for 20α-HSD activity and was shown to exhibit a K_m of 3.12 µM, and a V_max of 0.86 pmol/min per 10 µg protein towards progesterone. The levels of 20α-DHP measured in follicular fluid reflected this activity. Collectively, these results demonstrate for the first time that the gonadotropin-dependent induction of follicular luteinization is accompanied by an increase in AKR1C23 expression. Considering the 20α-HSD activity of AKR1C23, its regulated expression in luteinizing preovulatory follicles may provide a biochemical basis for the increase in ovarian 20α-DHP observed during gonadotropin-induced luteinization/ovulation. (The nucleotide sequence reported in this paper has been submitted to GenBank with accession number AY955082.)


**Introduction**

Enzymes capable of performing oxidoreductase activities are widespread. One superfamily comprises the aldo–keto reductases (AKRs), characterized as being monomeric, nicotinamide cofactor-dependent, cytosolic proteins of approximately 320 amino acids. These AKRs lack a Rossmann-fold motif required by short-chain dehydrogenase/reductase family members for NAD(P)H binding (Wilson et al. 1992, 1995, Hoog et al. 1994, el-Kabbani et al. 1995, Jornvall et al. 1995). They are found in a wide variety of organisms, from bacteria to mammals, and metabolize a wide variety of substrates, including aliphatic and aromatic aldehydes, isoflavonoids, monosaccharides, steroids, prostaglandins (PGs) and polycyclic aromatic hydrocarbons. Fourteen families of AKRs are currently known (AKR1–14), encompassing over 100 proteins, with the AKR1 family being the largest and containing the aldehyde reductases, the aldose reductases, the hydroxysteroid dehydrogenases (HSDs) and the 5β-reductases. Isoforms of the AKR1C family have been demonstrated to share at least 84% amino acid sequence identity, and to exhibit 3α-, 17β- and 20α-HSD activities (Penning et al. 2000). The current AKR1C3 has, in the past, also been named human liver 3α-HSD type II, 17β-HSD type V,
dihydrodiol dehydrogenase type X and PG F2α synthase (PGFS), thereby contributing to the confusion regarding nomenclature (Penning et al. 1996).

AKRs having 20α-HSD activity convert progesterone to 20α-hydroxy-4-pregnen-3-one (20α-DHP), a steroid considered inactive due to its lower affinity for the progesterone receptor (PR) (Ogle & Beyer 1982). However, levels of 20α-DHP have been demonstrated to increase after the coitus-induced preovulatory surge in luteinizing hormone (LH) in the rabbit, as well as after human chorionic gonadotropin (hCG) treatment in both rats and rabbits and in cultured rat granulosa cells (Lau et al. 1978, Nordenstrom & Johanson 1985, Lacy et al. 1993). Injection of equine chorionic gonadotropin (eCG) to immature rats with subsequent hCG treatment resulted in an increased production of progesterone and testosterone, as well as an increase in 20α-DHP levels surpassing those of progesterone prior to ovulation (Bauminger et al. 1977). 20α-DHP has been shown to induce ovulation when administered to immature rats after eCG treatment at doses 3 times that required by progesterone, an effect not seen with 20α-DHP's 5α-reduced metabolites (Gilles & Karavolas 1981). 20α-DHP has also been shown to induce a positive feedback effect on LH serum concentrations in an estrogen-primed eugonadal woman (Leyendecker et al. 1976) and has been shown to prolong the preovulatory LH discharge in the rabbit (Hilliard et al. 1967). When monolayer cultures of rat pituitaries were exposed to 20α-DHP, a negative feedback effect was observed on the basal secretion of follicle-stimulating hormone, whereas this progesterone metabolite increased the effect of gonadotropin-releasing hormone on LH secretion (Tang & Spies 1975).

In mammals, follicular luteinization is triggered by a surge in LH released by the anterior pituitary. At this time, a vast number of biological and structural changes occur: the steroidogenic enzymes responsible for 17β-estradiol production are downregulated, whereas those contributing to progesterone synthesis, a steroid required for the establishment of pregnancy, are induced. The regulation of genes responsible for progesterone synthesis and action have been studied in great detail during the periovulatory period (Park & Mayo 1991, Natraj & Richards 1993, Sugawara et al. 1997, Boerboom & Sirois 2001, Boerboom et al. 2003). However, no attempt has been made to study the regulation of proteins having progesterone-metabolizing activities, such as 20α-HSD, during the luteinization process. In the present study, the equine preovulatory follicle was used as a model to investigate the regulation of a novel AKR, AKR1C23, during hCG-induced ovulation/luteinization. The specific objectives were to clone equine AKR1C23 cDNA and determine the expression of its mRNA and protein in preovulatory follicles after hCG treatment.

Materials and methods

The nucleotide sequence reported in this paper has been submitted to GenBank with accession number AJ955082.

Materials

The Prime-a-Gene labeling system, pGEM-T Easy Vector System I, and the Access RT-PCR system were purchased from Promega Corp. (Madison, WI, USA). The [α-32P]dCTP was purchased from PerkinElmer Canada, Inc. (Woodbridge, ON, Canada), and the QuickHyb hybridization solution was obtained from Stratagene Cloning Systems (La Jolla, CA, USA). The TRIZol total RNA isolation reagent, SuperScript II reverse transcriptase, 1 kb DNA ladder, synthetic oligonucleotides, 5′-rapid amplification of cDNA ends (RACE) system (Version 2.0), pcDNA3.1+ vector, and LipofectAMINE PLUS were purchased from Invitrogen Life Technologies (Burlington, ON, Canada). The Qiagen OneStep RT-PCR System, the pQE-30 vector and the Ni-NTA Superflow beads were obtained from Qiagen, Inc. (Mississauga, ON, Canada). The pGEX-2T vector, protease-deficient E. coli BL-21 and glutathione-Sepharose beads were obtained from Amersham Pharmacia Biotech (Baie d’Urée, PQ, Canada). The Expand High Fidelity DNA Polymerase was purchased from Roche Diagnostics (Laval, PQ, Canada). Biotrans nylon membranes (pore size, 0.2 mm) were obtained from ICN Pharmaceuticals, Inc. (Montréal, PQ, Canada), and all electrophoretic reagents were purchased from Bio-Rad Laboratories (Richmond, CA, USA). The hCG was obtained from The Buttlcr Co. (Columbus, OH, USA). The Vectastain ABC kit was purchased from Vector Laboratories (Burlingame, CA, USA). The diaminobenzidine tetrahydrochloride, β-nicotinamide adenine dinucleotide phosphate (NADPH), and progesterone were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Cloning of the equine AKR1C23 cDNA

The equine AKR1C23 transcript was isolated in fragments using a multistep cloning strategy (Fig. 1). A 402 bp RT-PCR product (Fig. 1Aa) was initially cloned from pooled equine ovarian RNA samples isolated from preovulatory follicles isolated before (0 h) and after (36 h) hCG treatment. Ovarian tissues were isolated and RNA was extracted as previously described (Kerban et al. 1999). RT-PCR was performed using the Access RT-PCR kit (Promega) as directed by the manufacturer, using 500 ng RNA and oligonucleotide primers designed by sequence alignments of known AKR species homologs (Fig. 1B; primers 1 and 2). Following agarose gel electrophoresis, the RT-PCR product was excised.
**Induction of AKR1C23 in preovulatory follicles**

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Induction of AKR1C23 in preovulatory follicles was investigated using molecular and cellular techniques. The study was conducted in a local slaughterhouse from Standardbred and Thoroughbred mares previously described (Sirois & Doré 1997). Preovulatory follicles and corpora lutea were isolated at specific stages of the estrous cycle from Standardbred and Thoroughbred mares as previously described (Sirois & Doré 1997). Briefly, when preovulatory follicles reached 35 mm in diameter during estrus, the ovariectomy process was induced by injection of hCG (2500 IU, i.v.) and ovariectomies were performed via colpotomy using an ovariotome at 0, 12, 24, 30, 33, 36 or 39 h post-hCG (n = 4–6 mares/time point; ovulation occurring 39–42 h post-hCG) (Sirois & Doré 1997). Follicles were dissected into preparations of follicle wall (theca interna with attached granulosa cells) or further dissected into separate isolates of granulosa cells and theca interna. Ovariectomies were also performed on day 8 of the estrous cycle (day 0, day of ovulation) to obtain corpora lutea (n = 3 mares) (Sirois & Doré 1997).

Total RNA was isolated from tissues with TRIzol reagent (Invitrogen), according to manufacturer’s instructions using a Kinematica PT 1200C Polytron Homogenizer (Fisher Scientific, Montréal, PQ, Canada).

**Semi-quantitative RT-PCR and Southern analysis**

The Access RT-PCR System (Promega) was used for semi-quantitative analysis of AKR1C23 and ribosomal protein L7a (rPL7a) mRNA levels in equine tissues. Reactions were performed according to the manufacturer’s directions, using sense (5’-GAAGCAACAAAC TTGCCAGACAGGC-3’) and antisense (5’-CCACCTGG TTGCAGACATCC-3’) primers specific for equine AKR1C23, and sense (5’-ACAGGACATCCAGCCA ACG-3’) and antisense (5’-GCTCCTTTGTCTTCC GAGTTG-3’) primers specific for the equine control.
gene rpL7a. These reactions resulted in the production of AKR1C23 and rpL7a DNA fragments of 587 and 516 bp respectively. Each reaction was performed using 100 ng total RNA, and cycling conditions were one cycle of 48 °C for 45 min and 94 °C for 2 min, followed by a variable number of cycles of 94 °C for 30 s, 55 °C for 1 min and 68 °C for 2 min. The number of cycles used was optimized for each gene to fall within the linear range of PCR amplification and was 21 cycles for AKR1C23 and 18 cycles for rpL7a.

Following PCR amplification, samples were electrophoresed on 2% TAE-agarose gels, transferred to nylon membranes, and hybridized with corresponding radiolabeled AKR1C23 and rpL7a cDNA fragments using Prime-a-Gene labeling system (Promega) and QuickHyb hybridization solution (Stratagene). Membranes were exposed to a phosphor screen, and signals were quantified on a Storm imaging system using the ImageQuant software version 1.1 (Molecular Dynamics, Amersham Biosciences, Sunnyvale, CA, USA).

Production of an anti-equine AKR1C23 antibody

A pair of sense (5'-GATGGATCCGATCCCAAAGGT TGGCGTGT-3') and antisense (5'-CAGAATTCCCCT GCGGTTAAAGTGGACAC-3') primers that incorporated a BamHI and an EcoRI restriction site respectively were designed from the equine AKR1C23 open reading frame to generate a fragment (ΔAKR1C23) spanning the region from Asp² to Arg¹⁷¹. The fragment was amplified by PCR using the Expand High Fidelity polymerase (Roche Molecular Biochemicals) and following the manufacturer's protocol. The fragment was isolated after electrophoresis, digested with BamHI and EcoRI, subcloned into pGEX-2T in frame with the glutathione-S-transferase (GST) coding region (Amersham Pharmacia Biotech), and sequenced to confirm its identity. Protease-deficient E. coli BL-21 (Amersham Pharmacia Biotech) were transformed with the ΔAKR1C23/pGEX-2T construct, expression of the recombinant ΔAKR1C23/GST fusion protein was induced with isopropyl-1-thio-β-D-galactopyranoside (Fisher Scientific), and bacterial protein extracts were obtained after sonication and centrifugation. The ΔAKR1C23/GST fusion protein was purified by affinity on glutathione-Sepharose beads (Amersham Pharmacia Biotech), digested with thrombin to release the ΔAKR1C23, resolved by one-dimensional SDS-PAGE, transferred to nitrocellulose, and stained with Ponceau S Red (Brulé S et al. 2000). The ΔAKR1C23 band (Mr= 19 200) was cut and used to immunize rabbits as previously described (Brulé et al. 2000).

To demonstrate the specificity of the AKR1C23 antibody, the coding region of the AKR1C23 was subcloned into the mammalian expression vector pcDNA3-1+ (Invitrogen) and transient transfections were performed using the HEK293 cell line as previously described (Filion et al. 2001). Briefly, HEK293 cells were seeded in 75 cm² plates and transfected using 6 µg/pl of AKR1C23/pCDNA3-1 constructs and 36 µg LipofectAMINE PLUS in 1-7 ml MEM, in accordance with the manufacturer's protocol. Three hours after transfection, cells were incubated in fresh culture media for 24 h, collected, and protein extracted and analyzed by immunoblotting.

Cell extracts and immunoblotting analysis

Ovarian cell extracts were prepared as previously described (Filion et al. 2001). Briefly, tissues were homogenized and sonicated on ice in 20 mM Tris (pH 8·0), 50 mM EDTA and 0·1 mM diethyldithiocarbamic acid buffer containing 1-0% Tween. The sonicates were centrifuged at 16 000 g for 15 min at 4 °C. The recovered supernatant (whole cell extract) was stored at −80 °C until electrophoretic analyses were performed. Protein concentration was determined by the method of Bradford (1976) (Bio-Rad protein assay). Samples (50 µg protein) were resolved by one-dimensional SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes (Filion et al. 2001). Membranes were incubated with the polyclonal anti-AKR1C23 antibody (1:4000), and 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 (1:10 000 dilution) and the enhanced chemiluminescence system (ECL Plus), following the manufacturer’s protocol (Amersham Pharmacia Biotech).

Immunohistochemical localization of AKR1C23

Immunohistochemical staining was performed using the Vectastain ABC kit (Vector Laboratories), as previously described (Sirotis & Doré 1997). Briefly, formalin-fixed tissues were paraffin-embedded, and 3 µm-thick sections were prepared and deparaffinized through a graded alcohol series. 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-AKR1C23 antibody was diluted in PBS (1:1000 dilution) 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 dilution; Vector Laboratories) was applied, and sections were incubated for 45 min at room temperature.
temperature. Sections were washed in PBS for 10 min and incubated with avidin DH-biotinylated horseradish peroxidase H reagents (Vectastain ABC kit) for 45 min at room temperature. After washing with PBS for 10 min, the reaction was revealed using diaminobenzidine tetrahydrochloride as the chromogen. Sections were counterstained with Gill’s hematoxylin stain and mounted.

AKR1C23 expression, in vitro enzyme activity and measurement of 20α-DHP concentration in follicular fluid

A pair of sense (5′-CTCGGTACCATGGATCCAAAGGTTGCCGT-3′) and antisense (5′-TGCGTCGGATTTAATAATGCATGAAAATGG-3′) primers that incorporated a KpnI and a PstI restriction site respectively were designed from the equine AKR1C23 open reading frame to generate a full-length AKR1C23 protein. The cDNA was amplified by PCR using the Expand High Fidelity polymerase (Roche Molecular Biochemicals) and following the manufacturer’s protocol. The fragment was isolated after electrophoresis, digested with KpnI and PstI, subcloned into pQE-30 in frame with the His-tag coding region (Qiagen), and sequenced to confirm its identity.

E. coli M15 (Qiagen) were transformed with the AKR1C23/pQE-30 construct, expression of the His-tagged AKR1C23 fusion protein was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (Fisher Scientific), and bacterial protein extracts were obtained after lysis and centrifugation according to the manufacturer’s recommendations.

The His-AKR1C23 fusion protein was purified by affinity on Ni-NTA resin (Qiagen), and concentrated using a 30 kDa cutoff Amicon centrifugal filter device (Millipore Corp.).

The 20α-HSD activity was examined by following the decrease in NADPH absorbance at 340 nm on a Beckman Coulter DU800 spectrophotometer (Madore et al. 2003). Ten micrograms of purified AKR1C23 protein were assayed at 37 °C in 50 mM Tris–HCl (pH 7.5) with saturating NADPH (100 µM) at various progesterone concentrations.

The follicular fluid present in equine preovulatory follicles isolated between 0 and 39 h post-hCG was analyzed for 20α-DHP content by a gas chromatographic mass spectrometric (GC/MS) method developed to measure steroid hormone levels in rat and monkey serum (Bérubé et al. 2000). Briefly, 20α-DHP was extracted from follicular fluid by liquid–liquid and solid-phase extraction. Derivatization reactions were performed to improve the chromatographic and detection response of the steroids. Unconjugated steroids were quantified by means of GC/MS, using chemical ionization.

Statistical analysis

One-way ANOVA was used to test the effect of time after hCG administration on levels of AKR1C23 mRNA and on 20α-DHP concentration. AKR1C23 mRNA levels were normalized with the control gene rpL7a before analysis. When ANOVAs indicated significant differences (P<0.05), Dunnett’s test was used for multiple comparisons of individual means (P<0.05).

Results

Characterization of equine AKR1C23

To clone the equine AKR1C23 transcript, RT-PCR was performed on ovarian RNA using oligonucleotide primers designed by sequence alignment of known AKR1C species homologs. The resulting cDNA fragment (Fig. 1Aa) was sequenced and found to be highly homologous to 20α-HSD transcripts identified thus far. A combination of 5′- and 3′-RACE reactions yielded cDNA products corresponding to all remaining coding regions, as well as 3′- and 5′-untranslated regions (Fig. 1Ab and Ac). A RT-PCR product was generated to extend the length of the open reading frame, thereby confirming that all three products were derived from the same transcript (Fig. 1Ae). The deduced 1562 bp primary transcript encoded a 969 bp open reading frame (Fig. 1A, GenBank accession number AF459082), which predicted a protein of 322 amino acid residues.

The predicted protein is highly conserved, with a single amino acid deletion, when compared with human (AKR1C1) (Blouin et al. 2005), macaque (AKR1C1) (Higaki et al. 2002), rabbit (AKR1C5) (Lacy et al. 1993), cow (Madore et al. 2003) and rat (AKR1C8) (Albarracin et al. 1994) proteins suspected of having 20α-HSD activity. The equine AKR1C23 has 80·8% identity at the amino acid level and a 85·3% identity at the nucleic acid level when compared with human AKR1C1 (Fig. 2). All putative conserved amino acids implicated in AKR1C function appear to be present in the equine enzyme (Fig. 2).

Tissue distribution of equine AKR1C23 mRNA

To study the tissue distribution of equine AKR1C23, various equine tissues were obtained and the expression of AKR1C23 was examined by RT-PCR/Southern blotting. Results showed that the AKR1C23 transcript was expressed in many of the tissues studied (Fig. 3A).

Levels of AKR1C23 mRNA were highest in a preovulatory follicle isolated 36 h after hCG (i.e. approximately 3–6 h before ovulation) and testis; moderate in liver, skeletal muscle, heart, kidney and...
lung; low in brain, stomach and uterus; and very low in thymus, adrenal, spleen and skin. However, levels of the control gene rpL7a remained relatively constant in all tissues studied (Fig. 3B).

Regulation of AKR1C23 transcript in preovulatory follicles and corpora lutea

The regulation of AKR1C23 mRNA in preovulatory follicles was studied by RT-PCR/Southern blotting, using follicles isolated during estrus at 0, 12, 24 and 36 h after the administration of an ovulatory dose of hCG. Total RNA was extracted from the follicle wall (theca interna with attached granulosa cells), as well as from three corpora lutea obtained on day 8 of the estrous cycle. Levels of equine AKR1C23 mRNA were low in equine preovulatory follicles prior to treatment with hCG (0 h), but were clearly induced from 12 h to 36 h post-hCG (Fig. 4A). The AKR1C23 mRNA expression returned to basal levels in the corpus luteum at day 8 of the cycle (Fig. 4A). When results from multiple follicles and corpora lutea were expressed as ratios of AKR1C23 to rpL7a, a significant increase in AKR1C23 transcript

Figure 2 Deduced primary structure of the equine AKR1C23 protein and comparison with known 20α-HSDs and bovine PGFS. The predicted amino acid sequence of the equine (equ) AKR1C23 protein is aligned with human (hum; AKR1C1; GenBank accession number NP_001344), macaque (mcq; AKR1C1; Q95JH7), rabbit (rab; AKR1C5; P80508), and rat (AKR1C8; NP_612519) 20α-HSDs, as well as cow PGFS-like2 (cow; N/A; AAN11329). Identical residues are indicated by a printed period, numbers on the right refer to the last amino acid residue on that line. Bold underlined residues are highly conserved among AKRs, boxed residues are thought to be involved in NADPH cofactor binding, and residues with an asterisk are involved in substrate binding (Jez et al. 1997).

Figure 3 Expression of AKR1C23 mRNA in equine tissues. Total RNA was extracted from various equine tissues, and samples (100 ng) were analyzed for AKR1C23 and rpL7a (control gene) content by a semi-quantitative RT-PCR/Southern blotting techniques, as described in Materials and methods. (A) Expression of AKR1C23 mRNA in equine tissues. (B) Expression of rpL7a mRNA in equine tissues. The number of PCR cycles for each gene was within the linear range of amplification, and they represented 21 and 18 cycles for AKR1C23 and rpL7a respectively. Numbers on the right indicate the size of the PCR fragment.
was detected in follicles, reaching a plateau from 12 to 36 h post-hCG \( (P<0.05; \text{Fig. 5A}) \). Results demonstrated a slight yet significant induction of AKR1C23 mRNA in theca interna cells at 39 h post-hCG \( (P<0.05; \text{Fig. 5B}) \).

### Expression of AKR1C23 protein in equine preovulatory follicles

The hCG-dependent induction of AKR1C23 was studied at the protein level by immunoblotting and immunohistochemistry in follicles at 0 and 36 or 39 h post-hCG. The specificity of the antibody was confirmed, as it recognized the equine AKR1C23 protein overexpressed in HEK293 cells \( (M_r=37\ 000; \text{Fig. 6A}) \). Immunoblotting analyses were performed on protein extracts from granulosa cells and theca interna at 0 and 36 h post-hCG. An increase in levels of immunoreactive AKR1C23 was observed at 36 h post-hCG in granulosa cells (Fig. 6B). However, little or no signal was detected in theca interna samples (Fig. 6C). Immunohistochemical results demonstrated a marked change in AKR1C23 staining after hCG treatment (Fig. 7). Follicles isolated prior to hCG treatment (0 h) show a very compact granulosa cell layer and light staining (Fig. 7A). Although some darker staining is apparent in the periantral granulosa cells, this is probably due to the ‘edge effect’ observed when using this technique (Fig. 7A). The administration of hCG caused the granulosa cell layer to expand and an increase in AKR1C23 accumulation (Fig. 7B–D) was observed in follicles isolated 39 h post-hCG. Control sections of follicles isolated at 39 h post-hCG showed no staining when AKR1C23 antibody was omitted (Fig. 7F). The pattern of expression in testes demonstrated AKR1C23 expression in Leydig cells only (Fig. 7E).

### 20α-HSD activity and concentration of 20α-DHP in follicular fluid

The AKR1C23 recombinant protein was overexpressed in E. coli M15 cells and affinity-purified using an Ni-NTA column. Activity assays were done by monitoring the absorbance at 340 nm, at a temperature of 37 °C, and values were corrected for the background signal in the absence of substrate. Enzyme functionality was confirmed via its ability to reduce phenanthrenequinoine (data not shown). The use of various concentrations of progesterone established the 20α-HSD activity of the AKR1C23 enzyme. The Lineweaver–Burke plot for AKR1C23 reflected a \( K_m \) of 3-12 \( \mu \)M and a \( V_{max} \) of 0.86 pmol/min per 10 \( \mu \)g protein towards progesterone (Fig. 8A). When follicular fluid was examined, a significant increase in 20α-DHP was observed 30–36 h post-hCG (Fig. 8B).
Discussion

This study demonstrates for the first time that the process of follicular luteinization induced by hCG is accompanied by an increase in expression of AKR1C23, an AKR with many potential activities, including the conversion of progesterone to 20α-HSD. The process of luteinization/ovulation has previously been associated with dramatic changes in levels of steroidogenic enzymes in the different cellular compartments of the preovulatory follicle. Notably, a marked decrease or loss in the cytochrome P450 17α-hydroxylase/17,20-lyase and aromatase, which are key enzymes involved in androgen and estrogen biosynthesis, and the increase in steroidogenic acute regulatory protein and cytochrome P450 cholesterol side-chain cleavage expression, which contribute to enhanced progesterone synthesis (Fortune 1994, Richards 1994, Ronen-Fuhrmann et al. 1998, Sandhoff et al. 1998). Such changes have also been observed during hCG-induced luteinization/ovulation in the mare (Boerboom et al. 1999, Kerban et al. 1999, Boerboom & Sirois 2001). However, there has been no report on the regulation of enzymes with 20α-HSD activity during the periovulatory period. Previous investigations of the expression of 20α-HSD in the ovary have primarily been limited to the examination of its regulation during luteolysis prior to parturition by Northern blotting analysis and by tissue distribution analyses by in situ hybridization (Albarracin et al. 1994, Nishizawa et al. 2000, Pelletier et al. 2003). Collectively, these studies revealed that the 20α-HSD gene (AKR1C8) was highly expressed in the rat corpus luteum at the end of gestation, and that the human 20α-HSD (AKR1C1) promoter was functional in porcine luteinized granulosa cells in culture.

Results from the present study suggest that the induction of a progesterone-metabolizing enzyme such as AKR1C23 may provide the biochemical basis for the
increase in 20α-DHP observed during the periovulatory period. Indeed, an increase in 20α-DHP levels has been demonstrated to occur in a number of species, including the rat and rabbit (Lau et al. 1978, Nordenstrom & Johanson 1983, Lacy et al. 1993). This study establishes that such an increase in 20α-DHP also occurs in mares. However, the precise role of AKR1C23 during follicular luteinization/ovulation is intriguing and remains to be investigated. Its purpose as a progesterone-metabolizing enzyme remains perplexing since progesterone appears to be required for ovulation, as demonstrated by the anovulatory phenotype of PR-mutant mice (Lydon et al. 1995). AKR1C23’s physiological importance may involve its ability to produce 20α-DHP. This metabolite has been shown to promote ovulation and gonadotropin secretion, and has been shown to increase sexual receptivity when metabolized further by 5α-reductase in the brain (Frye & Leadbetter 1994).

The multifunctional nature of the AKR protein led investigators to question the predominant role of the enzyme under physiological conditions. However, the difficulties involved in substrate manipulation, product analysis and enzyme stability, make it hard to have a comparative view of all possible AKR activities under similar conditions. In this study, we demonstrate that the AKR1C23 enzyme does convert progesterone to 20α-DHP with an affinity that approaches that previously reported for the human enzyme (Zhang et al. 2000). AKRs of this type, like AKR1C3 and AKR1C7, have also been shown to harbor PGFS-like activity, where this enzyme converts PGD$_2$ into 9α,11β PGF$_2$α, a PGF$_{2α}$ isomer, as well as converting PGH$_2$ into PGF$_{2α}$ (Watanabe et al. 1986, Desmond et al. 2003). Considering that the levels of PGF$_{2α}$ have been demonstrated to increase following hCG treatment in equine preovulatory follicles (Sirois & Doré 1997), it is not unreasonable to think that AKR1C23 may contribute to this increase in PGF$_{2α}$. The concept of a protein having many enzymatic activities working in concert has previously been described (Madore et al. 2003), where an aldose reductase (AKR1B5) was shown to have both 20α-HSD and PGFS activities in cultures of bovine uterine endometrial cells. It was speculated that these concerted activities may lead to termination of the estrous cycle (Madore et al. 2003). Other possible activities of AKR1C23 such as 17β-HSD type V and 3α-HSD type II, which convert androstenedione to testosterone and 5α-dihydrotestosterone to androstanediol respectively have yet to be examined and their role, if any, during follicular luteinization needs to be elucidated.

The molecular control of AKR1C expression remains largely uncharacterized. Moreover, because of confusion regarding nomenclature, difficulties lie in identifying precisely which AKR was being studied in previous publications. Many reports addressed the regulation of AKRs speculated to have 20α-HSD activity in various tissues, including the ovary (Strauss & Stambaugh 1974, Lacy et al. 1993, Albarracin et al. 1994, Stocco et al. 2000, Pelletier et al. 2003). However, most ovarian studies remained largely at the level of the corpus luteum. Early investigations on the luteolytic effects of PGF$_{2α}$ showed that 20α-HSD activity is induced 150-fold in the rat ovary (Strauss & Stambaugh 1974), this being consistent with more recent findings that demonstrate that rat 20α-HSD (AKR1C8) is induced by PGF$_{2α}$ (Stocco et al. 2000). The transcription factor Nur77 was shown to play a key role in the PGF$_{2α}$-dependent induction of 20α-HSD (AKR1C8) in rat luteal cells (Stocco et al. 2000). The present study identifies high/ovulatory levels of gonadotropins as a physiological regulator of AKR1C23 in the preovulatory follicle, with the predominant regulation observed in granulosa cells. Interestingly, LH has previously been shown to upregulate Nur77 in rat granulosa cells (Park et al. 2003), thus providing a potential trans-activating factor for follicular AKR1C23 gene expression. Conversely, previous investigations suggest that prolactin is a repressor of 20α-HSD expression, since it was shown to
reduce the 20α-HSD (AKR1C8) protein level during corpus luteum regression in vivo, and in rat luteal cells in vitro (Albarracin et al. 1994).

In summary, this study is the first to characterize the primary structure of AKR1C23, to investigate the expression of the AKR1C23 gene in a developmental series of preovulatory follicles, and to identify ovulatory levels of gonadotropins as a positive regulator of AKR1C23 expression. The deduced primary structure of AKR1C23 is highly conserved when compared with species homologs with all putative conserved amino acids implicated in NADPH and substrate binding present (Jez et al. 1997). Although the precise molecular control of AKR1C23 induction in preovulatory follicles remains to be elucidated, it is interesting to note that the luteinization/ovulatory process is accompanied by an induction of PGF$_{2\alpha}$ and Nur77 (Siros & Doré 1997, Park et al. 2003). Given their putative role in 20α-HSD expression (Strauss & Stambaugh 1974, Stocco et al. 2000), it will be interesting to determine whether they

![Figure 7 immunohistochemical localization of AKR1C23 in equine preovulatory follicles.](image)

Immunohistochemistry was performed on formalin-fixed sections of preovulatory follicles isolated at 0 and 39 h after hCG treatment and of equine testicular tissues, as described in Materials and methods. Results show relatively weak, but detectable, AKR1C23 staining in granulosa (GC) and theca interna (TI) cells of a preovulatory follicle obtained 0 h after hCG administration (A), but a marked increase in signal intensity in both cell types of distinct follicles isolated 39 h after hCG treatment (B–D). (E) Intense AKR1C23 immunoreactivity is observed in Leydig (L) cells of equine testicular sections. (F) Control staining from the follicular tissue presented in (D) was negative when the primary antibody was omitted. Magnification, ×200 (A–C and E) and ×400 (D and F).
are intermediates in the gonadotropin-dependent induction of AKR1C23 in follicular cells. The precise physiological significance of AKR1C23 during ovulation and its role in regulating the bioactivity of progestins prior to follicular rupture should also remain the focus of future investigations.

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