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

K A Brown, D Boerboom, N Bouchard, M Doré¹, J G Lussier and J Sirois

Centre de Recherche en Reproduction Animale et Département de Biomédecine Vétérinaire, Faculté De Médecine Vétérinaire, Université de Montréal, 3200 Sicotte, Saint-Hyacinthe, Québec, Canada J2S 7C6

¹Département de Pathologie et Microbiologie, Faculté de Médecine Vétérinaire, Université de Montréal, Saint-Hyacinthe, Québec, Canada

(Requests for offprints should be addressed to J Sirois; E mail: jean.sirois@umontreal.ca)

Abstract

Aldo-keto reductases (AKRs) are multifunctional enzymes capable of acting on a wide variety of substrates, including sex steroids. AKRs having 20 α -hydroxysteroid dehydrogenase (20 α -HSD) activity can reduce progesterone to 20 α -hydroxy-4-pregnen-3-one (20 α -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 20 α -HSD activity and was shown to exhibit a K_M of 3.12 μ M, and a V_{max} of 0.86 pmol/min per 10 μ g protein towards progesterone. The levels of 20 α -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 20 α -HSD activity of AKR1C23, its regulated expression in luteinizing preovulatory follicles may provide a biochemical basis for the increase in ovarian 20 α -DHP observed during gonadotropin-induced luteinization/ovulation. (The nucleotide sequence reported in this paper has been submitted to GenBank with accession number AY955082.)

Journal of Molecular Endocrinology (2006) **36**, 449–461

Introduction

Enzymes capable of performing oxidoreductase activities are widespread. One superfamily comprises the ald-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 5 β -reductases. Isoforms of the AKR1C family have been demonstrated to share at least 84% amino acid sequence identity, and to exhibit 3 α -, 17 β - and 20 α -HSD activities (Penning *et al.* 2000). The current AKR1C3 has, in the past, also been named human liver 3 α -HSD type II, 17 β -HSD type V,

dihydrodiol dehydrogenase type X and PG F_{2α} synthase (PGFS), thereby contributing to the confusion regarding nomenclature (Penning *et al.* 1996).

AKRs having 20 α -HSD activity convert progesterone to 20 α -hydroxy-4-pregnen-3-one (20 α -DHP), a steroid considered inactive due to its lower affinity for the progesterone receptor (PR) (Ogle & Beyer 1982). However, levels of 20 α -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 20 α -DHP levels surpassing those of progesterone prior to ovulation (Bauminger *et al.* 1977). 20 α -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 20 α -DHP's 5 α -reduced metabolites (Gilles & Karavolas 1981). 20 α -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 20 α -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 17 β -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 20 α -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 AY955082.

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'Urfé, 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 Buttlar 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

Figure 1 Cloning of equine AKR1C23. (A) Cloned cDNA fragments. Each fragment is schematically represented, with its identity indicated on the right and its position in the deduced transcript sequence indicated in parentheses. Lines indicate untranslated regions (UTRs); open boxes designate the open reading frame (ORF). Lengths of the deduced transcript and its structural elements are indicated in bp. Arrows indicate the position and orientation of the oligonucleotides employed in the cloning processes, with numbers indicating their identity. (B) Oligonucleotides used in the various cloning procedures. Oligonucleotides 9 and 11 are components of the 5'-RACE kit (Invitrogen).

and ligated into the PGEM-T Easy plasmid vector (Promega), and proper recombinant plasmids were identified from transformed bacterial colonies using standard techniques (Sambrook *et al.* 1989). Sequencing of the insert was performed by the Service de Séquençage de l'Université Laval (Québec, PQ, Canada) using vector-based T7 and SP6 oligonucleotide primers. Sequences obtained from the initial RT-PCR product served as the basis for the design of specific oligonucleotides for 5'- and 3'-RACE procedures. The 3'-RACE was performed as previously described (Boerboom *et al.* 2000), except 5 µg pooled ovarian tissue RNA (as described above) were used as a template for the initial RT reaction (Fig. 1Ab). Briefly, an RT reaction was performed using a poly-dT oligonucleotide with anchor sequences at its 5' end (Fig. 1B; primer 3). This was followed by nested PCRs using oligonucleotide primers that bound to the anchor sequence in conjunction with AKR1C23-specific forward primers (Fig. 1B; primers 4–7). The product of the second PCR was isolated and sequenced as described above. 5'-RACE was performed using the 5'-RACE System, Version 2.0 kit (Invitrogen) as directed by the manufacturer, using 5 µg pooled ovarian tissue RNA (as

described above) and AKR1C23-specific primers for reverse transcription and PCR (Fig. 1B; primers 8, 10 and 12), along with forward primers supplied with the kit (Fig. 1B; primers 9 and 11). The longest product obtained (Fig. 1Ac) was isolated and sequenced as described above. A clone encompassing the entire coding region was isolated by RT-PCR (Fig. 1Ad), incorporating KpnI and XhoI restriction sites for subcloning into the eukaryotic expression vector pcDNA 3.1+ (Invitrogen), and found to correspond with the deduced primary AKR1C23 transcript reported herein (Fig. 1A). AKR1C23 nomenclature was attributed after the sequence was submitted to the AKR superfamily homepage (www.med.upenn.edu/akr) (Hyndman *et al.* 2003).

Equine tissues and RNA extraction

All animal procedures were approved by the institutional Animal Use and Care Committee.

Testicular tissues were obtained from the Large Animal Hospital of the Faculté de Médecine Vétérinaire (Université de Montréal) following a routine castration, whereas other non-ovarian tissues were collected at a local slaughterhouse.

Equine 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 ovulatory process was induced by injection of hCG (2500 IU, i.v.) and ovariectomies were performed via colpotomy using an ovariometer 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 AATGGATCCC-3') and antisense (5'-CCACCTGG TTGCAGACAGGC-3') primers specific for equine AKR1C23, and sense (5'-ACAGGACATCCAGCCCA AACG-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 radio-labeled 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'-GATGGATCCGATCCCAAGGT TGGCGTGT-3') and antisense (5'-CAGAATTCCTT TGGCGTTAAAGTTGGACAC-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 (Δ AKR1C23) 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 Δ AKR1C23/pGEX-2T construct, expression of the recombinant Δ AKR1C23/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 Δ AKR1C23/GST fusion protein was purified by affinity on glutathione-Sepharose beads (Amersham Pharmacia Biotech), digested with thrombin to release the Δ AKR1C23, resolved by one-dimensional SDS-PAGE, transferred to nitrocellulose, and stained with Ponceau S Red (Brûlé S *et al.* 2000). The Δ AKR1C23 band (M_r =19 200) was cut and used to immunize rabbits as previously described (Brûlé *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 μ g/plate of AKR1C23/pcDNA3.1 constructs and 36 μ g 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 μ g 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 (Sirois & Doré 1997). Briefly, formalin-fixed tissues were paraffin-embedded, and 3 μ m-thick sections were prepared and deparaffined 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. 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 20 α -DHP concentration in follicular fluid

A pair of sense (5'-CTCGGTACCATGGATCCCAAGGTTGGCGT-3') and antisense (5'-TGGCTGCAGTTAATAATCATCAGAAAATGG-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- β -thio-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 20 α -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 μ M) at various progesterone concentrations.

The follicular fluid present in equine preovulatory follicles isolated between 0 and 39 h post-hCG was analyzed for 20 α -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, 20 α -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 20 α -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 ($P < 0.05$). Statistical analyses were performed using JMP software (SAS Institute, Inc., Cary, NC, USA).

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 20 α -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 AY955082), 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 20 α -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

equ	MDPKGWRVELNDGHF	IHAL G F G TYAPNEVP	KSKAVEATKSAIDAG	FRHID * CAHLY * DNEKE	VGLAIRSKIQDGTVK	REDIFCTSK * LWATSL	90
hum	. .S. YQC. K.	MPV.A.	. . .L. . .L. .E. . . .	. . .S. . .N. .EQ.	. . .A.	. . .Y. . . .CN. H	90
mcq	. .S. HQC. K.	MPV.A.	. .N. .I. . .L. .E. . . .	. . .S. . .N. .EY.	. . .A.	. . .Y. . . .CN. H	90
rab	. . .FQ. .A. S.	.PV.E.	. .M. . .I.	. . .S. YF. K.	. . .A.	. . .Y. . . .C. FH	90
cow	. . .SQ. .K.	.PV.Q. .A. . . .	.RD. L. F. PF. .EV. . . .	A. Q. .EQ. I. Q. .	. .MA.	C. .F	90
rat	.NS. IQKM.S	.PV.TE. NL	RK. SM. S. .I. . .V. . . .	. . .S. . .Q. .EE. I. Q. .	. .V. . .E.	. . .Y. . . .S. .H	90
equ	RLQLVRPALEKSLKN	LQLDYV D LYIIHF P V	AVKPGEEHLPQDEQG	RMIFDFTV DLCAT W EA	MEKCKDAG L TKS I G V	SN F PNRRQLEMILNKP	180
hum	.PE.R.		S.VI. K. .N. .	KIL.	V.A.		180
mcq	.PEF.R.	L.	SL.LI. K. .N. .	KLL.	A.		180
rab	.PE.S. .D.		T. .L. .V. II. T. .H. .	KA.I.	A.		180
cow	.PE.S.	M. Y. L.	.L.LY. K. .N. .	KL. A. S. .F. L.	L.A.	. . .HK. . .K. . . .	180
rat	.PE.S. .N. .RK	.N.L.	SL.D. L. . . .H.	NL. L.D.	A.	K.	180
equ	GLKHK P V C N G V E CHP	YLNQSKLLDFCKSKD	IVLVAYSALGSQRE-	HWIDQSSPVLLDEPV	LCAMAKKYKRTPALI	ALRYQLQRGVVVLAK	269
hum	. .Y.	. .F. R.	H. .E.	P. V. PN.	. .L. .H.		270
mcq	. .Y.	. .R.	H. .K.	P. V. N.	. .L. .H.		270
rab	. .Y.	. .G. .E.G. . . .	H. .P.	E. V. .A.L. . . .	IG. L. .HQQ.	I.	270
cow	. .Y.	. .R.	H.	G. VNPNH.	.S. I. Q. H. K. A. .V. . .	. . .I.	270
rat	. .R.L	. .AY. .MN.	G. .T. .YK. . . .	YC. NEDT. . .D. .I. . . .	. .T.	. . .E. .I. T. V. . .	270
equ	[SYNEKR I KENVQAFG	FQLTSEDMKVL DGLN	RNLRYVTLEMFGHP	EYPFSDDY	322		
hum	. . .Q. .RQ. .V. E. . . .	. . .E. .AI.	. .V. .L. .DI. .P. .N. . .	. . .E.	323	80.8%	
mcq	. . .Q. .R. .MKV. E. . . .	. . .AI. .D.	. .I. .L. .DI. .P. .N. . .	. . .E.	323	80.8%	
rab	.FT.I. V. E. . . .	. .P.I. S. . . .	. .F. . . .ADFAI.	. . .E.	323	79.6%	
cow	GN. KEW. . .M. V. D. . . .	.E. .P. . .AI.	. .I. .CDFHPGV.	.F. .EE.	323	72.4%	
rat	.F. .E. .R. .L. V. D. . . .	.A. D. .EI. .N. D. . . .	. . .FPAN. .K.	NF. . .E.	323	71.2%	

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).

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 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.

Figure 4 Regulation of AKR1C23 transcript by hCG in equine preovulatory follicles. Preparations of follicle wall were obtained from preovulatory follicles isolated between 0 and 36 h after hCG, and corpora lutea (CL) were isolated on day 8 of the estrous cycle. Samples (100 ng) of total RNA were analyzed for AKR1C23 and rpL7a content by a semi-quantitative RT-PCR/Southern blotting technique, as described in Materials and methods. (A) Regulation of AKR1C23 mRNA in equine follicles (one representative follicle per time point). (B) Constitutive expression of rpL7a mRNA in the same follicles. Numbers on the right indicate the size of the PCR fragment. (C) Relative levels of intensity of AKR1C23 signal, normalized to the control gene rpL7a (mean $\pm$ S.E.M. $n=5$ or 6 distinct follicles (i.e. mares) per time point and $n=3$ different corpora lutea). Bars marked with an asterisk are significantly different from 0 h post-hCG ($P<0.05$).

was detected in follicles, reaching a plateau from 12 to 36 h post-hCG ($P<0.05$; Fig. 4C). No change in rpL7a transcript was detected after gonadotropin treatment (Fig. 4B).

In order to determine which cell type in the equine follicle wall was responsible for the expression of the AKR1C23 transcript, granulosa and theca interna cells were isolated from follicles obtained between 0 and 39 h post-hCG (Fig. 5). Results indicated that granulosa cells were the predominant site of AKR1C23 expression and that this cell type contributed more importantly to the increase in transcript observed in intact follicle wall preparations. In granulosa cells, this increase was

significant between 12 and 39 h post-hCG ($P<0.05$; Fig. 5A). Results demonstrated a slight yet significant induction of AKR1C23 mRNA in theca interna cells at 39 h post-hCG ($P<0.05$; 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 ($M_r=37\,000$; 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).

20 α -HSD activity and concentration of 20 α -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 phenanthrenequinone (data not shown). The use of various concentrations of progesterone established the 20 α -HSD activity of the AKR1C23 enzyme. The Lineweaver–Burke plot for AKR1C23 reflected a K_M of 3.12 μ M and a V_{max} of 0.86 pmol/min per 10 μ g protein towards progesterone (Fig. 8A). When follicular fluid was examined, a significant increase in 20 α -DHP was observed 30–36 h post-hCG (Fig. 8B).

A. Granulosa cells

B. Theca interna

Figure 5 Regulation of AKR1C23 mRNA in equine granulosa and theca interna cells. Preparations of granulosa cells (A) and theca interna (B) were isolated from equine preovulatory follicles between 0 and 39 h after hCG treatment, and samples (100 ng) of total RNA were analyzed for AKR1C23 and rpL7a content by a semi-quantitative RT-PCR/Southern blotting technique, as described in Materials and methods. The AKR1C23 signal was normalized with the control gene rpL7a, and results are presented as a ratio of AKR1C23 to rpL7a (means $\pm$ S.E.M.; $n=4$ samples (i.e. mares) per time point). Bars marked with an asterisk are significantly different from 0 h post-hCG ($P<0.05$). Insets show representative results for AKR1C23 and rpL7a mRNA levels from one sample per time point.

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 -DHP. 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 (Albarracín *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

Figure 6 AKR1C23 primary antibody specificity and regulation of AKR1C23 protein by hCG in equine granulosa and theca interna. Protein extracts were prepared from transiently transfected and mock-transfected (control) HEK293 cells (A), as well as from granulosa cells (B) and theca interna (C) isolated 0 and 36 h after hCG treatment ($n=2$ samples (i.e. mares) per time point) and were analyzed by one-dimensional SDS-PAGE and immunoblotting using a specific polyclonal antibody raised against a fragment of the equine AKR1C23 protein, as described in Materials and methods. Results from protein extracts (50 μ g/lane) are shown. Markers on the right indicate the migration of the molecular weight (M_r) standards (A) and expected position of AKR1C23 protein (B and C).

increase in 20 α -DHP observed during the periovulatory period. Indeed, an increase in 20 α -DHP levels has been demonstrated to occur in a number of species, including the rat and rabbit (Lau *et al.* 1978, Nordenstrom & Johanson 1985, Lacy *et al.* 1993). This study establishes that such an increase in 20 α -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 20 α -DHP. This metabolite has been shown to promote ovulation and gonadotropin secretion, and has been shown to increase sexual receptivity when metabolized further by 5 α -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 20 α -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 PGD₂ into 9 α ,11 β PGF_{2 α} , a PGF_{2 α} isomer, as well as converting PGH₂ into PGF_{2 α} (Watanabe *et al.* 1986, Desmond *et al.* 2003). Considering that the levels of PGF_{2 α} 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_{2 α} . 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 20 α -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 17 β -HSD type V and 3 α -HSD type II, which convert androstenedione to testosterone and 5 α -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 20 α -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_{2 α} showed that 20 α -HSD activity is induced 150-fold in the rat ovary (Strauss & Stambaugh 1974), this being consistent with more recent findings that demonstrate that rat 20 α -HSD (AKR1C8) is induced by PGF_{2 α} (Stocco *et al.* 2000). The transcription factor Nur77 was shown to play a key role in the PGF_{2 α} -dependent induction of 20 α -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 20 α -HSD expression, since it was shown to

Figure 7 Immunohistochemical localization of AKR1C23 in equine preovulatory follicles. 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, $\times 200$ (A–C and E) and $\times 400$ (D and F).

reduce the 20 α -HSD (AKR1C8) protein level during corpus luteum regression *in vivo*, and in rat luteal cells *in vitro* (Albarracín *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 α} and Nur77 (Sirois & Doré 1997, Park *et al.* 2003). Given their putative role in 20 α -HSD expression (Strauss & Stambaugh 1974, Stocco *et al.* 2000), it will be interesting to determine whether they

A. Lineweaver–Burk plot

B. 20 α -DHP in Follicular Fluid

Figure 8 Enzyme kinetics of AKR1C23 and steroid levels in follicular fluid. (A) Lineweaver–Burk plot of 20 α -HSD activity of equine AKR1C23. Various concentrations of progesterone were assayed at 37 °C by monitoring absorbance at 340 nm. (B) Concentrations of 20 α -DHP were measured in follicular fluid of preovulatory follicles isolated between 0 and 36 h after hCG treatment (mean $\pm$ S.E.M. $n=4-6$ samples per time point (i.e. mares)). Data were transformed to logarithms before analysis as heterogeneity of variance was observed. Bars marked with asterisks are significantly different from 0 h post-hCG ($P<0.05$).

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.

Acknowledgements

We would like to thank Dr Alain Bélanger and Mr René Bérubé from the Molecular Endocrinology and Oncology Research Centre of the Centre Hospitalier de l'Université Laval (Québec, QC, Canada) for their precious help in measuring the steroid levels in our follicular fluid samples. This study was supported by Natural Sciences and Engineering Research Council of Canada (NSERC) Grant OPG0171135 (to J S), and by a NSERC Postgraduate Scholarship (to K A B), a Canadian Institutes of Health Research (CIHR) fellowship (to D B), and a CIHR Investigator Award (to J S), and the authors state that no conflict of interest exists that would prejudice this study's impartiality.

References

- Albarracín CT, Parmer TG, Duan WR, Nelson SE & Gibori G 1994 Identification of a major prolactin-regulated protein as 20 α -hydroxysteroid dehydrogenase: coordinate regulation of its activity, protein content, and messenger ribonucleic acid expression. *Endocrinology* **134** 2453–2460.
- Bauminger S, Eckstein B & Lindner HR 1977 Changes in steroid concentration in the ovaries of immature rats treated with pregnant mare serum gonadotrophin and human chorionic gonadotrophin. *Journal of Endocrinology* **75** 43–48.
- Bérubé R, Malenfant J, Gauvin D, Blais M, Gagnon E, Dumas R, Racine M, Bourque J & Bélanger A 2000 Quantitation of androgenic and estrogenic steroids in rat and monkey serum using gas chromatography and negative chemical ionization mass spectrometry. *ASMS Conference on Mass Spectrometry and Allied Topics*, June 11–15, Long Beach, CA.
- Blouin K, Blanchette S, Richard C, Dupont P, Luu-The V & Tchernof A 2005 Expression and activity of steroid aldoketoreductases 1C in omental adipose tissue are positive correlates of adiposity in women. *American Journal of Physiology. Endocrinology and Metabolism* **288** E398–E404.
- Boerboom D & Sirois J 2001 Equine P450 cholesterol side-chain cleavage and 3 β -hydroxysteroid dehydrogenase/ δ (5)- δ (4) isomerase: molecular cloning and regulation of their messenger ribonucleic acids in equine follicles during the ovulatory process. *Biology of Reproduction* **64** 206–215.
- Boerboom D, Kerban A & Sirois J 1999 Dual regulation of promoter II- and promoter I α -derived cytochrome P450 aromatase transcripts in equine granulosa cells during human chorionic gonadotropin-induced ovulation: a novel model for the study of aromatase promoter switching. *Endocrinology* **140** 4133–4141.
- Boerboom D, Pilon N, Behdjani R, Silversides DW & Sirois J 2000 Expression and regulation of transcripts encoding two members of the NR5A nuclear receptor subfamily of orphan nuclear

- receptors, steroidogenic factor-1 and NR5A2, in equine ovarian cells during the ovulatory process. *Endocrinology* **141** 4647–4656.
- Boerboom D, Russell DL, Richards JS & Sirois J 2003 Regulation of transcripts encoding ADAMTS-1 (a disintegrin and metalloproteinase with thrombospondin-like motifs-1) and progesterone receptor by human chorionic gonadotropin in equine preovulatory follicles. *Journal of Molecular Endocrinology* **31** 473–485.
- Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72** 248–254.
- Brûlé S, Rabahi F, Faure R, Beckers JF, Silversides DW & Lussier JG 2000 Vacuolar system-associated protein-60: a protein characterized from bovine granulosa and luteal cells that is associated with intracellular vesicles and related to human 80K-H and murine beta-glucosidase II. *Biology of Reproduction* **62** 642–654.
- Desmond JC, Mountford JC, Drayson MT, Walker EA, Hewison M, Ride JP, Luong QT, Hayden RE, Vanin EF & Bunce CM 2003 The aldo-keto reductase AKR1C3 is a novel suppressor of cell differentiation that provides a plausible target for the non-cyclooxygenase-dependent antineoplastic actions of nonsteroidal anti-inflammatory drugs. *Cancer Research* **63** 505–512.
- el-Kabbani O, Judge K, Ginell SL, Myles DA, DeLucas LJ & Flynn TG 1995 Structure of porcine aldehyde reductase holoenzyme. *Nature Structural Biology* **2** 687–692.
- Filion F, Bouchard N, Goff AK, Lussier JG & Sirois J 2001 Molecular cloning and induction of bovine prostaglandin E synthase by gonadotropins in ovarian follicles prior to ovulation in vivo. *Journal of Biological Chemistry* **276** 34323–34330.
- Fortune JE 1994 Ovarian follicular growth and development in mammals. *Biology of Reproduction* **50** 225–232.
- Frye CA & Leadbetter EA 1994 5-Alpha-reduced progesterone metabolites are essential in hamster VTA for sexual receptivity. *Life Sciences* **54** 653–659.
- Gilles PA & Karavolas HJ 1981 Effect on ovulation of 20 alpha-hydroxy-4-pregnen-3-one and its 5 alpha-reduced metabolites in immature rats treated with pregnant mare serum gonadotrophin. *Journal of Endocrinology* **88** 289–292.
- Higaki Y, Kamiya T, Usami N, Shintani S, Shiraishi H, Ishikura S, Yamamoto I & Hara A 2002 Molecular characterization of two monkey dihydrodiol dehydrogenases. *Drug Metabolism and Pharmacokinetics* **17** 348–356.
- Hilliard J, Penardi R & Sawyer CH 1967 A functional role for 20-alpha-hydroxypregn-4-en-3-one in the rabbit. *Endocrinology* **80** 901–909.
- Hoog SS, Pawlowski JE, Alzari PM, Penning TM & Lewis M 1994 Three-dimensional structure of rat liver 3 alpha-hydroxysteroid/dihydrodiol dehydrogenase: a member of the aldo-keto reductase superfamily. *PNAS* **91** 2517–2521.
- Hyndman D, Bauman DR, Heredia VV & Penning TM 2003 The aldo-keto reductase superfamily homepage. *Chemico-Biological Interactions* **143–144** 621–631.
- Jez JM, Bennett MJ, Schlegel BP, Lewis M & Penning TM 1997 Comparative anatomy of the aldo-keto reductase superfamily. *Biochemical Journal* **326** 625–636.
- Jornvall H, Persson B, Krook M, Atrian S, Gonzalez-Duarte R, Jeffery J & Ghosh D 1995 Short-chain dehydrogenases/reductases (SDR). *Biochemistry* **34** 6003–6013.
- Kerban A, Boerboom D & Sirois J 1999 Human chorionic gonadotropin induces an inverse regulation of steroidogenic acute regulatory protein messenger ribonucleic acid in theca interna and granulosa cells of equine preovulatory follicles. *Endocrinology* **140** 667–674.
- Lacy WR, Washenick KJ, Cook RG & Dunbar BS 1993 Molecular cloning and expression of an abundant rabbit ovarian protein with 20 alpha-hydroxysteroid dehydrogenase activity. *Molecular Endocrinology* **7** 58–66.
- Lau IF, Saksena SK & Chang MC 1978 Periovalutary steroid concentrations in HCG-treated rabbits. *Hormone Research* **9** 26–30.
- Leyendecker G, Wildt L, Gips H, Nocke W & Plotz EJ 1976 Experimental studies on the positive feedback effect of progesterone, 17 alpha-hydroxyprogesterone and 20 alpha-dihydroprogesterone on the pituitary release of LH and FSH in the human female. The estrogen priming of the progesterone feedback on pituitary gonadotropins in the eugonadal woman. *Archiv für Gynakologie* **221** 29–45.
- Lydon JP, DeMayo FJ, Funk CR, Mani SK, Hughes AR, Montgomery CA Jr, Shyamala G, Conneely OM & O'Malley BW 1995 Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. *Genes and Development* **9** 2266–2278.
- Madore E, Harvey N, Parent J, Chapdelaine P, Arsh JA & Fortier MA 2003 An aldose reductase with 20 alpha-hydroxysteroid dehydrogenase activity is most likely the enzyme responsible for the production of prostaglandin f2 alpha in the bovine endometrium. *Journal of Biological Chemistry* **278** 11205–11212.
- Natraj U & Richards JS 1993 Hormonal regulation, localization, and functional activity of the progesterone receptor in granulosa cells of rat preovulatory follicles. *Endocrinology* **133** 761–769.
- Nishizawa M, Nakajima T, Yasuda K, Kanzaki H, Sasaguri Y, Watanabe K & Ito S 2000 Close kinship of human 20 alpha-hydroxysteroid dehydrogenase gene with three aldo-keto reductase genes. *Genes to Cells* **5** 111–125.
- Nordenstrom K & Johanson C 1985 Steroidogenesis in isolated rat granulosa cells—changes during follicular maturation. *Acta Endocrinologica* **108** 550–556.
- Ogle TF & Beyer BK 1982 Steroid-binding specificity of the progesterone receptor from rat placenta. *Journal of Steroid Biochemistry* **16** 147–150.
- Park JI, Park HJ, Lee YI, Seo YM & Chun SY 2003 Regulation of NGFI-B expression during the ovulatory process. *Molecular and Cellular Endocrinology* **202** 25–29.
- Park OK & Mayo KE 1991 Transient expression of progesterone receptor messenger RNA in ovarian granulosa cells after the preovulatory luteinizing hormone surge. *Molecular Endocrinology* **5** 967–978.
- Pelletier G, Luu-The V, Li S, Ren L & Labrie F 2003 Sex-related expression of 20 alpha-hydroxysteroid dehydrogenase mRNA in the adult mouse. *Journal of Histochemistry and Cytochemistry* **51** 1425–1436.
- Penning TM, Pawlowski JE, Schlegel BP, Jez JM, Lin HK, Hoog SS, Bennett MJ & Lewis M 1996 Mammalian 3 alpha-hydroxysteroid dehydrogenases. *Steroids* **61** 508–523.
- Penning TM, Burczynski ME, Jez JM, Hung CF, Lin HK, Ma H, Moore M, Palackal N & Ratnam K 2000 Human 3 alpha-hydroxysteroid dehydrogenase isoforms (AKR1C1-AKR1C4) of the aldo-keto reductase superfamily: functional plasticity and tissue distribution reveals roles in the inactivation and formation of male and female sex hormones. *Biochemical Journal* **351** 67–77.
- Richards JS 1994 Hormonal control of gene expression in the ovary. *Endocrine Reviews* **15** 725–751.
- Ronen-Fuhrmann T, Timberg R, King SR, Hales KH, Hales DB, Stocco DM & Orly J 1998 Spatio-temporal expression patterns of steroidogenic acute regulatory protein (StAR) during follicular development in the rat ovary. *Endocrinology* **139** 303–315.
- Sambrook J, Fritsch EF & Maniatis T 1989 *Molecular Cloning: a Laboratory Manual*, edn 2. Cold Spring Harbor Laboratory: Cold Spring Harbor, NY.
- Sandhoff TW, Hales DB, Hales KH & McLean MP 1998 Transcriptional regulation of the rat steroidogenic acute regulatory protein gene by steroidogenic factor 1. *Endocrinology* **139** 4820–4831.
- Sirois J & Doré M 1997 The late induction of prostaglandin G/H synthase-2 in equine preovulatory follicles supports its role as

- a determinant of the ovulatory process. *Endocrinology* **138** 4427–4434.
- Stocco CO, Zhong L, Sugimoto Y, Ichikawa A, Lau LF & Gibori G 2000 Prostaglandin F₂ alpha-induced expression of 20 alpha-hydroxysteroid dehydrogenase involves the transcription factor NUR77. *Journal of Biological Chemistry* **275** 37202–37211.
- Strauss JF III & Stambaugh RL 1974 Induction of 20 alpha-hydroxysteroid dehydrogenase in rat corpora lutea of pregnancy by prostaglandin F-2 alpha. *Prostaglandins* **5** 73–85.
- Sugawara T, Kiriakidou M, McAllister JM, Holt JA, Arakane F & Strauss JF III 1997 Regulation of expression of the steroidogenic acute regulatory protein (StAR) gene: a central role for steroidogenic factor 1. *Steroids* **62** 5–9.
- Tang LK & Spies HG 1975 Effects of gonadal steroids on the basal and LRF-induced gonadotropin secretion by cultures of rat pituitary. *Endocrinology* **96** 349–355.
- Watanabe K, Iguchi Y, Iguchi S, Arai Y, Hayaishi O & Roberts LJ II 1986 Stereospecific conversion of prostaglandin D2 to (5Z,13E)-(15S)-9 alpha-11 beta,15-trihydroxyprosta-5,13-dien-1-oic acid (9 alpha,11 beta-prostaglandin F₂) and of prostaglandin H₂ to prostaglandin F₂ alpha by bovine lung prostaglandin F synthase. *PNAS* **83** 1583–1587.
- Wilson DK, Bohren KM, Gabbay KH & Quiocho FA 1992 An unlikely sugar substrate site in the 1·65 Å structure of the human aldose reductase holoenzyme implicated in diabetic complications. *Science* **257** 81–84.
- Wilson DK, Nakano T, Petrash JM & Quiocho FA 1995 1·7 Å structure of FR-1, a fibroblast growth factor-induced member of the aldo-keto reductase family, complexed with coenzyme and inhibitor. *Biochemistry* **34** 14323–14330.
- Zhang Y, Dufort I, Rheault P & Luu-The V 2000 Characterization of a human 20 alpha-hydroxysteroid dehydrogenase. *Journal of Molecular Endocrinology* **25** 221–228.

Received in final form 30 January 2006

Accepted 27 February 2006