

—Original Article—

Expression of Aldo-keto Reductase 1C23 in the Equine Corpus Luteum in Different Luteal Phases

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Abstract. Regression of the corpus luteum (CL) is characterized by a decay in progesterone (P_4) production (functional luteolysis) and disappearance of luteal tissues (structural luteolysis). In mares, structural luteolysis is thought to be caused by apoptosis of luteal cells, but functional luteolysis is poorly understood. 20α -hydroxysteroid dehydrogenase (20α -HSD) catabolizes P_4 into its biologically inactive form, 20α -hydroxyprogesterone (20α -OHP). In mares, aldo-keto reductase (AKR) 1C23, which is a member of the AKR superfamily, has 20α -HSD activity. To clarify whether AKR1C23 is associated with functional luteolysis in mares, we investigated the expression of AKR1C23 in the CL in different luteal phases. The luteal P_4 concentration and levels of 3β -hydroxysteroid dehydrogenase (3β -HSD) mRNA were higher in the mid luteal phase than in the late and regressed luteal phases ($P<0.05$), but the level of 3β -HSD protein was higher in the late luteal phase than in the regressed luteal phase ($P<0.05$). The luteal 20α -OHP concentration and the level of AKR1C23 mRNA were higher in the late luteal phase than in the early and mid luteal phases ($P<0.05$), and the level of AKR1C23 protein was also highest in the late luteal phase. Taken together, these findings suggest that metabolism of P_4 by AKR1C23 is one of the processes contributing to functional luteolysis in mares.

Key words: 20α -hydroxysteroid dehydrogenase, Corpus luteum, Functional luteolysis, Mares, Progesterone (J. Reprod. Dev. 60: 150–154, 2014)

The corpus luteum (CL) is a mammalian endocrine organ that is formed in the ovary after ovulation. The CL produces progesterone (P_4), which is essential for the establishment and maintenance of pregnancy. In the absence of pregnancy, the CL regresses following the pulsatile release of uterine prostaglandin $F_{2\alpha}$ (PGF). Regression of the CL is characterized by a decay in P_4 production (functional luteolysis) and the disappearance of luteal tissue (structural luteolysis) [1]. In mares, structural luteolysis has been suggested to be caused by apoptosis of luteal cells [2], while functional luteolysis is poorly understood.

P_4 is catabolized into its biologically inactive form, 20α -hydroxyprogesterone (20α -OHP), by 20α -hydroxysteroid dehydrogenase (20α -HSD). In rodents, ovarian 20α -HSD plays a role in functional luteolysis at term of pseudopregnancy as well as

during the estrous cycle [3, 4]. Recently, it has also been demonstrated in the bovine CL that 20α -HSD is expressed throughout the estrous cycle and that 20α -HSD expression is remarkably higher in the late stage than in the other stages of the estrous cycle [5].

Aldo-keto reductases (AKRs) are multifunctional enzymes that act on a wide range of substrates including steroid hormones [6]. In mares, AKR1C23 has 20α -HSD activity and converts P_4 to 20α -OHP [7]. Although it is reported that AKR1C23 is expressed in the equine CL [7], the role of AKR1C23 in the equine CL remains unclear. In the present study, to clarify the possible mechanism of functional luteolysis in mares, we investigated the 20α -OHP concentrations and expression of AKR1C23 in the equine CL in the different luteal phases.

Materials and Methods

Collection of equine CLs

Fifty-eight Anglo-Norman mares of various ages and weighing approximately 1,000 kg, which were imported from Canada and then fattened at a ranch adjacent to an abattoir ($\sim N32^\circ$) in Kumamoto, Japan, for at least two years before exsanguination, were utilized in

Received: November 6, 2013

Accepted: December 27, 2013

Published online in J-STAGE: February 4, 2014

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the present study. Fifty-eight ovaries containing a CL were collected from randomly designated cyclic nonpregnant mares at the abattoir in accordance with protocols approved by the local institutional animal care and use committee from April until the end of August. Mares possessing ovaries with a macroscopic abnormality including anovulatory hemorrhagic follicles were eliminated. After midsagittal sectioning, the CLs were classified as being in the early, mid, late or regressed luteal phase ($n=3-8$ /phase) by macroscopic observation of the CLs and follicles (Table 1) as described previously [8, 9]. Following determination of the phases, CL tissues were immediately separated from the ovaries, rinsed with cold sterile saline, frozen rapidly in liquid nitrogen and then stored at -80 C until being processed for P_4 , 20α -OHP, RNA and protein extraction.

P₄ and 20 α -OHP extraction

P_4 and 20α -OHP were extracted from luteal tissues as described previously [10]. Briefly, 2 ml of ethanol was added to the minced luteal tissue samples (approximately 0.2 g). The tissues were then homogenized 5 times for 10 sec on ice by a tissue homogenizer (Phycotron; NITI-ON, Chiba, Japan; NS-50) and 3 times for 1 min on ice by an ultrasonic homogenizer (VCX400 Vibra-Cell; Sonics & Materials, Newtown, CT, USA). After centrifugation (1,700 rpm for 30 min at 4 C), the supernatant was evaporated in a vacuum oven at 50 C, and the residue was dissolved in 2 ml of enzyme immunoassay (EIA) buffer (7.12 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 8.5 g NaCl in 1,000 ml ultrapure water, pH 7.2).

P₄ and 20 α -OHP determination

The concentrations of P_4 in the tissue extracts were determined directly by EIA as described previously [11]. The P_4 standard curve ranged from 0.391 to 100 ng/ml, and the ED50 of the assay was 2.0 ng/ml. The intra- and interassay coefficients of variation were on average 5.4 and 7.0%, respectively. The cross-reactivity of the antibody was 100% for P_4 and 0.03% for 20α -OHP.

The concentrations of 20α -OHP in the tissue extracts were determined directly by EIA. The 20α -OHP EIA was based on the EIA for P_4 with some modifications. Standards or samples (20 μ l) were incubated with 50 μ l polyclonal antibody (raised in a rabbit against 20α -OH-Progesterone-3-CMO-BSA; Cosmo Bio, Tokyo, Japan) solution (1:800,000) and 50 μ l 20α -OH-Progesterone-3-CMO-HRP (1:400,000; Cosmo Bio) for 16 h at 4 C. The standard curve ranged from 0.0976 to 50 ng/ml, and the ED50 of the assay was 2.5 ng/ml. The intra- and interassay coefficients of variation were on average 6.8 and 8.6%, respectively. The cross-reactivity of the antibody was 100% for 20α -OHP, 1.22% for P_4 , 0.99% for pregnenolone and 0.35% for testosterone.

RNA isolation and cDNA synthesis

Total RNA was prepared from luteal tissues using TRIzol Reagent according to the manufacturer's directions (no. 15596-026; Invitrogen, Carlsbad, CA, USA). Total RNA (1 μ g) was reverse transcribed using a ThermoScript RT-PCR System (no. 11146-016; Invitrogen).

Real-time PCR

Gene expression was determined by real-time PCR using a MyiQ system (no. 170-9740, Bio-Rad Laboratories, Melville, NY, USA)

and SYBR[®] *Premix Ex Taq*[™] II (RR081B, TaKaRa, Shiga, Japan) starting with 2 ng of reverse-transcribed total RNA as described previously [12]. Briefly, for quantification of the mRNA expression levels, the primer length (20–25 bp) and GC contents of each primer (50–60%) were synthesized (Table 2) and were chosen using an online software package [13]. To determine the most stable internal control gene under our experimental conditions, three potential housekeeping genes were initially considered (β 2-microglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β -actin), and geNorm analysis was performed using the geNorm software (version 3.5). The M values of B2M, GAPDH and β -actin were 0.784, 0.505 and 0.501, respectively. Therefore, β -actin was identified as the most stable gene. The primers for 3β -HSD, AKR1C23 and β -actin generated specific 132-bp, 148-bp and 113-bp products, respectively. Each PCR yielded only a single amplification product. PCR was performed under the following conditions: 95 C for 3 min, followed by 45 cycles of 94 C for 15 sec, 55 C for 20 sec and 72 C for 15 sec. Use of SYBR[®] *Premix Ex Taq*[™] II at elevated temperatures resulted in reliable and sensitive quantification of the RT-PCR products with high linearity (Pearson correlation coefficient $r > 0.99$). To correct for differences in RNA quality and quantity between samples, the expression levels of target gene mRNA were normalized by dividing the quantity of the target gene by the quantity of β -actin in each sample.

Western blot analysis

Each protein in the luteal tissues was assessed by Western blot analysis. The luteal tissues were homogenized on ice in homogenization buffer (25 mM Tris-HCl, 300 mM sucrose, 2 mM EDTA, Complete [protease inhibitor cocktail; no. 11 697 498 001; Roche Diagnostics, Mannheim, Germany], pH 7.4) by the tissue homogenizer, followed by filtration with a metal wire mesh (150 μ m). For protein analysis, tissue homogenates were centrifuged at $600 \times g$ for 10 min at 4 C. The supernatants were then centrifuged at $9,000 \times g$ for 30 min at 4 C, and the resulting supernatants were used as cytoplasmic fraction. Protein concentrations were determined by the BCA method [14] using BSA as a standard.

The protein samples (50 μ g protein) were then solubilized in SDS gel-loading buffer (50 mM Tris-HCl, 2% SDS [no. 31607-94; Nacalai Tesque, Kyoto, Japan], 10% glycerol, 1% β -mercaptoethanol [no. 137-06862; Wako Pure Chemical Industries, Osaka, Japan], pH 6.8), heated at 95 C for 10 min and subjected to 15% SDS-PAGE for 1 h at 250 V. Separated proteins were electrophoretically transferred to polyvinylidene difluoride membranes (RPN303F; GE Healthcare, Milwaukee, WI, USA) for 40 min at 200 mA using a Trans-Blot[®] SD Semi-Dry Electrophoretic Transfer Cell (no. 170-3940, Bio-Rad Laboratories). The membranes were incubated in blocking buffer (5% nonfat dry milk in TBS [25 mM Tris-HCl, 137 mM NaCl, pH 7.5] with 0.1% Tween 20 [TBST]) for 1 h at room temperature and then incubated separately with specific primary antibodies to 3β -HSD (40 kDa; 1:6,000; ab75710; Abcam plc., Cambridge, UK), AKR1C1 (37 kDa; 1:5,000; LS-B6269; LifeSpan BioSciences, Seattle, WA, USA) and β -actin (42 kDa; 1:4,000; A2228; Sigma-Aldrich, St. Louis, MO, USA) in TBST overnight at 4 C. After primary antibody incubation, the membranes were washed in TBST for 10 min 3 times at room temperature. After washing, the membranes were incubated

Table 1. Macromorphological criteria of the ovary for classifying the luteal phase in mares

Tissue	Criteria	Early luteal phase (Corpus hemorrhagicum)	Mid luteal phase	Late luteal phase	Regressed luteal phase
CL	Appearance	A large central cavity is filled with blood clot	CL is irregular, mushroom shaped or gourd shaped	CL is irregular, mushroom shaped or gourd shaped	CL has a small and oblong structure
	Color	Dark red	Flesh colored	Buff colored	Straw colored
Follicle	Diameter of the largest follicle (mm)		15–20	30–35	>35

Table 2. Primers used in real-time PCR

Gene	Primer	Sequence	Accession No.	Product size
<i>3β-HSD</i>	Forward	5'-TGGCATCCTGACACACAAC-3'	D89666	132 bp
	Reverse	5'-AACTGTCCTTGGATGCTTGG-3'		
<i>AKR1C23</i>	Forward	5'-CATGAAAGTCCTAGATGGCCTAAAC-3'	AY955082	148 bp
	Reverse	5'-CACTATCCACACACAGGGCTTC-3'		
<i>β-actin</i>	Forward	5'-ATGGGCCAGAAGGACTCATA-3'	NM_001081838	113 bp
	Reverse	5'-TTCTCCATGTCGTCCAGTT-3'		

with secondary antibody (anti-mouse, HRP-linked whole antibody produced in sheep [NA931; Amersham Biosciences, San Francisco, CA, USA]; 1: 6,000 for 3β-HSD, 1: 10,000 for AKR1C1, 1: 40,000 for β-actin) in TBST for 1 h at room temperature, washed in TBST for 10 min 3 times at room temperature and then incubated with Immobilon Western Chemiluminescent HRP Substrate (WBKLS0500; Merck KGaA, Darmstadt, Germany). Images were captured using a ChemiDoc™ XRS+ System (#170-8265; Bio-Rad Laboratories). The intensity of the immunological reaction was estimated by measuring the optical density in the defined area by computerized densitometry using NIH Image (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

The results are expressed as mean ± SEM values obtained from 3–8 luteal tissues/phase. The statistical significance of differences in luteal P₄ and 20α-OHP concentrations, the levels of 3β-HSD and AKR1C23 mRNA, and 3β-HSD and AKR1C23 protein levels was analyzed by a one-way analysis of variance (ANOVA). The comparisons between phases were performed using the Tukey-Kramer multiple comparison test. P values < 0.05 were considered significant.

Results

Luteal P₄ and 20α-OHP concentration in the different luteal phases

The luteal P₄ concentration was higher in the mid luteal phase than in the late and regressed luteal phases (Fig. 1a: P<0.05). The luteal 20α-OHP concentration was higher in the late luteal phase than in any other luteal phases (Fig. 1b: P<0.05).

Expressions of 3β-HSD mRNA and protein in the equine CL in the different luteal phases

Specific transcripts for 3β-HSD were detected in the equine CL throughout the luteal phase. The level of 3β-HSD mRNA was higher

in the early and mid luteal phases than in the late and regressed luteal phases (Fig. 2a: P<0.05). 3β-HSD protein was expressed in the equine CL throughout the luteal phase. The level of 3β-HSD protein was higher in the late luteal phase than in the regressed luteal phase (Fig. 2b: P<0.05).

Expressions of AKR1C23 mRNA and protein in the equine CL in the different luteal phases

Specific transcripts for AKR1C23 were detected in the equine CL throughout the luteal phase. The level of AKR1C23 mRNA was higher in the late luteal phase than in the early and mid luteal phases (Fig. 3a: P<0.05). AKR1C23 protein was expressed in the equine CL throughout the luteal phase. The level of AKR1C23 protein was higher in the late luteal phase than in the early luteal phase (Fig. 3b: P<0.05).

Discussion

In the present study, P₄ concentrations in the luteal tissues were high in the mid luteal phase and low in the late and regressed luteal phases. This result is consistent with previous reports that showed changes in circulating P₄ concentration throughout the luteal phase in mares [15, 16], indicating that functional luteolysis occurs in the late luteal phase. As in the case of the P₄ concentrations in the luteal tissues, the mRNA expression of 3β-HSD, which converts pregnenolone into P₄, was higher in the early and mid luteal phases than in the late and regressed luteal phases. However, unexpectedly, the level of 3β-HSD protein was highest in the late luteal phase. In cattle [17, 18] and sheep [19, 20], the level of mRNA for 3β-HSD decreased in parallel with circulating P₄ concentrations during PGF-induced luteolysis, but 3β-HSD protein expression or activity in the CL did not change. Based on the above findings, 3β-HSD activity in the CL may not play a role in functional luteolysis in mares.

The 20α-OHP concentrations in the luteal tissues were higher in

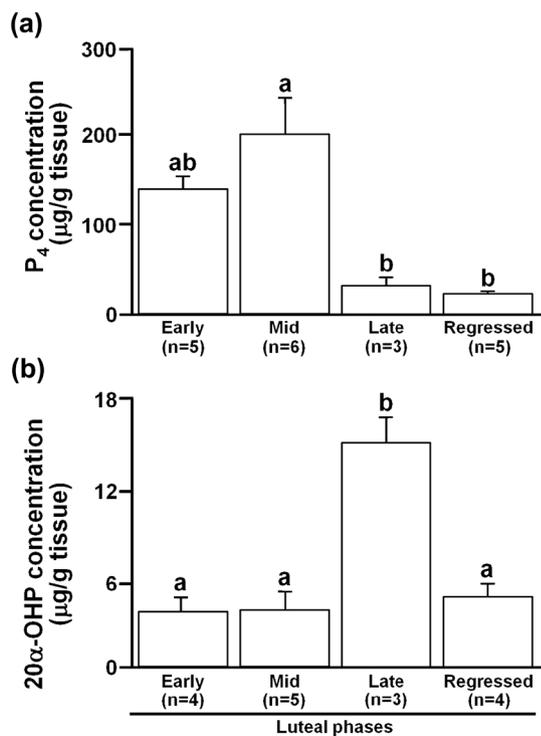


Fig. 1. Luteal P₄ (a) and 20α-OHP (b) concentrations in the different luteal phases. All experimental data are shown as means ± SEM. Different letters indicate significant differences ($P < 0.05$) as determined by ANOVA followed by the Tukey-Kramer multiple comparison test.

the late luteal phase than in any other luteal phases. This finding strongly suggests that P₄ is converted into 20α-OHP in the late luteal tissues. In rodents, 20α-HSD expressed in luteal cells catabolizes P₄ into 20α-OHP at the termination of pregnancy to allow parturition to occur [21]. In mares, AKR1C23 has 20α-HSD activity; i.e., it converts P₄ into 20α-OHP [7]. In the present study, the mRNA and protein expressions of AKR1C23 were highest in the late luteal phase when the 20α-OHP concentration in the luteal tissues reached a peak. These results are consistent with previous observations in the bovine CL [5]. Together, our findings suggest that AKR1C23 converts P₄ into 20α-OHP in the late luteal phase, resulting in a decrease in the luteal P₄ concentration (Fig. 1a). In mares, PGF secreted from the uterus in the late luteal phase induces luteolysis, i.e., a decrease in circulating P₄ concentration [22]. In addition, Fas ligand (FASL) expressed in the late CL reduces P₄ production and stimulates PGF production by equine luteal cells [23]. In rodents, PGF has been demonstrated to induce 20α-HSD expression in the CL, which is accompanied by a significant reduction in the circulating levels of P₄ and a rise in the levels of 20α-OHP [21]. Based on the above findings, AKR1C23 expression in the equine CL may be induced by PGF and FASL, leading to functional luteolysis. Further study is needed to clarify the mechanism of the regulation of AKR1C23 expression in the equine CL.

The overall findings in the present study strongly suggest that

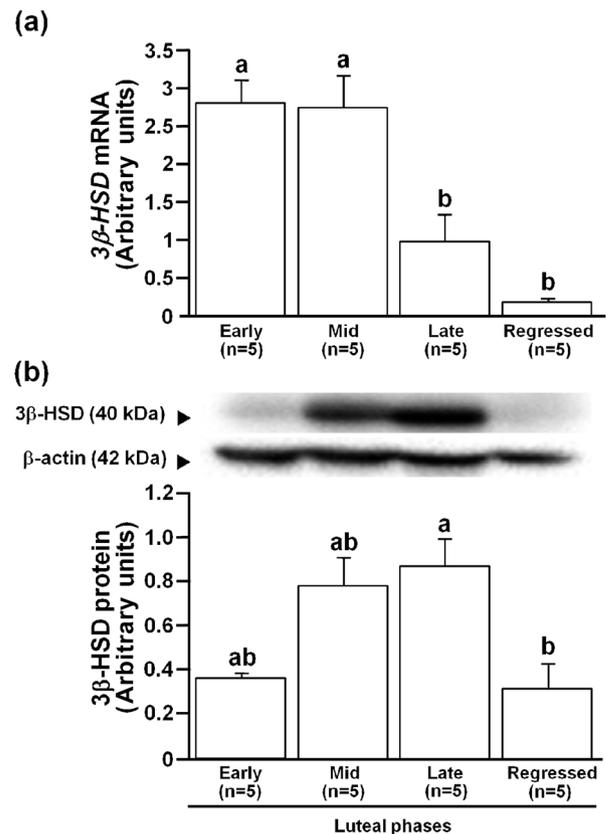


Fig. 2. Expressions of 3β-HSD mRNA (a) and protein (b) in the equine CL in the different luteal phases. All experimental data are shown as means ± SEM. Different letters indicate significant differences ($P < 0.05$) as determined by ANOVA followed by the Tukey-Kramer multiple comparison test.

conversion of P₄ into 20α-OHP by AKR1C23 is one of the processes contributing to functional luteolysis in mares.

Acknowledgments

This study was supported by the Japanese-Polish Joint Research Project under an agreement between the Japan Society for the Promotion of Science and Polish Academy of Sciences and was supported in part by the Equine Research Institute, Japan Racing Association.

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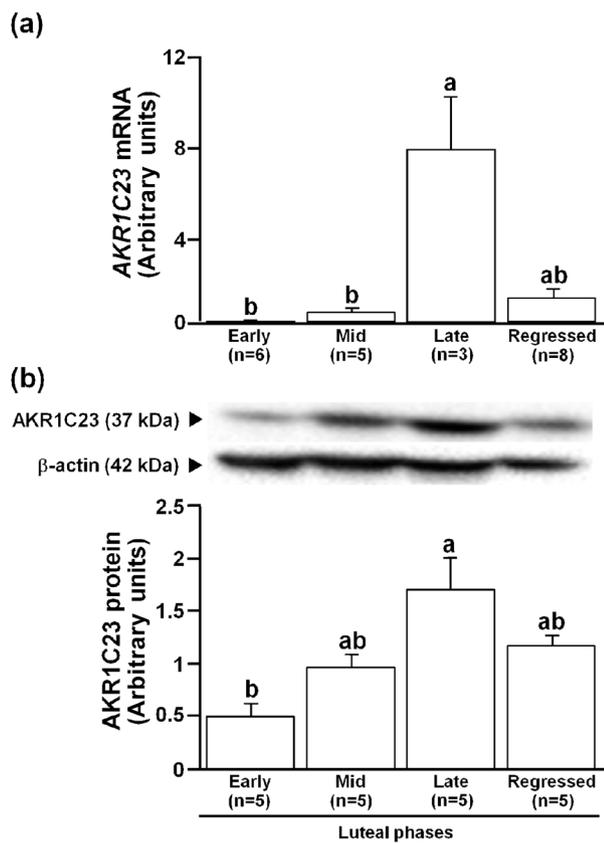


Fig. 3. Expressions of AKR1C23 mRNA (a) and protein (b) in the equine CL in the different luteal phases. All experimental data are shown as means \pm SEM. Different letters indicate significant differences ($P < 0.05$) as determined by ANOVA followed by the Tukey-Kramer multiple comparison test.

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