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—Original Article—

Gene Expression of 11β-HSD and Glucocorticoid Receptor in the Bovine (*Bos taurus*) Follicle During Follicular Maturation and Atresia: The Role of Follicular Stimulating Hormone

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Abstract. Glucocorticoids modulate ovarian function in cattle. However, their regulatory mechanisms have not been fully elucidated. In the present study, we examined gene expression of two glucocorticoid-metabolizing enzymes, a bidirectional 11β-HSD type 1 (11HSD1) and a dehydrogenase 11β-HSD type 2 (11HSD2), and glucocorticoid receptor (GR) in bovine follicles during follicular maturation and atresia. Granulosa cells (GCs) and theca interna layers (TIs) were harvested from follicles classified as small growing, dominant, preovulatory, early atretic and late atretic follicles. The expression levels of 11HSD1, 11HSD2 and GR mRNA were quantified by real-time PCR. In the healthy follicles, expression of 11HSD1 mRNA increased as follicles matured, both in GCs and TIs. A significant negative correlation was found between the concentration of cortisol in follicular fluid and the level of 11HSD1 mRNA in GCs. The expression of 11HSD2 and GR was either very low or largely unchanged during follicular maturation. In the atretic follicles, a drastic increase in the expression of 11HSD2 was observed both in GCs and TIs. To assess the effect of FSH on the expression of 11HSDs and GR, GCs were cultured with FSH (0–100 ng/ml) for up to 6 days. FSH increased 11HSD1 mRNA in a dose-dependent manner, but not 11HSD2, nor GR. Taken together, these results suggest that developmentally-regulated 11HSD1 plays a pivotal role in modulating the local glucocorticoid environment in maturing bovine follicles.

Key words: Bovine, Cortisol, 11β-HSD, Follicle, Glucocorticoid receptor, mRNA

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The ovary is a glucocorticoid target organ, and actions of glucocorticoids on ovarian functions have been reported in several species [1]. Glucocorticoids appear to exert both stimulatory and suppressive effects on follicular functions. Glucocorticoids enhance FSH-stimulated progesterone synthesis in cultured granulosa cells (GCs) of rats and cattle [2–5] and thecal androgen production in cattle [5, 6]. Oxytocin production is also stimulated by glucocorticoids in cultured bovine GCs [7]. On the other hand, glucocorticoids have been shown to suppress P450 aromatase (P450arom) activity and decrease the number of LH receptors (LH-R) in rats [2, 4], cattle [5] and pigs [8]. These results indicate that glucocorticoid action has to be appropriately regulated to ensure healthy follicular development. The ovary appears to cope with this problem by expressing two glucocorticoid metabolizing enzymes, 11β-HSD type 1 (11HSD1) and type 2 (11HSD2). Research has shown that 11HSD1 is predominantly a reductase that acts as an activator of glucocorticoids (converts inactive glucocorticoids, such as cortisone to cortisol), while 11HSD2 is a dehydrogenase acting as an inactivator [9]. In human and rat preovulatory follicles, GCs predominantly express 11HSD2, which may protect maturing follicles from suppressive effects of glucocorticoids [10–12]. The pattern of 11HSD expression appears to be different in cattle, where GCs in preovulatory follicles express 11HSD1 but not 11HSD2 [13]. In cattle, 11HSD1 might act as a dehydrogenase rather than a reductase, as a negative correlation was observed between gene expression of 11HSD1 and concentrations of cortisol in follicular fluid [13]. In that study, however, 11HSD expression was examined only in GCs collected from follicles larger than 10 mm and no developmental change in gene expression was examined.

Thus, in the present study, we examined the pattern of gene expression for 11HSD1, 11HSD2 and glucocorticoid receptor (GR) in bovine GCs and theca interna layers (TIs) during follicular maturation and atresia. To determine the developmental regulation of these genes, we also examined the effect of FSH on the expression of these genes in cultured GCs.

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Materials and Methods

Sample collection and storage

Paired ovaries were obtained from Holstein × Japanese Black F1

Table 1. Systematic classification of the bovine follicles used in the present study

Follicular status	Follicular size (mm)	E2/P4	CL stage	Absence of follicle	Glucose (mg/dl)
GF	<8.5	N/A	I – III	POF/DF	N/A
DF	≥8.5	≥1	I – III	N/A	N/A
POF	≥8.5	≥1	IV	N/A	N/A
EAF	≥8.5	<1	I – IV	N/A	≥45
LAF	≥8.5	<1	I – IV	N/A	<45

GF, growing follicle; DF, dominant follicle; POF, preovulatory follicle; EAF, early atretic follicle; LAF, late atretic follicle.

heifers (*Bos taurus*) at a local slaughterhouse. Only healthy ovarian pairs were used in the present study. Follicular fluid (FF) was aspirated from follicles using a syringe fitted with a 20-G needle and then kept at –30 C. The aspirated follicles were dissected from ovaries and placed in RNAlater™ (Ambion®, Austin, TX, USA) and frozen at –30 C. In the laboratory, GCs were carefully harvested from the follicular walls using a spatula. The remaining follicular walls were further cleared of the surrounding stroma and theca externa as well as remaining GCs under a stereomicroscope to obtain TIs.

Classification of corpora lutea and follicles

Corpora lutea (CLs) were macroscopically assessed for color, vascularity and consistency using published criteria [14] and were classified into four stages (stage I, days 1–4 post ovulation; stage II, days 5–10; stage III, days 11–17; and stage IV, days 18–20).

Follicular diameter was estimated from the volume of FF using the equation reported by Murasawa *et al.* [15]. Follicles were classified into five groups based on diameter (≥8.5 mm or <8.5 mm), relative concentrations of estradiol (E2) and progesterone (P4) in FF (E2:P4≥1 or <1), concentrations of glucose in FF (≥45 mg/dl or <45 mg/dl), and the stages of accompanying CLs and follicles (growing follicle, GF; dominant follicle, DF; preovulatory follicle, POF; early atretic follicle, EAF; late atretic follicle, LAF) [16] (Table 1).

Granulosa cell culture

Ovaries were obtained from Holstein cows at a local slaughterhouse. Granulosa cells were harvested from follicles <8 mm in diameter by aspiration using a 10-ml syringe fitted with an 18-G needle. The collected cells were pooled and washed three times with culture medium. The culture system was based on the method reported by Gutierrez *et al.* [17] with some modifications; 2×10^5 viable cells were cultured in 0.5 ml DMEM/F12 with bicarbonate supplemented with 20 mM HEPES, 3 mM L-glutamine, 100 IU/ml penicillin, 50 mg/ml streptomycin, 0.1% bovine serum albumin, 2.5 µg/ml transferrin, 4 ng/ml selenium and 100 ng/ml insulin at 37 C in 5% CO_2 in air. The culture medium was supplemented with fetal calf serum for the first two days, then culture was continued without serum for up to 6 days with 0–100 ng/ml highly purified bovine FSH (AFP-5332B; provided by Dr AF Parlow, NIDDK, USA) and 100 nmol/l androstenedione (Sigma). The spent medium was replaced with fresh medium every 2 days.

RNA extraction

Tissue and cell samples were homogenized in a denaturing solution containing 4 M guanidium thiocyanate (Wako Pure Chemical Industries, Osaka, Japan), 25 mM sodium citrate (Wako), 0.5% sarkosyl (Sigma Chemical, St. Louis, MO, USA) and 0.1 M β-mercaptoethanol (Kanto Chemical, Tokyo, Japan). Total RNA was extracted with phenol-chloroform [18], further purified and treated with DNase using a commercial kit (SV Total RNA Isolation System: Promega, Madison, WI, USA) and then stored at –30 C in THE RNA Storage Solution (Ambion).

Reverse transcription (RT) and quantitative PCR

Single-strand cDNA was generated from total RNA (0.5–1.0 µg) using a commercial kit (Omniscript® Reverse Transcription Kit: QIAGEN GmbH, Hilden, Germany) and a random primer. The reverse transcription cycle consisted of a 120-min cDNA synthesis at 37 C and a 5-min inactivation at 94 C.

Messenger RNA encoding 11HSD1, 11HSD2, GR, P450arom and β-actin were quantified by real-time PCR (Light Cycler, Roche Diagnostics, Indianapolis, IN, USA) using a commercial kit (QuantiTect™ SYBR® Green PCR, QIAGEN). The primers were designed using Primer3 [19] based on reported bovine sequences (Table 2). The amplification program consisted of an initial 15-min activation at 95 C followed by 40 cycles of PCR steps (15 sec denaturation at 94 C, 25 sec annealing at 58 C, 20 sec extension at 72 C for 11HSD1, 11HSD2, P450arom and β-Actin; 15 sec denaturation at 94 C, 25 sec annealing at 52 C, 25 sec extension at 72 C for GR).

For quantification of the target genes, a series of standards was constructed by amplifying a fragment of DNA that contained the target sequence for real-time PCR (Table 2). The intra- and interassay coefficients of variations for these quantifications were less than 15%. The values were normalized using β-actin as the internal standard.

Steroid hormone assay

Concentrations of E2 and P4 in the FF samples were determined by an enzyme immunoassay (EIA) as published previously [20]. The concentrations of cortisol were determined by using a commercial kit (Enzyme Immunoassay for Cortisol, EA65, Oxford Biomedical Research, Oxford, MI, USA). The intra- and interassay coefficients of variation for these assays were less than 10%.

Glucose assay

Concentrations of glucose in the FF samples were determined by

Table 2. Primers for quantitative real-time PCR

Gene (size: bp)	Primer	Sequence	GenBank no.[a]	Position[b]
11HSD1 (111)	Sense	aagcagaccaacgggagcatt	AF548027	532–552
	Antisense	ggagaagaacccatccagagca	AF548027	621–642
11HSD2 (123)	Sense	cgagcacttgaatgggcagtt	AF074706	1033–1053
	Antisense	cctgggtaatagcggcggagt	AF074706	1135–1155
GR (100)	Sense	gggtatgacagctcggttcca	AY238475	1058–1078
	Antisense	cctttgcccatttcactgctg	AY238475	1137–1157
P450arom (151)	Sense	ttgcaaagcatccccaggtt	Z32741	1147–1166
	Antisense	aggtccacaacgggctggta	Z32741	1278–1297
β-actin (228)	Sense	tgccatgtatgtggccatcc	K00622	162–181
	Antisense	cgctcggctgtggtggtaa	K00622	371–389

[a] GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide). [b] Nucleotide position in the reported sequence.

Table 3. Characteristics of each follicular category: diameter and concentrations of estradiol (E2), progesterone (P4), cortisol and glucose in follicular fluid

Group (n)	Follicle diameter (mm)	E2 (ng/ml)	P4 (ng/ml)	Cortisol (ng/ml)	Glucose (mg/dl)
GF (10)	7.3 ± 0.3^{a}	2.6 ± 1.0^{ad}	9.0 ± 1.7^{a}	31.6 ± 9.2	57.8 ± 2.4^{a}
DF (23)	11.9 ± 0.4^{b}	33.7 ± 3.4^{b}	18.9 ± 1.8^{bc}	18.9 ± 3.2	58.2 ± 3.0^{ab}
POF (10)	14.2 ± 1.0^{b}	70.2 ± 12.6^{c}	22.5 ± 2.6^{cd}	19.8 ± 3.0	74.4 ± 2.1^{b}
EAF (34)	13.4 ± 0.4^{b}	10.1 ± 2.1^{ad}	113.5 ± 28.1^{d}	19.4 ± 3.0	71.6 ± 2.3^{b}
LAF (8)	11.6 ± 0.9^{b}	2.6 ± 1.2^{d}	260.8 ± 51.0^{c}	12.9 ± 5.7	33.8 ± 4.2^{c}

Mean ± SEM. GF, growing follicle; DF, dominant follicle; POF, preovulatory follicle; EAF, early atretic follicle; LAF, late atretic follicle. Values within a row with different superscripts are significantly different (P<0.05).

a colorimetric method using a commercial kit (Glucose CII-Test Wako, Wako). The intra- and interassay coefficients of variation were less than 5%.

Statistical analysis

The data were analyzed using a one- or two-way ANOVA followed by the Steel-Dwass (*in vivo* data) or Tukey-Kramer multiple comparison test (*in vitro* data). The relationships between the levels of mRNA and size of follicles were analyzed by a regression analysis. All data were presented as means ± SEM with statistical significance set at P<0.05.

Results

Characteristics of follicles

A total of 85 follicles from 63 animals were used in the present study. The diameters of follicles and concentrations of steroids and glucose in FF in each group are shown in Table 3. There were significant differences in steroid concentrations among follicle classes. The concentrations of glucose in FF were comparable to the levels reported in bovine follicular fluid (30–72 mg/dl) [21]. Despite a precipitous decrease in the E2 concentration in the EAFs, the glucose concentrations in these follicles were maintained as high as that in healthy dominant follicles (i.e., POFs and DFs). On the other hand, low glucose concentrations were always associated with low E2 concentrations (i.e., LAFs).

Gene expressions of 11HSD1, 11HSD2 and GR in follicular tissues during follicular maturation and atresia

The mRNAs encoding 11HSD1, 11HSD2 and GR were all detected in bovine follicles. Expression of 11HSD1 mRNA increased in both GCs and TIs as follicular maturation progressed from the GF to the DF/POF (P<0.05, Fig. 1A). The expression of 11HSD1 mRNA was largely unchanged in the atretic follicles and was higher in TIs than in GCs in healthy follicles (GF, DF and POF: P<0.05, Fig. 1A).

Only negligible levels of 11HSD2 mRNA were detected in the GCs in GFs. The expression increased slightly but significantly in DFs and POFs, then dramatically increased in atretic follicles (P<0.05, Fig. 1B). Comparably higher levels of 11HSD2 mRNA were observed in TIs. The levels were unchanged throughout follicular development and in EAFs but increased 3-fold in LAFs (P<0.05, Fig. 1B).

The expression of GR mRNA was unchanged throughout follicular development in GCs and TIs, and increased in atretic follicles (Fig. 1C). The level of GR mRNA was much higher in TIs than in GCs regardless of the follicular stage (P<0.01).

Relationships between indices of follicular maturation and levels of gene expression in healthy follicles

To examine the effect of follicular maturation on gene expression, we correlated the levels of 11HSD and GR mRNA with either follicular diameter (i.e., morphological index of follicular maturation) or concentration of E2 in FF (i.e., functional index of

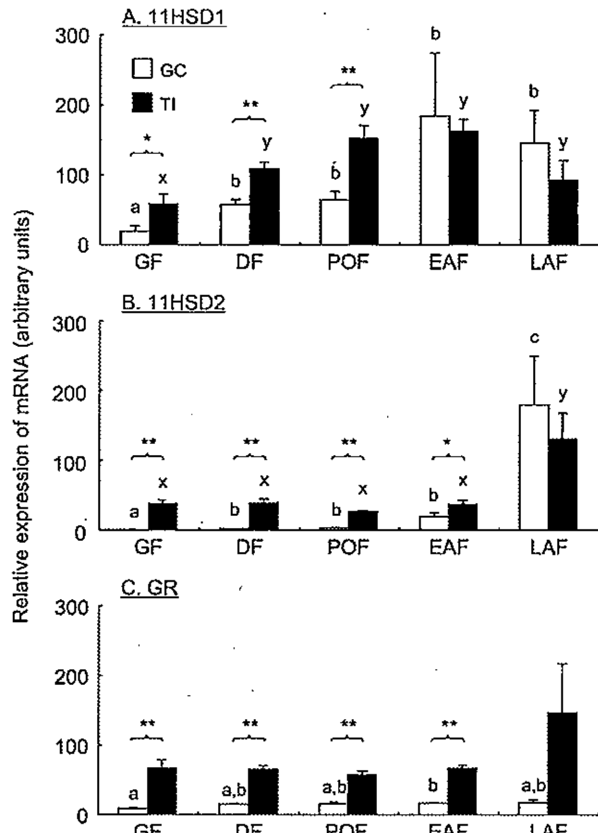

Fig. 1. Relative expression of mRNA encoding 11β-HSD type 1 (11HSD1: A), 11β-HSD type 2 (11HSD2: B) and glucocorticoid receptor (GR: C) in bovine granulose cells (GCs) and theca interna layers (TIs) during follicular maturation and atresia. Mean ± SEM. GF, growing follicle; DF, dominant follicle; POF, preovulatory follicle; EAF, early atretic follicle; LAF, late atretic follicle. [a,b; x,y] Significantly different from each other ($P<0.05$). * $P<0.05$, ** $P<0.01$.

follicular maturation) in healthy follicles (Table 4). Significant positive correlation was observed between the levels of 11HSD1 mRNA and either follicular diameter ($r \geq 0.50$, $P<0.005$) or the concentration of E2 ($r \geq 0.48$, $P<0.001$) for both GCs and TIs. Significant positive correlation was also observed between the levels of 11HSD2 mRNA and either follicular diameter ($r \geq 0.45$, $P<0.005$) or the concentration of E2 ($r \geq 0.45$, $P<0.005$) for GCs but not TIs. Weak positive correlation was found between the gene expression of GR and follicular diameter in GCs ($r=0.32$, $P<0.05$).

Relationships between levels of gene expression and the concentration of cortisol in follicular fluid in healthy follicles

Using exponential regression, significant negative correlation was detected between the levels of 11HSD1 mRNA in GCs and the concentration of cortisol in follicular fluid ($r=-0.44$, $P<0.005$). No such correlation was found for 11HSD2. Weak correlation was detected between the expression of GR mRNA in GCs and the concentration of cortisol ($r=-0.39$, $P<0.05$). No correlation was found between the expression of any genes and follicular cortisol in atretic follicle.

Time-dependent effect of FSH on the gene expressions of 11HSD1, 11HSD2, GR and P450arom in cultured granulosa cells

Cultured granulosa cells were treated with 10 ng/ml FSH for up to 6 days, and the levels of mRNA were quantified for 11HSD1, 11HSD2 and GR along with a typical FSH target gene, P450arom (Fig. 2). The treatment with FSH increased the gene expression of 11HSD1 as well as P450arom (Fig. 2A, D, $P<0.05$). Four days of FSH treatment elicited maximum levels of gene expression of 11HSD1, whereas an extra 2 days of treatment resulted in a farther 15-fold increase in P450arom mRNA. In the control culture, the basal levels of these FSH-responsive genes were maintained for up to 4 days, then decreased by day 6 ($P<0.05$). The expressions of 11HSD2 and GR mRNA were not affected by FSH treatment but gradually increased as the culture proceeded in both control and treated cells (Fig. 2B, C, $P<0.05$). Treatment with FSH brought about gradual increases in the concentrations of E2 in the culture

Table 4. Correlations between the levels of 11HSDs or GR mRNA and diameter of follicles, concentration of estradiol (E2) or cortisol in follicular fluid (FF) in granulosa cells (GC) and the theca interna (TI) of healthy bovine follicles

Gene: tissue	Follicular diameter	E2 in FF	Cortisol in FF
11HSD1: GC	r=0.50 P<0.0005	r=0.48 P<0.001	r=–0.44 P<0.005
11HSD1: TI	r=0.55 P<0.0001	r=0.53 P<0.0005	r=0.06 n.s.
11HSD2: GC	r=0.45 P<0.005	r=0.51 P<0.0005	r=–0.12 n.s.
11HSD2: TI	r=–0.30 n.s.	r=–0.30 n.s.	r=0.02 n.s.
GR: GC	r=0.32 P<0.05	r=0.19 n.s.	r=–0.39 P<0.05
GR: TI	r=0.16 n.s.	r=0.02 n.s.	r=0.08 n.s.

Healthy follicles include growing, dominant and preovulatory follicles (n=43). n.s.: not significant.

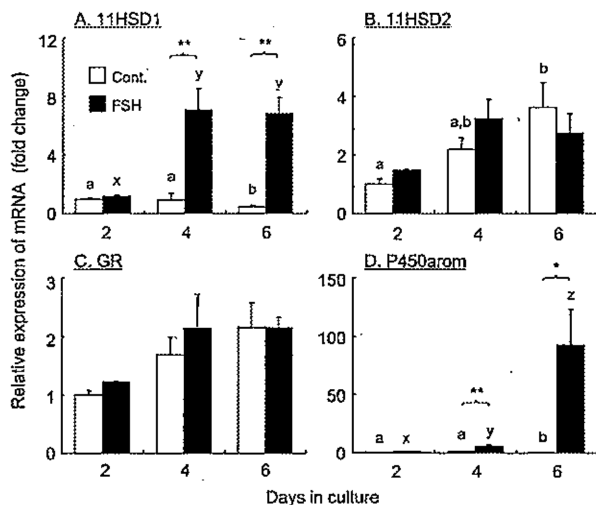

Fig. 2. Time-dependent effect of FSH on the gene expressions of 11β-HSD type 1 (11HSD1), 11β-HSD type 2 (11HSD2), glucocorticoid receptor (GR) and P450 aromatase (P450arom) in cultured bovine granulose cells (GCs). Granulosa cells (2×10^5 viable cells) were cultured in 0.5 ml DMEM/F12 supplemented with FSH (10 ng/ml) for 2, 4 or 6 days and then harvested for mRNA quantification. Mean ± SEM (n=4). [a,b; x,y,z] Significantly different from each other ($P<0.05$). * $P<0.05$, ** $P<0.01$.

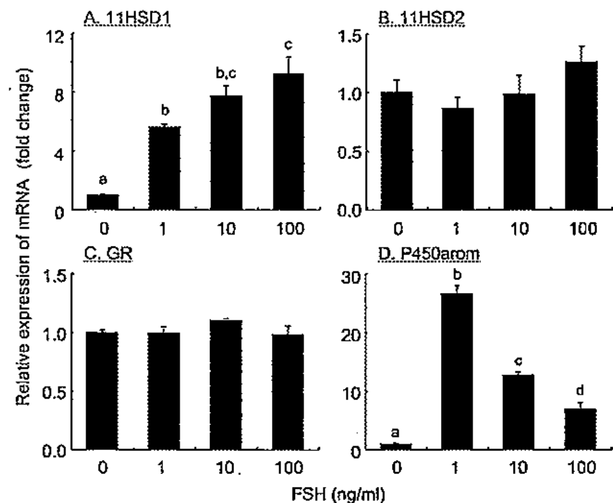

Fig. 3. Dose-dependent effect of FSH on the gene expressions of 11β-HSD type 1 (11HSD1), 11β-HSD type 2 (11HSD2), glucocorticoid receptor (GR) and P450 aromatase (P450arom) in cultured bovine granulose cells (GCs). Granulosa cells (2×10^5 viable cells) were cultured in 0.5 ml DMEM/F12 supplemented with/without FSH (0–100 ng/ml) for 4 days and then harvested for mRNA quantification. Mean ± SEM (n=3). [a,b,c,d] Significantly different from each other ($P<0.05$).

media collected at days 2, 4 and 6 (3.8 ± 1.9, 7.3 ± 0.4 and 45.9 ± 2.6 ng/ml, respectively). There was a significant positive correlation between the level of P450arom mRNA and concentration of E2 ($r=0.73$, $P<0.01$) but not between the concentration of E2 and level of 11HSD1 mRNA.

Dose-dependent effect of FSH on gene expression of 11HSD1, 11HSD2, GR and P450arom in cultured granulosa cells

Granulosa cells were treated with FSH (0–100 ng/ml) for 4 days, and the levels of gene expressions were quantified for 11HSD1 and 11HSD2, GR and P450arom (Fig. 3). Among the three doses tested (1, 10, 100 ng/ml), the maximum response was achieved by 1 ng/ml for P450arom (Fig. 3D). The levels of P450arom expression steadily decreased as the dose of FSH increased (Fig. 3D, $P<0.05$). The gene expression of 11HSD1 was increased in a dose-dependent manner by the FSH treatment (Fig. 3A, $P<0.05$). The expressions of 11HSD2 and GR were not affected by the FSH treatment (Fig. 3B, C). Treatment with FSH brought about a dose-dependent decrease in the concentrations of E2 in the culture media collected at day 4 (0.3 ± 0.1, 34.0 ± 3.8, 22.5 ± 2.0 and 13.8 ± 1.1 ng/ml for 0, 1, 10 and 100 ng/ml FSH, respectively). There was highly significant positive correlation between the level of P450arom mRNA and concentration of E2 ($r=0.90$, $P<0.0001$) but not between the concentration of E2 and level of 11HSD1 mRNA.

Discussion

In the present study, we demonstrated that maturing bovine follicles express both 11HSD1 and 11HSD2 mRNA. The expression of 11HSD1 increased as follicular maturation progressed both in GCs and TIs, whereas that of 11HSD2 was very low in GCs and unchanged in TIs, indicating 11HSD1 is the dominant 11β-HSD in mature bovine follicles. Significant negative correlation between the level of 11HSD1 mRNA in GCs and the concentration of cortisol in FF confirms the findings of our previous study [13] and further implies that 11HSD1 may act as a dehydrogenase rather than a reductase in these follicles.

Glucocorticoids are not produced by the ovary and are derived from the adrenal gland in an endocrine manner. In cattle, adrenal secretion of glucocorticoids is under circadian control, being high in the morning and low in the evening, with strong ultradian rhythms [22]. The secretion of glucocorticoids is also increased by mental and physical stresses [23]. Thus, the ovary is continuously exposed to periodic and sporadic waves of glucocorticoids. Increases in glucocorticoids may exert deleterious effects on bovine follicular maturation by suppressing P450arom activity and LH-R expression [5]. Therefore, upregulation of 11β-HSD dehydrogenase that inactivates glucocorticoids might be an important mechanism to protect maturing follicles from circulating glucocorticoids. In rats and humans, 11HSD2 appears to play this protective role [10, 11]. Maturing follicles in these species expressed high levels of 11HSD2 in granulosa and theca/interstitial cells, whereas the expression of 11HSD1 was low or negligible. Recently, Sunak *et al.* [24] demonstrated that both cortisol oxidation and NADP(+)-dependent 11HSD1 dehydrogenase activity in porcine GCs increase as follicular development progresses. Since no 11-ketosteroid reductase activity was observed in the GCs, the authors concluded that 11HSD1 acts as a dehydrogenase in mature

porcine follicles [24]. The results of the present study, as well as that of our previous study [13], parallel the results obtained in the pig and indicate that the 11HSD1-mediated glucocorticoid inactivating mechanism is also functional in bovine maturing follicles.

In the glucocorticoid target organs, 11HSD1 acts primarily as a NADPH-dependent reductase [9, 25]. However, the direction of metabolism is altered by the availability of cofactors and carbohydrate substrates [25–31]. In rat Leydig cells, 11HSD1 acts as a dehydrogenase and protects steroidogenesis from inhibitory effects exerted by the active glucocorticoid, corticosterone [26, 32]. Likewise, 11HSD1 was shown to predominantly act as a dehydrogenase in the ovine placenta, where it protects the fetus from adverse effects of maternal glucocorticoids [33].

As follicles mature, androgen production in TIs and its conversion to estrogen in GCs are enhanced [34]. Because both reactions require NADPH as a hydrogen donor, the intracellular ratio of NADPH to NADP(+) is likely to be lower in preovulatory follicles. This may be the reason why 11HSD1 acts as dehydrogenase in these follicles [28]. If this is the case, 11HSD1 benefits P450 17α-hydroxylase and P450arom by not only supplying cofactors, but also by preventing antagonistic effects of cortisol on estrogen biosynthesis. To confirm this hypothesis, however, well-designed experiments are necessary.

The present study demonstrated that the expression of 11HSD1 mRNA steadily increased as follicular development progressed both in GCs and TIs. FSH appears to be responsible for the upregulation of 11HSD1 gene expression in bovine GCs, as has been shown in cultured human and rat GCs [12, 35]. The expression of 11HSD2, on the other hand, was not affected by FSH, implying that the alteration of glucocorticoids metabolism associated with follicular maturation is primarily regulated by FSH-responsive 11HSD1 in bovine GCs. In the present study, highly significant positive correlations were found between the expression of 11HSD1 and the concentration of E2 in FF, both for GCs and TIs. It is not likely, however, that E2 upregulates 11HSD1 mRNA in GCs. In the time-dependent experiment, the expression of 11HSD1 was maximally stimulated by day 4, at which point the E2 concentration in the medium was still low, whereas the expression was not altered by the presence of high levels of E2 in the medium at day 6. Likewise, high doses of FSH decreased GC E2 output dose dependently, while the expression of 11HSD1 steadily increased. Moreover, no correlation was found between the expression of 11HSD1 mRNA and the concentration of E2 in the media in these experiments.

The dose-dependent experiment clearly showed that the gene expressions of 11HSD1 and P450arom are differentially regulated by high levels of FSH. This situation resembles the preovulatory period after the LH surge, in which the gene expression of 11HSD1 increases [10, 11] while that of P450arom precipitously decreases [36]. During this period, 11HSD1 appears to act predominantly as a reductase and increases local concentrations of cortisol in FF [12]. Therefore, this differential regulation may be the mechanism that allows 11HSD1 to preferentially utilize NADPH, thus acting as a reductase. Using a microdialysis perfusion system embedded in the follicular wall, we demonstrated that a temporal increase in follicular cortisol occurs around the preovulatory LH surge in cattle [37]. Since no increase in circulating cortisol was observed in plasma, we concluded that the local mechanism that increases cortisol at a response to the preovulatory LH surge is also functional in cattle. Whether this increase in cortisol output is mediated by 11HSD1 is not known. Further study is necessary to clarify the exact role of 11HSD1 in bovine periovulatory follicles.

In the present study, 11HSD2 mRNA was expressed stably in TIs throughout follicular maturation. This result implies that the 11HSD2 mediating protection system mainly operates in the TIs of bovine follicles. However, the11HSD2 expression in GCs, albeit low, might still play a physiological role in these follicles. It has been shown that bovine GCs express both 11HSD2 mRNA and protein, and NAD(+)-dependent dehydrogenase activity (i.e., 11HSD2 activity) was evident in GCS homogenate prepared from preovulatory follicles [38]. In rat Leydig cells, the gene expression of 11HSD2 was shown to be about 1000-fold lower than that of 11HSD1, yet significant NAD(+)-dependent 11HSD2 dehydrogenase activity was observed [32]. Thus, upregulation of 11HSD2 gene expression in GCs found in the present study may play an important role in maturing follicles. The presence of a high affinity 11HSD2 along with low affinity 11HSD1 may be beneficial for GCs to cope with a wide range of glucocorticoids as suggested for the Leydig cells of rats [32].

In the present study, neither follicular maturation nor FSH treatment affected the expression of GR mRNA. These results are in accordance with our previous study in which the treatment with eCG increased the expression of 11HSD1 mRNA but not GR in immature rat ovaries [11]. Taken together, these results suggest that the transcriptional regulation of GR is not a part of the ovarian glucocorticoid regulatory mechanism in cattle.

Without a preovulatory gonadotropin surge, dominant follicles lose their ability to ovulate and undergo an atretic process characterized by a rapid decrease in estrogen synthesis [39]. Gene expression of steroidogenic enzymes and gonadotropin receptors is also downregulated in atretic follicles [39, 40]. In the present study, however, the expressions of 11HSDs and GR mRNA were either dramatically upregulated or maintained in the atretic follicles. Thus, the expressions of 11HSDs and GR are spared from the atresia that disrupts follicular estrogen biosynthesis. Although the physiological significance of this phenomenon is not clear, the dramatic upregulation of 11HSD2 mRNA in GCs implies a physiological role for this enzyme in the atretic process. Likewise, 11HSD1 may act as a dehydrogenase under the hypoglycemic condition found in LAFs. It has been reported that depletion of glucose and pyruvate in the culture medium suppressed 11HSD1 reductive activity to nearly undetectable levels in cultured rat Leydig cells [26]. In LAFs, the concentrations of cortisol in FF tended to decrease; however, no correlation was found between the expression of 11HSD mRNA and the concentration of follicular cortisol. Further study is necessary to clarify this point.

In conclusion, the results of the present study showed that the gene expression of 11HSD1, but not that of 11HSD2, is developmentally regulated in maturing bovine follicles. Thus, 11HSD1 plays a pivotal role in modulating the local glucocorticoid environment in bovine preovulatory follicles.

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References

1. Tetsuka M. Actions of glucocorticoid and their regulatory mechanisms in the ovary. *Anim Sci J* 2007; 78: 112–120.
2. Hsueh AJW, Erickson GF. Glucocorticoid inhibition of FSH-induced estrogen production in cultured rat granulosa cells. *Steroids* 1978; 32: 639–648.
3. Adashi EY, Jones PB, Hsueh AJ. Synergistic effect of glucocorticoids on the stimulation of progesterone production by follicle-stimulating hormone in cultured rat granulosa cells. *Endocrinology* 1981; 109: 1888–1894.
4. Schoonmaker JN, Erickson GF. Glucocorticoid modulation of follicle-stimulating hormone-mediated granulosa cell differentiation. *Endocrinology* 1983; 113: 1356–1363.
5. Kawate N, Inaba T, Mori J. Effects of cortisol on the amounts of estradiol-17β and progesterone secreted and the number of luteinizing hormone receptors in cultured bovine granulosa cells. *Anim Reprod Sci* 1993; 32: 15–25.
6. Spicer LJ, Chamberlain CS. Influence of cortisol on insulin- and insulin-like growth factor 1 (IGF-1)-induced steroid production and on IGF-1 receptors in cultured bovine granulosa cells and thecal cells. *Endocrine* 1998; 9: 153–161.
7. Luck MR. Enhanced secretion of oxytocin from bovine granulosa cells treated with adrenal steroids. *J Reprod Fertil* 1988; 83: 901–907.
8. Danisova A, Sebokova E, Kolena J. Effect of corticosteroids on estradiol and testosterone secretion by granulosa cells in culture. *Exp Clin Endocrinol* 1987; 89: 165–173.
9. Krozowski Z, Li KX, Koyama K, Smith RE, Obeyesekere VR, Stein-Oakley A, Sasano H, Coulter C, Cole T, Sheppard KE. The type I and type II 11β-hydroxysteroid dehydrogenase enzymes. *J Steroid Biochem Mol Biol* 1999; 69: 391–401.
10. Tetsuka M, Thomas FJ, Thomas MJ, Anderson RA, Mason JI, Hillier SG. Differential expression of messenger ribonucleic acids encoding 11β-hydroxysteroid dehydrogenase types 1 and 2 in human granulosa cells. *J Clin Endocrinol Metab* 1997; 82: 2006–2009.
11. Tetsuka M, Milne M, Simpson GE, Hillier SG. Expression of 11β-hydroxysteroid dehydrogenase, glucocorticoid receptor and mineralocorticoid receptor genes in rat ovary. *Biol Reprod* 1999; 60: 330–335.
12. Yong PY, Thong KJ, Andrew R, Walker BR, Hillier SG. Development-related increase in cortisol biosynthesis by human granulosa cells. *J Clin Endocrinol Metab* 2000; 85: 4728–4733.
13. Tetsuka M, Yamamoto S, Hayashida N, Hayashi KG, Hayashi M, Acosta TJ, Miyamoto A. Expression of 11β-hydroxysteroid dehydrogenases in bovine follicle and corpus luteum. *J Endocrinol* 2003; 177: 445–452.
14. Ireland JJ, Murphee RL, Coulson PB. Accuracy of predicting stages of bovine estrous cycle by gross appearance of the corpus luteum. *J Dairy Sci* 1980; 63: 155–160.
15. Murasawa M, Takahashi T, Nishimoto H, Yamamoto S, Hamano S, Tetsuka M. Relationship between ovarian weight and follicular population in heifers. *J Reprod Dev* 2005; 51: 689–693.
16. Nishimoto H, Hamano S, Hill GA, Miyamoto A, Tetsuka M. Classification of bovine follicles based on the concentrations of steroids, glucose and lactate in follicular fluid and the status of accompanying follicles. *J Reprod Dev* 2009; 55: 219–224.
17. Gutierrez CG, Campbell BK, Webb R. Development of a long-term bovine granulosa cell culture system: induction and maintenance of estradiol production, response to follicle- stimulating hormone, and morphological characteristics. *Biol Reprod* 1997; 56: 608–616.
18. Chomczynski P, Sacchi N. Single step method of RNA isolation by acid guanidinium thiocyanate phenol chloroform extraction. *Anal Biochem* 1987; 162: 156–159.
19. Rozen S, Skaletsky HJ. Primer3 on the WWW for general users and for biologist programmers. *In*: Krawetz S, Misener S (eds.), Bioinformatics Methods and Protocols: Methods in Molecular Biology. Totowa, NJ: Humana Press; 2000: 365–386.
20. Miyamoto A, Okuda K, Schweigert FJ, Schams D. Effects of basic fibroblast growth factor, transforming growth factor- beta and nerve growth factor on the secretory function of the bovine corpus luteum *in vitro*. *J Endocrinol* 1992; 135: 103–114.
21. Leroy JL, Vanholder T, Delanghe JR, Opsomer G, Van Soom A, Bols PE, de Kruif A. Metabolite and ionic composition of follicular fluid from different-sized follicles and their relationship to serum concentrations in dairy cows. *Anim Reprod Sci* 2004; 80: 201–211.
22. Lefcourt AM, Bitman J, Kahl S, Wood DL. Circadian and ultradian rhythms of peripheral cortisol concentrations in lactating dairy cows. *J Dairy Sci* 1993; 76: 2607–2612.
23. Dobson H, Smith RF. What is stress, and how does it affect reproduction? *Anim Reprod Sci* 2000; 60–61: 743–752.
24. Sunak N, Green DF, Abeydeera LR, Thurston LM, Michael AE. Implication of cortisol and 11β-hydroxysteroid dehydrogenase enzymes in the development of porcine (*Sus scrofa domestica*) ovarian follicles and cysts. *Reproduction* 2007; 133: 1149–1158.
25. Tomlinson JW, Walker EA, Bujalska IJ, Draper N, Lavery GG, Cooper MS, Hewison M, Stewart PM. 11β-hydroxysteroid dehydrogenase type 1: a tissue-specific regulator of glucocorticoid response. *Endocr Rev* 2004; 25: 831–866.
26. Ge RS, Hardy MP. Initial predominance of the oxidative activity of type I 11β-hydroxysteroid dehydrogenase in primary rat Leydig cells and transfected cell lines. *J Androl* 2000; 21: 303–310.
27. Draper N, Walker EA, Bujalska IJ, Tomlinson JW, Chalder SM, Arlt W, Lavery GG, Bedendo O, Ray DW, Laing I, Malunowicz E, White PC, Hewison M, Mason PJ, Connell JM, Shackleton CH, Stewart PM. Mutations in the genes encoding 11β-hydroxysteroid dehydrogenase type 1 and hexose-6-phosphate dehydrogenase interact to cause cortisone reductase deficiency. *Nat Genet* 2003; 34: 434–439.
28. Michael AE, Thurston LM, Rae MT. Glucocorticoid metabolism and reproduction: a tale of two enzymes. *Reproduction* 2003; 126: 425–441.
29. Atanasov AG, Nashev LG, Schweizer RA, Frick C, Odermatt A. Hexose-6-phosphate dehydrogenase determines the reaction direction of 11β-hydroxysteroid dehydrogenase type 1 as an oxoreductase. *FEBS Lett* 2004; 571: 129–133.
30. McCormick KL, Wang X, Mick GJ. Evidence that the 11β-hydroxysteroid dehydrogenase (11β-HSD1) is regulated by pentose pathway flux. Studies in rat adipocytes and microsomes. *J Biol Chem* 2006; 281: 341–347.
31. White PC, Rogoff D, McMillan DR, Lavery GG. Hexose 6-phosphate dehydrogenase (H6PD) and corticosteroid metabolism. *Mol Cell Endocrinol* 2007; 265–266: 89–92.
32. Ge RS, Dong Q, Niu EM, Sottas CM, Hardy DO, Catterall JF, Latif SA, Morris DJ, Hardy MP. 11β-Hydroxysteroid dehydrogenase 2 in rat leydig cells: its role in blunting glucocorticoid action at physiological levels of substrate. *Endocrinology* 2005; 146: 2657–2664.
33. Yang K, Langlois DA, Campbell LE, Challis JR, Krkosek M, Yu M. Cellular localization and developmental regulation of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) gene expression in the ovine placenta. *Placenta* 1997; 18: 503–509.
34. Tian XC, Berndtson AK, Fortune JE. Differentiation of bovine preovulatory follicles during the follicular phase is associated with increases in messenger ribonucleic acid for cytochrome P450 side-chain cleavage, 3 beta-hydroxysteroid dehydrogenase, and P450 17 alpha-hydroxylase, but not P450 aromatase. *Endocrinology* 1995; 136: 5102–5110.
35. Tetsuka M, Haines LC, Milne M, Simpson GE, Hillier SG. Regulation of 11β-hydroxysteroid dehydrogenase type 1 gene expression by LH and interleukin-1beta in cultured rat granulosa cells. *J Endocrinol* 1999; 163: 417–423.
36. Komar CM, Berndtson AK, Evans AC, Fortune JE. Decline in circulating estradiol during the periovulatory period is correlated with decreases in estradiol and androgen, and in messenger RNA for p450 aromatase and p450 17α-hydroxylase, in bovine preovulatory follicles. *Biol Reprod* 2001; 64: 1797–1805.
37. Acosta TJ, Tetsuka M, Matsui M, Shimizu T, Berisha B, Schams D, Miyamoto A. *In vivo* evidence that local cortisol production increases in the preovulatory follicle of the cow. *J Reprod Dev* 2005; 51: 483–489.
38. Thurston LM, Abayasekara DR, Michael AE. 11β-Hydroxysteroid dehydrogenase expression and activities in bovine granulosa cells and corpora lutea implicate corticosteroids in bovine ovarian physiology. *J Endocrinol* 2007; 193: 299–310.
39. Burke CR, Cardenas H, Mussard ML, Gasser CL, Day ML. Steroidogenic changes and steady state amount of messenger RNA encoding steroidogenic enzymes, gonadotropin receptors and cell-death signalling in the dominant ovarian follicle during estradiol-induced atresia in cattle. *Anim Reprod Sci* 2007; 99: 244–257.
40. Bao B, Garverick HA. Expression of steroidogenic enzyme and gonadotropin receptor genes in bovine follicles during ovarian follicular waves: a review. *J Anim Sci* 1998; 76: 1903–1921.