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—Original Article—

Expression of genes encoding mineralocorticoid biosynthetic enzymes and the mineralocorticoid receptor, and levels of mineralocorticoids in the bovine follicle and corpus luteum

Memory MUKANGWA[1)], Koki TAKIZAWA[1)], You AOKI[1)], Seizo HAMANO[2)] and Masafumi TETSUKA[1)]

[1)]*Department of Life and Food Science, Obihiro University of Agriculture and Veterinary Medicine, Hokkaido 080-8555, Japan*

[2)]*Maebashi Institute of Animal Science, Livestock Improvement Association of Japan Inc., Gunma 371-0121, Japan*

Abstract. Unlike sex steroids, mineralocorticoids have attracted limited attention in ovarian physiology. Recent studies on primates have indicated possible local synthesis and action of mineralocorticoids in the ovary. Here, we examined developmental changes in the levels of mineralocorticoids and expression of genes encoding their biosynthetic enzymes and receptor in the bovine ovary. The follicles and corpora lutea (CL) were collected from F1 heifers. Expression levels of 21α-hydroxylase (*CYP21A2*), 11β-hydroxylase-1 (*CYP11B1*), and the mineralocorticoid receptor (*NR3C2*) in granulosa cells (GC), thecal layers (TL), and CL tissues were quantified by real-time PCR, whereas mineralocorticoids in the follicular fluid were measured by enzyme immunoassay (EIA). TL and GC expressed *CYP21A2* and *NR3C2*, whereas *CYP11B1* was expressed at very low or undetectable levels. The expression levels of these genes were not significantly different among small/large and healthy/atretic follicles but were higher in TL than in GC. *CYP21A2* and *NR3C2* were expressed in all CL stages with higher expression observed in the mid-stage. *CYP11B1* expression was only apparent in the mid-stage CL. Aldosterone was detected in all follicles, and its concentration was not significantly different among the follicular groups. In paired large-healthy/atretic follicles, the concentration of deoxycorticosterone, a precursor of aldosterone, was approximately ten-fold higher than that of aldosterone and not significantly different between healthy and atretic follicles. In conclusion, the presence of mineralocorticoids and expression of *NR3C2* in the bovine follicle together with the developmental change in the expression of *CYP21A2*, *CYP11B1,* and *NR3C2* in the CL suggest possible endocrine/paracrine/autocrine roles of mineralocorticoids in the bovine ovary.

Key words: Aldosterone, Corpus luteum, Follicle, Mineralocorticoid, Mineralocorticoid receptor

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Mineralocorticoids belong to the C21 corticosteroids produced in the adrenal cortex and play a crucial role in controlling electrolyte and fluid balance in target organs such as the kidney. Mineralocorticoids are synthesized from progesterone (P4) through a series of reactions. In the first step, mediated by 21-hydroxylase (CYP21A2), P4 is converted to deoxycorticosterone (DOC), an active but less potent mineralocorticoid. Subsequently, DOC is converted to corticosterone through 11β-hydroxylation, which in turn, through 18-hydroxylation and 18-methyl oxidation, is converted into the prime mineralocorticoid – aldosterone (Aldo). In primates and rodents, the last two reactions are mediated by 11β-hydroxylase-1 (CYP11B1) and 11β-hydroxylase-2 (CYP11B2), respectively [1–5], whereas in livestock species such as cattle, sheep, and pigs, CYP11B1 mediates both these processes [6–8]. Therefore, the expression of these enzymes is a prerequisite for local mineralocorticoid synthesis in an organ.

Mineralocorticoids are classically thought to be produced only in the adrenal cortex; however, evidence indicating extra-adrenal mineralocorticoid production has accumulated in recent years [9]. Likewise, the ovary has been shown to produce mineralocorticoids in various species. In macaques, cultured granulosa cells (GC) exposed to human chorionic gonadotropin (hCG) express CYP21A2 and produce DOC [10]. In cattle, GC and theca cells were shown to express functional CYP11B1 [11]. In humans, concentrations of Aldo and its precursor corticosterone in preovulatory follicles stimulated by an ovulatory dose of hCG are higher than those in the plasma [12]. In addition, luteinized GC have been shown to express CYP21A2 [13]. In addition, the presence of the mineralocorticoid receptor (NR3C2) has been reported in the ovary of rats [14, 15], macaques [10], cattle [16], and humans [12, 13]. Altogether, these results indicate that the ovary is constantly exposed to circulating and possibly locally synthesized mineralocorticoids. Despite these observations, only a few studies have examined the effects of mineralocorticoids on ovarian functions. In macaques, Fru and co-workers demonstrated that locally synthesized DOC acts through NR3C2 to stimulate P4 production in cultured granulosa cells [10]. Recently, we showed that Aldo enhances bovine oocyte maturation [17]. These results

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Correspondence: M Tetsuka (e-mail: mtetsuka@obihiro.ac.jp)

indicate that mineralocorticoids act not only as endocrine factors but also as autocrine/paracrine factors to regulate ovarian physiology.

To our knowledge, developmental changes in the expression of mineralocorticoid biosynthetic enzymes and NR3C2 have not been reported in the bovine follicle and corpus luteum (CL). Therefore, in this study, we examined the expression levels of *CYP21A2*, *CYP11B1,* and *NR3C2* in bovine follicles and CL of various physiological states and developmental stages. Moreover, we examined the concentrations of the corticosteroids DOC, Aldo, and cortisol together with those of the prime ovarian steroids estradiol (E2) and P4 in the follicular fluid collected from healthy and atretic follicles.

Materials and Methods

Sample collection and preparation

Pairs of ovaries were collected from Holstein-Japanese Black F1 heifers at a local slaughterhouse. The ovaries were kept on ice and transported to the laboratory within 30 min of slaughter. The ovaries were macroscopically inspected, and the number and size of the follicles and corpora lutea (CL) were recorded. The follicular fluid (FF) was aspirated from the follicles using a disposable syringe fitted with a 20G needle, and its weight was recorded [18]. The aspirated follicles and CL were dissected from ovaries and stored in RNAlater (Ambion, Austin, TX, USA) and kept at −30°C until further preparation of the samples.

Granulosa cells (GC) were carefully scraped off from follicular walls using a spatula, pelleted, and lysed with TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA). The follicular walls were further cleared of the remaining GC and stroma to obtain thecal layers (TL) [18]. The TL were cut into small pieces and kept in TRIzol Reagent at −30°C until RNA extraction. The adrenal gland and kidney were also harvested from a Holstein cow to be used as positive controls in mRNA analysis and kept in RNAlater at −30°C until RNA extraction.

Corpus luteum and follicle classification

The CL were macroscopically examined for color and vascularity and further classified into four stages (luteal phase: stages I–III; follicular phase: stage IV) according to the criteria reported by Ireland *et al*. [19]. Diameters of the follicles were estimated based on the weight of the follicular fluid using the equation $y = 12.96x^{0.31}$, where y is the diameter of the follicle (mm) and x is the weight of the follicular fluid (g) [20]. Based on the relative concentrations of estradiol (E2) and progesterone (P4) in the follicular fluid, all follicles were initially classified as healthy (E2/P4 ≥ 1) and atretic (E2/P4 < 1) as reported by Nishimoto *et al*. [21]. The follicles were further classified into seven groups based on the diameter (small: < 8.5 mm or large: ≥ 8.5 mm) and stage and status of the accompanying CL and follicles, respectively [21]. Accordingly, large healthy follicles were classified either as dominant (DF) or preovulatory follicles (POF), whereas large atretic follicles were classified as early atretic (EAF), mid-atretic (MAF), or late atretic follicles (LAF). Small follicles were classified as small growing follicles (SGF) or small atretic follicles (SAF) (Table 1).

Total RNA extraction and cDNA synthesis

Total RNA was extracted from GC, TL, CL, and positive control samples using TRIzol Reagent according to the manufacturer's instruction. Pelleted RNA was dissolved in THE RNA Storage Solution (Thermo Fisher Scientific) at a concentration of 1 µg/µl and kept at −80°C. Removal of contaminating genomic DNA and cDNA synthesis were performed by using the QuantiTect Reverse Transcription kit (Qiagen, Hilden, Germany) with 2 µg RNA per sample according to the manufacturer's instruction.

Quantitative PCR

The levels of mRNAs encoding CYP21A2, CYP11B1, NR3C2, 11β-hydroxysteroid dehydrogenase type 2 (HSD11B2), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified by real-time quantitative PCR using LightCycler Nano (Roche Diagnostics, Mannheim, Germany) and FastStart Essential DNA Green Master (Roche). The primers were designed using the National Centre for Biotechnological Information (NCBI) primer designing tool Primer-BLAST [22] based on the reported bovine sequences (Table 2), and were purchased from Sigma. The amplification program consisted of an initial activation for 10 min at 95°C, followed by 45 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 10 sec, and extension at 72°C for 15 sec. The authenticity of the PCR products was verified by monitoring the melting curve and the size of the PCR products. All primer pairs produced a clear single peak melting curve profile and amplicons at the expected size. A positive control (adrenal gland for *CYP21A2*, *CYP11B1,* and *GAPDH*; kidney for *NR3C2* and *HSD11B2*) and a negative control (dH_2O) were included in each PCR run. Amplifications were performed in duplicates. For all quantifications, the intra- and inter-assay coefficients of variation (CV) were less than 10% and 15%, respectively. For normalization of mRNA data, GAPDH was used as an internal control. Relative gene expression was calculated by the $2^{-\Delta Ct}$ method.

Table 1. Follicular classification criteria

Follicular class	Size (mm)	E2/P4 (w/w)	Accompanying follicles	Corpora lutea (CL) stage
Small atretic (SAF)	< 8.5	< 1	DF/POF	I–IV
Small growing (SGF)	< 8.5	≥ 1	No DF/POF	I–III
Dominant (DF)	≥ 8.5	≥ 1	SAF/MAF	I–III
Preovulatory (POF)	≥ 8.5	≥ 1	LAF	IV
Early atretic (EAF)	≥ 8.5	< 1	SGF, no DF/POF	II–III
Mid-atretic (MAF)	≥ 8.5	< 1	DF	II–III
Late atretic (LAF)	≥ 8.5	< 1	EAF/POF	III–IV

Table 2. Primers used for quantitative real-time PCR

Gene	Primer	Sequence (5′-3′)	GenBank no.	Position
CYP21A2	Forward	CCTGGAGCTGTTCGTGGTG	NM_174639.1	1442–1460
	Reverse	GCTGGACCTTGAGGTTGACA	NM_174639.1	1544–1563
CYP11B1	Forward	CCATCGAAGCCAGCACCTTA	NM_174638.3	592–611
	Reverse	CTGGGCACAAACATGAGCTG	NM_174638.3	711–730
NR3C2	Forward	CCGTACCCACGGAGCAGTC	NM_001191349.2	216–235
	Reverse	CTTGCTGGAGGCAAGGGAGT	NM_001191349.2	98–116
HSD11B2	Forward	CGAGCACTTGAATGGGCAGTT	AF074706	1033–1053
	Reverse	CCTGGGTAATAGCGGCGGAGT	AF074706	1135–1155
GAPDH	Forward	GCGCCAAGAGGGTCATCATC	NM_001034034.2	409–428
	Reverse	AGTCCCTCCACGATGCCAAA	NM_001034034.2	567–586

Steroid assay

Commercial enzyme immunoassay (EIA) kits were obtained from Cayman Chemical Company (Ann Arbor, MI, USA) for measuring E2, P4, Aldo, and cortisol, and from MyBioSource (San Diego, CA, USA) for measuring DOC. For measuring E2, P4, and cortisol, the follicular fluid samples were diluted with EIA buffer at dilution factors of 1:100–1:10,000, and EIAs were performed according to the manufacturers' instructions. Undiluted follicular fluid samples were used to measure Aldo, whereas, for DOC measurement, the samples were diluted with PBS at a dilution factor of 1:5. In order to construct standard curves for Aldo assays, a steroid-free follicular fluid was used for standard preparations. For this purpose, steroids were removed from pooled follicular fluid by incubating the fluid with activated charcoal (Sigma) according to the manufacturer's instructions. E2, P4, and Aldo were measured in all samples, whereas DOC and cortisol were measured only in paired samples. The intra- and inter-assay CV were 6.1% and 9.8% for E2, 8.0%, and 17.1% for P4, and 5.2% and 7.3% for Aldo, respectively. In paired samples, the intra-assay CV for DOC and cortisol were 9.3% and 3.4%, respectively.

Statistical analysis

Data analysis was performed by the software R 3.5.2 for Windows (R Studio Team, 2016). After failing the normality test, the difference among groups was examined by the Kruskal test, followed by the Wilcoxon rank-sum test. In order to examine the differences between the paired follicular samples, the Wilcoxon signed-rank test was used. The results of data analyses were expressed as medians (interquartile range), and differences between the groups were considered significant at $P < 0.05$. The relationship between the concentration of cortisol and expression of *HSD11B2* was analyzed using Spearman's rank correlation coefficient.

Results

Concentrations of E2, P4, and Aldo in the follicular fluid of classified follicles

Follicular fluid samples from 56 classified follicles belonging to 35 animals were analyzed for steroid concentrations. There were clear and significant differences among follicular groups in E2 and E2/P4 ratio except between DF and EAF. There was no significant difference in the P4 concentration, although it substantially increased in LAF (Table 3). The median concentration of Aldo in all the follicular fluid samples was 95.2 (67.5–158.5) pg/ml, and, at most, three orders of magnitude lower than that of its precursor P4. Moreover, the Aldo levels were not significantly different among the follicular categories (Table 3).

Gene expression of CYP21A2, CYP11B1, and NR3C2 in the classified follicles

CYP21A2 was expressed in the TL and GC (Fig. 1A). The expres-

Table 3. Characteristics of follicles

Follicular category (n)	Size (mm)	E2 (ng/ml)	P4 (ng/ml)	E2/P4 ratio	Aldo (pg/ml)
SGF (8)	7.9 (7.6–8.0) [a]	61.1 (23.6–97.0) [a]	23.0 (14.4–28.1) [a]	3.3 (2.0–4.2) [a]	105.7 (81.4–118.2)
DF (7)	13.4 (12.5–13.9) [b]	90.0 (53.1–111.5) [a]	46.7 (41.8–77.3) [a,b]	1.3 (1.1–3.7) [a,d]	80.2 (40.0–113.9)
POF (8)	15.9 (14.8–17.3) [b]	773.4 (511.1–1471.5) [b]	60.8 (48.0–68.5) [a,b]	15.4 (9.9–21.5) [b]	127.8 (91.8–161.4)
SAF (8)	7.9 (7.3–8.1) [a]	3.0 (2.4–5.7) [c]	258.0 (127.7–340.7) [b]	0.0 (0.0–0.0) [c,d]	196.3 (114.3–216.5)
EAF (8)	13.4 (11.1–13.9) [b]	22.3 (15.4–36.4) [a]	113.4 (33.5–241.3) [a,b]	0.4(0.1–0.7) [d]	123.7 (66.9–182.7)
MAF (9)	13.2 (11.1–13.5) [b]	3.0 (2.6–5.6) [c]	60.1 (30.7–137.0) [a,b]	0.0 (0.0–0.2) [c,d]	71.6 (67.4–94.0)
LAF (8)	12.6 (11.3–13.7) [b]	1.8 (1.4–2.4) [c]	424.9 (190.5–589.0) [b]	0.0 (0.0–0.0) [c]	89.3 (41.8–153.4)

All the values are shown as medians (interquartile range). Values with different superscripts within a column are significantly different at $P < 0.05$.
SGF, small growing; DF, dominant; POF, preovulatory; SAF, small atretic; EAF, early atretic; MAF, mid-atretic; LAF, late atretic.

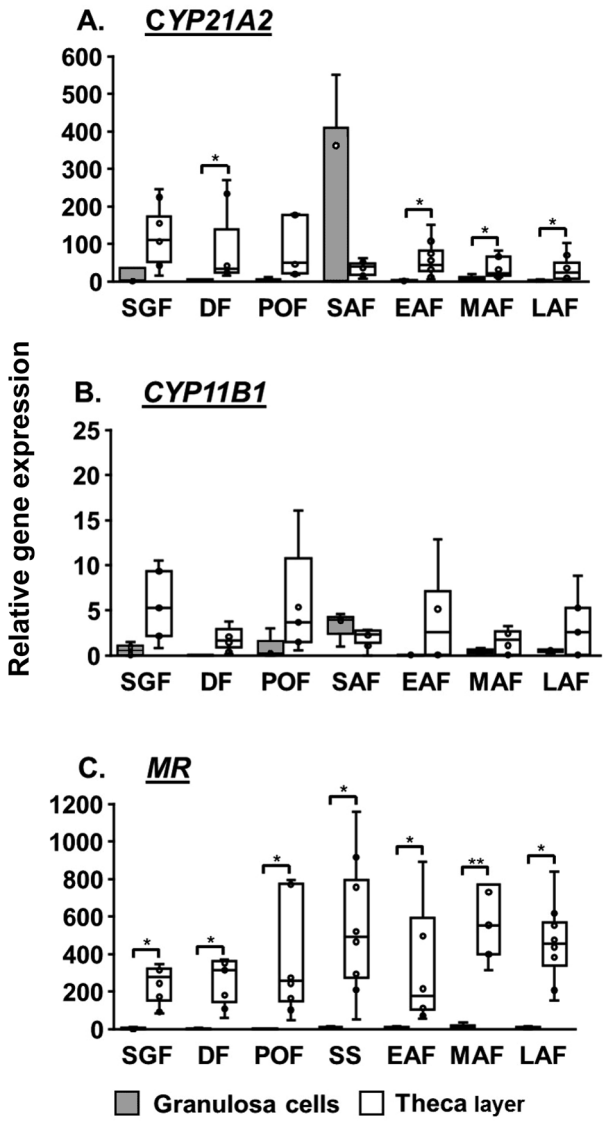

Fig. 1. Relative expression of *CYP21A2* (A), *CYP11B1* (B), and *NR3C2* (C) in bovine granulosa cells (GC) and theca layer (TL) during follicular maturation and atresia. SGF, small growing follicle; DF, dominant follicle; POF, preovulatory follicle; SAF, small atretic follicle; EAF, early atretic follicle; MAF, mid-atretic follicle; LAF, late atretic follicle. Values are shown as medians and interquartile ranges with bars displaying minimum and maximum values. * $P < 0.05$, ** $P < 0.01$.

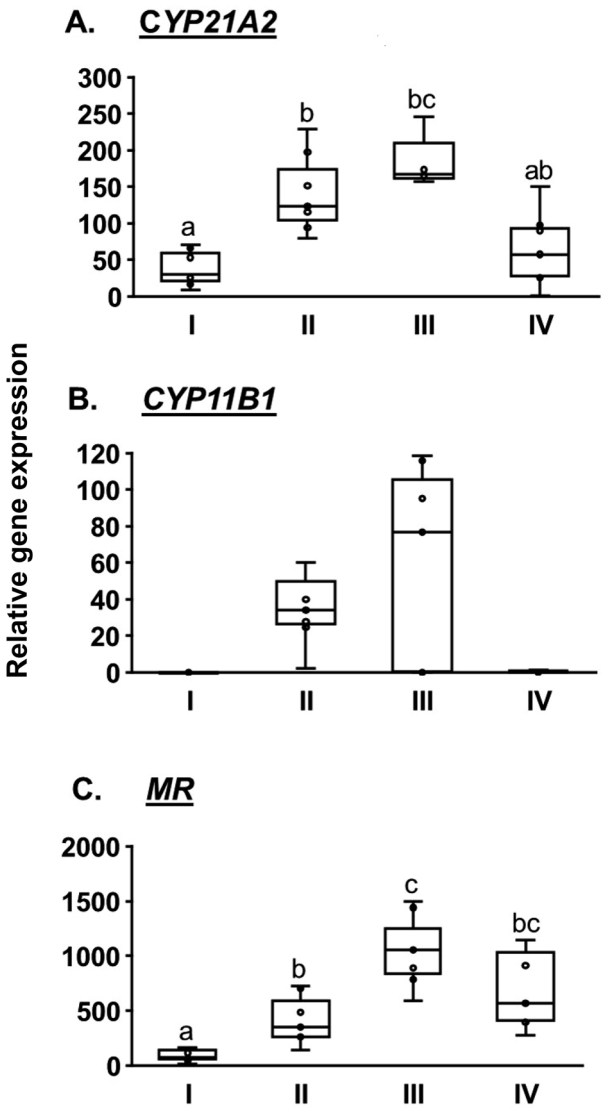

Fig. 2. Relative expression of *CYP21A2* (A), *CYP11B1* (B), and *NR3C2* (C) in bovine corpora lutea (CL) classified into four developmental stages (I–IV). Values are shown as medians and interquartile ranges with bars displaying minimum and maximum values. Different letters indicate significant differences ($^{a, b, c}$ $P < 0.05$).

sion levels were not significantly different among the follicular classes but were much higher in the TL than in the GC. The expression of *CYP11B1* was relatively lower and indiscernible in some of the GC and TL follicles (Fig. 1B). Both the TL and GC expressed *NR3C2,* and the expression was higher in the TL than in the GC. No significant difference was observed among follicular classes in the expression of *NR3C2* in the GC and TL (Fig. 1C).

Gene expression of CYP21A2, CYP11B1, and NR3C2 in the CL

Altogether, 28 CL from 28 animals were analyzed for gene expression. The expression pattern of *CYP21A2* followed the production profile of P4, with the highest expression at stage III and a rapid decrease at stage IV (Fig. 2A). The expression levels at stage II and III were significantly higher than at stage I ($P < 0.05$). The expression levels of *CYP11B1* were undetectable at stages I and IV. The levels increased at stages II and III, although no significant difference was observed among the stages (Fig. 2B). The expression of *NR3C2* increased at stage II and reached the highest value at stage III, then declined at stage IV. The expression was significantly higher at

stages II–IV than at stage I ($P < 0.05$; Fig. 2C).

Concentrations of DOC, Aldo, and cortisol in large paired healthy and atretic follicles

We compared the concentrations of steroids between large healthy (DF and POF) and atretic (MAF and LAF) follicles collected in pairs from 14 animals. Despite apparent differences in the concentrations of E2 and P4 ($P < 0.01$), no difference was observed in the concentrations of Aldo and DOC. In contrast, the concentration of cortisol was significantly lower ($P < 0.05$) in the atretic follicles (Fig. 3). The concentration of Aldo in these follicles was approximately 10- and 1000-fold lower than those of its precursors DOC and P4, respectively, and nearly 150-fold lower than that of cortisol.

Relationship between the concentration of cortisol and gene expression of HSD11B2

The GC and TL of the paired follicles were examined for expression of *HSD11B2,* the enzyme that converts cortisol to cortisone. The gene expression was significantly higher in the atretic follicles than in the healthy follicles in both GC and TL ($P < 0.05$) (Fig. 4). Although the gene expression was significantly higher in TL than in GC of the healthy follicles ($P < 0.01$), the difference was not significantly different from that observed in atretic follicles. The follicular cortisol concentration strongly and negatively correlated with *HSD11B2* expression in TL ($\rho = -0.69$, $P < 0.001$).

Discussion

In cattle, sheep, and pigs, mineralocorticoids are synthesized from P4 through a series of reactions mediated by *CYP21A2* and *CYP11B1*. Therefore, the expression of these enzymes is a prerequisite for *de novo* synthesis of mineralocorticoids in the bovine follicle and CL. In this study, we have shown that the bovine follicle expresses *CYP21A2*. However, the expression of *CYP11B1* was much lower than that of *CYP21A2* and hardly discernible in some follicular samples.

The follicles in humans [13], macaques [10], cattle [11], horses [23], and mice [24] have been shown to express the mineralocorticoid biosynthetic enzymes; however, the expression pattern varied among these species. In macaques, GC primed with FSH expressed *CYP21A2*, whereas *CYP11B1* and *CYP11B2*, the enzymes mediating the final steps of Aldo synthesis in primates, were undetected before and after an ovulatory stimulus with hCG [10]. Similarly, human GC primed with FSH and exposed to hCG expressed *CYP21A2* [13]. Conversely, an ovulatory stimulus with LH/hCG induced the expression of *CYP11B1* in murine ovaries [24]. Recently, Amweg *et al*. (2017) demonstrated the expression of *CYP11B1* in the bovine granulosa and theca cells harvested from small (< 5 mm), medium (5–10 mm), and large (> 10 mm) non-atretic follicles [11]. The authors also reported that cultured follicular walls of large antral follicles produced a substantial amount of cortisol, and that ACTH stimulated its synthesis. The synthesis was suppressed by metyrapone, an inhibitor of CYP11B1 [11, 25]. In these studies, neither 11-deoxycortisol, the immediate precursor of cortisol, nor serum, which may contain 11-deoxycortisol, was added to the medium. Thus, these results indicate that the bovine follicle is capable of synthesizing cortisol from *de novo* synthesized P4. The reason why *CYP11B1* could not be detected in some follicular samples is not known. However, the *CYP11B1* expression reported by Amweg *et al*. (2017) may also contain very low levels of gene expression as indicated by the large error bars (SD appeared to be

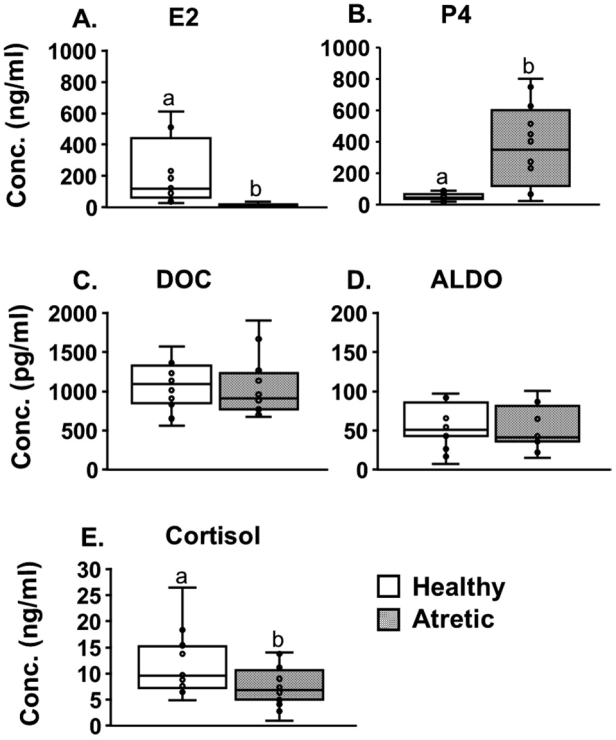

Fig. 3. Concentrations (Conc.) of estradiol (E2) (A), progesterone (P4) (B), deoxycorticosterone (DOC) (C), aldosterone (Aldo) (D), and cortisol (E) in the follicular fluid collected from paired healthy and atretic follicles. Values are shown as medians and interquartile ranges with bars displaying minimum and maximum values. Different letters indicate significant differences ($^{a, b}$ $P < 0.05$, $^{a, c}$ $P < 0.01$).

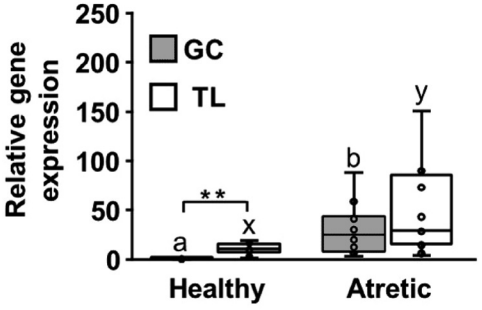

Fig. 4. Relative expression of *HSD11B2* in bovine granulosa cells (GC) and theca layer (TL) from paired large (> 10 mm) follicles (healthy and atretic) within an animal. Values are shown as medians and interquartile ranges with bars displaying minimum and maximum values. Different letters within the same tissue indicate significant differences ($^{a, b; x, y}$ $P < 0.05$). ** $P < 0.01$.

far larger than mean values in some groups) [11].

In the present study, despite the significant difference in size and physiological status among the follicular categories, no significant difference was observed in the gene expression of *CYP21A2* and *CYP11B1*. This lack of difference is due to significant variations in gene expression, as observed by Amweg *et al*. (2017) [11]. Likewise, Aldo concentrations in the follicular fluid varied substantially and did not differ among the follicular categories. These results imply that the concentrations of Aldo in the follicular fluid are not affected by the status of follicles but by the circulating levels of Aldo. To verify this hypothesis, we collected healthy and atretic follicles in pairs from 14 cows. While E2 and P4 levels were different between the healthy and atretic follicles, DOC and Aldo levels did not differ between these follicles, indicating that follicular levels of DOC and Aldo are not affected by the physiological status of the follicle.

To our knowledge, only one study has reported mineralocorticoid concentrations in the bovine follicular fluid to date [26]. Our results obtained by EIA are comparable to their findings, obtained through high-performance liquid chromatography-tandem mass spectrometry for Aldo, but ten-fold lower for DOC. In the present study, we could not collect blood samples matching the follicular samples due to sampling limitations at the slaughterhouse. This factor prevented us from comparing the concentrations of mineralocorticoids between the serum and the follicular fluid, as done previously in humans [12]. Nevertheless, the follicular fluid concentrations of DOC and Aldo in the present study are somewhat lower or comparable to serum concentrations reported for DOC in pregnant heifers (1–70 ng/ml) [27], and for Aldo in lactating cows and heifers (approximately 100 pg/ml) [28]. Altogether, these results suggest that DOC and Aldo in the bovine follicular fluid are mainly, if not completely, derived from the circulation. However, the appreciable levels of *CYP21A2* expression observed in this study, and activity of *CYP11B1* reported by Amweg *et al*. (2017), indicate possible local synthesis of mineralocorticoids in the bovine follicle [11]. In macaques, the concentrations of DOC in the follicular fluid as well as the synthesis of DOC by cultured GC sharply increased after hCG treatment [10]. Likewise, the concentrations of Aldo in the follicular fluid were reported to be much higher than those in the plasma of women who received an ovulatory hCG stimulus [12]. As all our samples were collected before the LH-surge, we may have missed the increase in mineralocorticoid synthesis in the POF triggered by the LH-surge. A further study is necessary to elucidate this possibility.

As CYP21A2 and CYP11B1 also mediate the production of cortisol, we measured the concentrations of cortisol in the paired follicles. Unlike mineralocorticoids, the cortisol level was significantly lower in the atretic follicles compared to that in the healthy follicles. This difference in cortisol levels appeared to be caused by the increased expression of *HSD11B2*, a dehydrogenase that converts cortisol into inert cortisone, in GC and TL of atretic follicles [18, present study] rather than by changes in the expression levels of *CYP21A2* and *CYP11B1*. The highly significant negative correlation observed in this study between the concentration of cortisol and the expression of *HSD11B2* supports this hypothesis.

The presence of mineralocorticoids and the expression of *NR3C2* in the bovine follicle indicate that mineralocorticoids may act as a local regulator of follicular functions. As mentioned above, hCG was shown to stimulate DOC production in cultured macaque GC [10]. In that study, P4 production was also stimulated by hCG, and this effect was almost completely negated by the concomitant treatment with spironolactone, an NR3C2 antagonist [10]. These results indicate that the locally produced DOC acts through NR3C2, as an autocrine factor, to stimulate P4 production.

Sneeringer *et al*. (2011) also suggested that in humans, an increase in the follicular Aldo before ovulation might stimulate oocyte maturation through NR3C2 expressed by the oocyte [12]. The bovine oocyte also expresses *NR3C2* [16] as well as *HSD11B2*, the dehydrogenase that protects NR3C2 from non-specific activation by cortisol [29]. Co-expression of these genes is a typical feature of mineralocorticoid target cells. Thus, we tested this possibility by adding Aldo to the bovine *in vitro* maturation medium. Aldo enhanced oocyte maturation, and this effect was reversed by eplerenone, a specific NR3C2 antagonist [17]. These results imply that Aldo, as an endocrine, and possibly as a paracrine/autocrine factor, plays an important role in the bovine follicle destined for ovulation.

This study also demonstrated that the expression of *CYP21A2* and *NR3C2* was much higher in TL than in GC. This notion indicates that the synthesis and action of mineralocorticoids may occur at the outer layer of follicles where the vascular network resides. The bovine theca cells have been shown to express two types of receptors for angiotensin II (Ang II) [30], a vital factor of the renin-angiotensin system, which is a well-known regulatory system of Aldo synthesis in the adrenal gland. For example, Ang II, acting through Ang II receptor type 1, increases the expression of CYP11B2, and thereby stimulates Aldo production in an adrenocortical cell line [31]. The expression of Ang II receptor type 2 was also increased by the GnRH treatment of the bovine theca cells in the preovulatory follicles [32]. Likewise, an ovulatory dose of gonadotropin has been shown to increase the levels of Ang II in the follicular fluid [33]. These findings parallel the gonadotropin-induced increase in follicular mineralocorticoids and *CYP21A2* expression observed in primates [10, 12, 13]. In summary, the expression of *CYP21A2* and *NR3C2* in the presence of renin-angiotensin system in the bovine follicle suggests a localized regulation system for the synthesis of mineralocorticoids.

The CL is renowned for producing P4 in a developmentally regulated fashion, with the highest production at stage III (fully developed CL), followed by a rapid reduction at stage IV (regressing CL). In the present study, the expression of *CYP21A2* and *NR3C2* paralleled the P4 production pattern, whereas *CYP11B1* was only temporarily expressed at stages II and III. These results suggest developmentally regulated synthesis and action of mineralocorticoids in the bovine CL. As mentioned above, an ovulatory stimulus; for example, gonadotropin surge or equivalent hCG treatment, appears to induce mineralocorticoid synthesis in the follicle. In this regard, increases in the expression of mineralocorticoid biosynthetic enzymes along with the development of CL might be triggered by the LH-surge and maintained by the pulsatile secretion of LH thereafter. If this is the case, locally synthesized mineralocorticoid may play an essential role in the luteinizing process, such as increasing P4 synthesis, as reported in the cultured macaque GC [10].

In conclusion, bovine follicles contain appreciable levels of mineralocorticoids, irrespective of their size and physiological status. Although they are likely to be derived from circulation, the expression

of *CYP21A2* and *CYP11B1* in the follicle implies possible *de novo* synthesis of mineralocorticoids. The expression of *NR3C2* in the follicle also indicates local action of mineralocorticoids. On the other hand, the expression of *CYP21A2*, *CYP11B1,* and *NR3C2* appear to be developmentally regulated in the bovine CL. Collectively, the present results suggest that the bovine ovary is an extra-adrenal organ for the synthesis of mineralocorticoids and a viable target for action.

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