ウシ卵巣における糖質コルチコイドの生理的役割の評価

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はしがき

哺乳動物の卵巣生理に副腎皮質由来の糖質コルチコイドが影響を与えることは、過去多くの動物種で示されてきた。しかし卵巣局所レベルで糖質コルチコイドの作用がどのように調節されているかは明確にされていなかった。近年、ラットおよびヒトの卵巣で糖質コルチコイドの活性化、非活性化を司る２種類の酵素が発現していること、この酵素の発現比率によって卵巣局所の糖質コルチコイドレベルが制御されていることが解明された。

我々はウシ卵巣でも、ヒトやラットと同様、糖質コルチコイドレベルを局所的に調節する機構が存在するとの仮説のもとに、一連の実験を行った。得られた主な結果は次のとおりである。

１．ウシの卵胞や黄体で糖質コルチコイドレセプターと糖質コルチコイドの代謝酵素である２種類の 11β-HSD が発現していること（代謝と受容からなる糖質コルチコイドシステムの存在）。

２．これらの代謝酵素の発現が卵胞発育、黄体形成と退行に伴って変動すること（生理状態の変化に伴う糖質コルチコイド環境の変動）。

３．成熟卵胞における代謝酵素の発現レベルと卵胞液中コルチゾール（活性型糖質コルチコイド）濃度の間に負の相関関係が認められたこと（代謝酵素による糖質コルチコイドの非活性化）。

４．卵胞液中コルチゾールと成熟卵胞の指標であるエストロジェン濃度の間に負の相関関係が認められたこと（糖質コルチゾールによる卵胞成熟の阻害）。

これらの結果から卵巣が体内を循環する糖質コルチコイドにただ曝されているだけではなく、それを代謝することで、合目的的に卵巣内糖質コルチコイド環境を調節している可能性が示唆された。

本研究の期間中に発生した BSE により、予定していた実験のいくつかが実行不可能になったことは残念なことである。しかし本研究の枠内で卵巣における糖質コルチコイド調節機構が一部解明されたことで今後の研究の下地ができたことは意義深い。ウシ等の家畜では糖質コルチコイドがストレス性の卵巣機能不全や卵胞のう腫などの卵巣疾患に関与していることが示唆されている。糖質コルチコイドとその調節機構がこうした症例にどのように関与しているのか解明すべく、今後取り組んで行く所存である。

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研究発表

（1）学会誌等

1. Tetsuka M, Yamamoto S, Hayashida N, Hayashi KG, Hayashi M, Acosta TJ, Miyamoto A. 2003（平成 15 年） Expression of 11β-hydroxysteroid dehydrogenases in bovine follicle and corpus luteum. *Journal of Endocrinology* (in press：http://journals.endocrinology.org/joe/fca/pdf/JOE05183.pdf).

（2）口答発表

1. 山本沙和，手塚雅文，林田典子，林憲悟，林美里，Tomas J Acosta，宮本明夫，福井豊　2001（平成 13 年）ウシ黄体における糖質コルチコイドの代謝と受容　第 94 回　日本繁殖生物学会　2001 年 9 月

2. 林田典子，手塚雅文，鹿内亜季野，山本沙和，松浦淳子，福井豊　2001（平成 13 年）ウシ卵胞細胞および卵子におけるエストロジェンレセプターの発現　第 94 回　日本繁殖生物学会　2001 年 9 月

3. 手塚雅文，山本沙和，林田典子，Tomas J Acosta，林憲悟，林美里，宮本明夫，福井豊　2001（平成 13 年）ウシ顆粒層細胞におけるエストロジェンレセプターmRNA の発現動態　第 94 回　日本繁殖生物学会　2001 年 9 月

4. 手塚雅文，鹿内亜希野，林田典子，Tomas J Acosta，林憲悟，林美里，宮本明夫　2002（平成 14 年）ウシ卵胞内コルチゾールは卵胞のアンドロジェン・エストロジェン合成に影響を与えない？　第95回　日本繁殖生物学会　2002 年 9 月

5. Tetsuka M, Yamamoto S, Hayashida N, Hayashi K, Hayashi M, Acosta TJ, Miyamoto A. 2002（平成 14 年）Expression of 11β-hydroxysteroid dehydrogenases in bovine follicle and corpus luteum.　6th International Symposium on Reproduction in Domestic Ruminants 2002 年 8 月

Expression of 11β-hydroxysteroid dehydrogenases in bovine follicle and corpus luteum

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Abstract

In glucocorticoid target organs, local concentrations of active glucocorticoid are determined by a relative expression of two 11β-HSDs: bi-directional 11β-HSD type1 (11HSD1) that mainly activates cortisone to cortisol, and dehydrogenase 11β-HSD type2 (11HSD2) that inactivates cortisol to cortisone. In this study, we examined the expression of mRNA encoding these two 11β-HSDs in bovine granulosa cells harvested from preovulatory follicles and corpora lutea (CL). Ovaries were obtained from Holstein cows in a local slaughterhouse. Follicles larger than 10 mm in diameter and CL were dissected and follicular fluid and granulosa cells were taken. Corpora lutea were weighed and their stages were morphologically assessed (stage I: Day1-4, stage II: Day5-10, stage III: Day 11-17, stage IV: Day18-20). Follicles were classified into four groups according to their hormonal status (E2:P >1: oestrogen active, E2:P <1: oestrogen inactive) and stages of oestrous cycle (luteal or follicular phase). Total RNA was extracted with phenol-chloroform and subjected to a semi-quantitative RT-PCR for 11HSD1, 11HSD2 and β-actin. Concentrations of steroids in follicular fluid were determined by an EIA. In granulosa cells, only 11HSD1 mRNA was detected. There was a negative correlation between expression of 11HSD1 and concentration of cortisol in follicular fluid ($P<0.05$), indicating 11HSD1 may act as a dehydrogenase in the bovine follicle. Both types of 11β-HSDs were

expressed in CL. The levels of mRNA for the both isozymes were high in the stage I and II, and decreased in the stage III CL. In the stage IV CL, the expression of 11HSD2 but not 11HSD1 mRNA increased. These results indicate that the bovine granulosa cells and CL express 11HSD1 and 11HSD2, and they may play an important physiological role in the bovine ovary through modulating local glucocorticoid environment.

Introduction

The ovary expresses glucocorticoid receptor (GR) and direct action of glucocorticoid on various ovarian functions has been reported in several species (Hsueh & Erickson 1978, Luck 1988, Fitzpatrick & Richards 1991, Kawate *et al.* 1993, Urban *et al.* 1994, Spicer & Chamberlain 1998, Gaytan *et al.* 2002, Yang *et al.* 2002). The ovary does not produce glucocorticoid per se and the access of active glucocorticoid (i.e. cortisol) to GR is regulated by a relative expression of two 11β-HSDs: a bi-directional 11β-HSD type 1 (11HSD1), that mainly activates cortisone to cortisol (11-dehydrocorticosterone to corticosterone in rodents), and a dehydrogenase 11β-HSD type 2 (11HSD2) that inactivates cortisol to cortisone (Krozowski *et al.* 1999). Recently it was shown that the expression of 11HSD1 is dramatically up-regulated while that of 11HSD2 is down-regulated in human and rat follicles during hCG-induced ovulation (Tetsuka *et al.* 1997, 1999). In human, this change in the expression results in a temporal rise in cortisol in follicular fluid (Harlow *et al.* 1997). The physiological importance of this phenomenon is not clear. It is suggested, however, that the increased level of cortisol may enhance oocyte maturation (Fateh *et al.* 1989, Michael *et al.* 1999) or suppress inflammation associated with ovulation, thereby promoting rapid healing of the wound left by follicular rupture (Hillier & Tetsuka 1998). In pregnant rat corpora lutea (CL), the expression of 11HSD2 was shown to be dramatically up-

regulated which was coincident with the rise in circulating glucocorticoid just prior to parturition (Waddell *et al.* 1996). Regarding an anti-luteolytic nature of glucocorticoid, the expression of 11HSD2 appears to be important for the prepartal luteal regression, which is a prerequisite for parturition (Waddell *et al.* 1996). Thus, these results indicate that the magnitude of glucocorticoid action in the ovary is mainly determined by a relative expression of these two 11β-HSDs in these species.

In cattle, glucocorticoid is both stimulatory and inhibitory to ovarian functions. It has been shown to enhance progesterone (P4)(Kawate *et al.* 1993) and oxytocin production (Luck 1988) in cultured granulosa cells, and IGF-stimulated P4 and androstenedione production in cultured thecal cells (Spicer & Chamberlain 1998). Whereas it was shown to inhibit production of oestradiol-17β (E2) and expression of LH-receptor in cultured granulosa cells (Kawate *et al.* 1993). For a healthy follicular development, therefore, it is necessary that the expression of these factors is properly regulated in the face of circulating glucocorticoid.

We hypothesized that the 11HSD is present in bovine preovulatory follicles and CL and may play an important role in promoting follicular maturation and maintaining CL functions through attenuating local levels of glucocorticoid. In this study, we tested this hypothesis by examining the expression of mRNA encoding 11HSD1 and 11HSD2 in bovine granulosa cells harvested

from preovulatory follicles and CL of various physiological stages.

Materials and methods

Collection of tissue samples

Ovaries were obtained from Holstein cows in a local slaughterhouse. The ovaries were kept in pairs in ice cold PBS and brought in the laboratory within one hour after the slaughter. Follicles larger than 10 mm in diameter and CL were dissected from the ovaries. Follicular fluid was aspirated from each follicle, centrifuged briefly at 800g to remove follicular debris and kept at -20°C until steroid hormone assays. Granulosa cells were harvested by gently scraping follicular wall with a spatula and centrifuged them briefly at 800g. The resultant pellet was lysed in ice-cold denaturing solution containing 4M guanidium thiocyanate (Wako Pure Chemical Industories Ltd., Osaka, Japan), 25 mM sodium citrate (Wako), 0.5% (w:v) sarcosyl (Sigma Chemical Co., St. Louis, MO, USA), and 0.1 M β-mercaptoethanol (Kanto Chemical Co. Inc, Tokyo, Japan). The remained follicular wall was washed with PBS to remove as much as granulosa cells and served as a thecal tissue. Corpora lutea were weighed and morphologically assessed their stages to one of four categories (stage I: Day1-4, stage II: Day5-10, stage III: Day 11-17, stage IV: Day18-20) according to Ireland *et al.* (1980). They were sliced into small

pieces and frozen in liquid nitrogen. The follicles were classified into four groups according to their hormonal status (E2:P4 >1: oestrogen active: EA, E2:P4 <1: oestrogen inactive: EI) and the stages of oestrous cycle estimated by the status of accompanied CL (luteal phase: stages I-III or follicular phase: stage IV). Under this criterion, oestrogen active follicles are preovulatory follicles destined to ovulation (follicular phase) or non-ovulatory dominant follicles (luteal phase), while EI follicles in both phases are either subordinate follicles destined to atresia or dominant follicles from the previous follicular wave in static or regressing phase. Of total, 36 preovulatory follicles from 28 cows were used in the present study.

Uterine caruncular and inter-caruncular tissues were also obtained from a non-pregnant Holstein cow in the slaughterhouse. Other tissue samples, including the liver, kidney, adrenal and spleen were obtained from a Holstein steer killed in the university farm. Leukocytes were obtained from pooled blood samples collected from cyclic non-pregnant cows.

RNA Isolation and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Tissue samples were homogenized in the denaturing solution. Total RNA was extracted from granulosa cell lysates and tissue homogenates with phenol-chloroform (Chomczynski & Sacchi 1987). The resultant total RNA was further purified and DNase treated using a SV total RNA

extraction kit (Promega Co., Madison, WI, USA). The total RNA was quantified and purity assessed by a spectrophotometry. The integrity of the RNA was confirmed by a gel electrophoresis to visualize 28S and 18S rRNA. While the RNA samples obtained from granulosa cells were exclusively granulosa origin, those from CL were mixture of RNAs from luteal, endothelial and small amount of blood cells.

Single-strand cDNA was reverse transcribed from total RNA (0.5 μg) using MMLV-reverse transcriptase (Promega) and a oligo dT primer (Promega) at 37°C for 60 min. One twentieth of the resultant cDNA was used for PCR amplifications (26 cycles for actin, 30 cycles for 11β-HSDs) using Taq-DNA polymerase (Promega) in 20μl reaction. Each PCR cycle consisted: 45 sec denaturing at 94°C, 45 sec annealing at 55°C (actin) or 60°C (11β-HSDs), and 60 sec extension at 72°C with the final extension for 10 min. A set of serially diluted total RNA from the liver (11HSD1: 2~500ng), adrenal gland (11HSD2: 2~500ng) or CL (actin: 8~2000ng) was included to each RT-PCR trial to construct a standard curve. The primers for 11β-HSDs and β-actin were designed using an online primer design tool, Primer 3 (Rozen & Skaletsky 2000: http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) based on the bovine sequences for 11HSD2 and β-actin and the ovine sequence for 11HSD1 (Table 1). The amplified

DNA was resolved on 2% agarose gel containing ethidium bromide and visualized under UV illumination. Signal intensities were analyzed by a computerized densitometry (Luminous Imager version 2.0G, Aisin Cosmos RD Co. Ltd., Aichi, Japan) and relative abundance of the target mRNA was estimated using the standard curve. The values were normalized using β-actin as an internal standard. A second set of the RNA samples was subjected to amplifications without the reverse transcriptase to monitor possible contamination of the RNA samples with genomic DNA. No signal was detected in these amplifications. The PCR products were either sequenced (11HSD1: GenBank accession AF548027) or cut by restriction enzymes (11HSD2 and β-actin) and their authenticity were confirmed. The sequenced bovine 11HSD1 cDNA fragment is 554 bp in length and shares 96%, 77% and 63% sequence identity with sheep (P51975), human (XP005516) and rat (NP 058776) 11HSD1 respectively.

Steroid hormone assay

Concentrations of P_4, E_2 and cortisol in the follicular fluid samples were determined by an enzyme immunoassay (EIA) as mentioned previously (Miyamoto *et al.* 1992, Wijayagunawardane *et al.* 1998, Acosta *et al.* 2002).

The range of standard curve and the ED_{50} of the assay were 0.8-80 ng/ml and 2.5 ng/ml for

cortisol, 2-2,000 pg/ml and 110 pg/ml for E2 and 0.05-25 ng/ml and 2.4 ng/ml for P4 respectively. The intra- and inter-assay coefficients of variation were 6.8 and 8.6% for cortisol, 4.8 and 7.5% for P4, and 6.3 and 8.5% for E2 respectively. The cross-reactivities of the cortisol antibody were 100% for cortisol, 11.5% for 11-deoxycortisol, 4% for cortisone, 2% for corticosterone, 0.035% for P4, 0.06% for androstenedione, 0.02% for testosterone, and less than 0.01% for aldosterone, pregnenolone, dehydroepiandrosterone and E2 (Acosta *et al.* 2002).

Statistical analysis

Data were transformed to logarithms before analysis when necessary. The effects of hormonal status (EA vs EI) and phase of the oestrous cycle (follicular vs luteal) on the expression of 11HSD1 in granulosa cells and levels of cortisol in follicular fluid were analyzed using a two-way analysis of variance (ANOVA). Comparisons among group means were carried out using an one-way ANOVA, followed by a Tukey-Kramer test. The relationships between the levels of 11HSD1 expression and follicular cortisol, and between levels of 11HSD1 and 11HSD2 mRNA in CL were analysed by a regression analysis.

Results

The expression of 11HSD1 mRNA was detected in the liver, kidney, adrenal, spleen, CL, granulosa cells, thecal, uterine caruncle and inter-caruncular tissues whereas 11HSD2 mRNA was detected in the kidney, adrenal, CL and uterine tissues with a weak signal also detected in the spleen (Fig. 1). The expression of 11HSD1 mRNA in the CL, granulosa cells and thecal tissue was approximately 20, 10 and 5% of that in the liver respectively. While the expression of 11HSD2 mRNA in the CL was approximately 10% of that in the kidney.

Only 11HSD1 mRNA was detected in granulosa cells while the both 11HSD1 and 11HSD2 were detected in CL (Fig. 2). The expression of 11HSD1 mRNA in the stage II CL (84.4 ± 35.5, mean ± s.e.m. arbitrary unit, n=5) was significantly higher than that in granulosa cells harvested from EA follicles in follicular phase (8.6 ± 6.0, mean ± s.e.m. arbitrary unit, n=8, $P<0.01$).

The expression of 11HSD1 mRNA varied among follicles in different hormonal status (Fig. 3a). The ANOVA revealed a significant phase effect on the expression of 11HSD1 mRNA ($P<0.01$), while no status effect was found. A significant interaction between the phase and status of follicles was also found ($P<0.01$). There were significant differences in levels of 11HSD1 mRNA between the luteal phase EI and the luteal phase EA, and the luteal phase EI and the follicular phase EI ($P<0.05$). Cortisol was detected in follicular fluid from all follicles. There

was no significant phase or status effect on the levels of follicular cortisol (Fig. 3b). In all four groups, the levels of cortisol tended to decrease as the levels of 11HSD1 mRNA increased, although the correlation between these two indices did not reach a significant level except lutel phase EA: follicular phase EA (R^2=0.32, P=0.14, n=8), follicular phase EI (R^2=0.49, P=0.12, n=6), luteal phase EA (R^2=0.34, $P<0.05$, n=13), luteal phase EI (R^2=0.32, P=0.11, n=9). When all data were combined, a significant negative correlation was found between the levels of 11HSD1 mRNA and cortisol (R^2=0.18, $P<0.05$, n=36: Fig. 4).

The levels of mRNA for 11HSD1 and 11HSD2 decreased in the stage III CL compared with those in stage I and II CL ($P<0.05$ and $P<0.01$ for 11HSD1 and 11HSD2 respectively: Fig. 5a). While the expression of 11HSD1 mRNA continued to decrease, the expression of 11HSD2 rebounded in the stage IV CL and was significantly higher than that in the stage III CL ($P<0.01$: Fig. 5a). When all data were combined, a significant positive correlation was found between levels of 11HSD1 and 11HSD2 mRNA (R^2=0.42, $P<0.01$, n=20: Fig. 5b).

Discussion

In this study we have shown that, 1) the bovine ovarian tissues express 11β-HSD mRNA at a level comparable to that in classic glucocorticoid target organs, such as liver, 2) preovulatory follicles only express 11HSD1 while CL express both 11HSD1 and 11HSD2 mRNA, 3) in preovulatory follicles, 11HSD1 may decrease local concentration of cortisol, and 4) in CL the expression of 11HSD1 and 11HSD2 fluctuates during the luteal development and regression.

Because the glucocorticoid is only synthesized in the adrenal cortex and delivered systemically to the whole body, the presence of 11β-HSD is crucial for glucocorticoid target organs such as the liver and lung for modulating local concentration of active glucocorticoid (Krozowski *et al.* 1999). The expression of 11HSD1 and 11HSD2 in the bovine ovarian tissues, together with reported direct glucocorticoid action in cultured follicular cells (Luck 1988, Kawate *et al.* 1993, Spicer & Chamberlain 1998), indicates that the bovine ovary is a glucocorticoid target organ which is also capable to modulate local glucocorticoid environment.

Because the circulating levels of cortisol are largely unchanged throughout the bovine oestrous cycle (Garverick *et al.* 1971, Roussel *et al.* 1983, Lyimo *et al.* 2000), this system may play an important role in regulating ovarian glucocorticoid action.

In human, relative expression of 11HSD1 and 11HSD2 has been shown to determine levels of

cortisol in follicles (Yong *et al.* 2001). In maturing preovulatory follicles before the mid-cycle surge of gonadotrophins, the expression of 11HSD2 dominates over that of 11HSD1, resulting in lower levels of cortisol in the follicular fluid compared with that in the serum (Yding Andersen *et al.* 1999). This mechanism is likely to be beneficial for maturing follicles, as active glucocorticoids have been shown to inhibit oestrogen production in cultured granulosa cells (Hsueh & Erickson 1978, Kawate *et al.* 1993).

The preovulatory gonadotrophin surge dramatically induces the expression of 11HSD1 while it decreases the expression of 11HSD2 in follicles (Tetsuka *et al.* 1997, Yong *et al.* 2001). This results in a rapid increase in follicular cortisol just before follicular rupture (Harlow *et al.* 1997). The physiological importance of this event is not clear but it may enhance oocyte maturation (Fateh *et al.* 1989, Michael *et al.* 1999) or prevent an excess inflammation caused by ovulation (Hillier & Tetsuka 1998). In the present study, however, all follicles were collected before the gonadotrophin surge and thus the presence of a similar mechanism in the bovine preovulatory follicles is still to be clarified.

Interestingly, the bovine preovulatory follicles were shown to express 11HSD1 only. This is different from the results found in human and rat follicles where 11HSD2 is a predominant 11β-HSD before the preovulatory gonadotrophin surge (Tetsuka *et al.* 1997, Tetsuka *et al.* 1999).

The expression of follicular 11HSD1 was found to be highest in luteal phase EI follicles. The mechanism that regulates 11HSD1 expression is not clear. Nevertheless, follicles of all categories expressed comparable levels of 11HSD1, indicating that the enzyme expression is not much affected by a physiological status of follicles. The concentrations of cortisol in follicular fluid found in this study are in accord with those reported in bovine follicular fluid (Spicer & Zinn, 1987). In this study, however, a significant negative correlation was found between levels of the 11HSD1 mRNA and follicular cortisol. This result implies that 11HSD1 may act as a dehydrogenase rather than a reductase. In human and rodents, the 11HSD1 is known to be a bi-directional enzyme with a predominant reductase activity (Li & Wagner 1983). In ovine placenta, on the other hand, it was shown that 11HSD1 acts predominantly as a dehydrogenase and protects a fetus from adverse effects of maternal glucocorticoid (Yang *et al.* 1997). Because the bovine 11HSD1 shares a high sequence identity with that of sheep, the bovine isozyme can be also predominantly a dehydrogenase. If this is the case, the expression of 11HSD1 may create a favorable environment for increasing oestrogen production by decreasing levels of cortisol which has shown to inhibit oestrogen production and LH-receptor expression in cultured bovine granulosa cells (Kawate *et al.* 1993). Otherwise this phenomenon might be attributable to the activity of a third 11β-HSD isozyme (11β-HSD type3), a dehydrogenase, of

which presence was implicated in the sheep kidney (Gomez-Sanchez *et al.* 1997). Nevertheless a further study is necessary to characterize the bovine 11HSD1.

In the bovine CL, both 11HSD1 and 11HSD2 were detected and their expression levels were much higher than those in the granulosa cells. The expression of both isozymes was high in the developing CL (stages I and II) and decreased in the stage III CL, in which progesterone production reaches a maximum level. The expression of these two isozymes appeared to be similarly regulated except in the regressing CL (stage IV) where 11HSD2 rebounds while 11HSD1 remains low. These results indicate that a local glucocorticoid environment is stage specifically regulated in the bovine CL. It is reported that the bovine CL produces prostaglandins and they act as important local regulators of CL functions (Milvae 1986, Kobayashi & Miyamoto 2000). Administration of an inhibitor of prostaglandin synthesis, indomethacin, has been shown to inhibit CL development if given early in the oestrous cycle (Milvae 1986). On the other hand, indomethacin given later in the oestrous cycle resulted in an extended CL life span (Milvae 1986). Repeated administrations of a synthetic glucocorticoid, betamethasone, during luteal phase have been also shown to prolong luteal life span in cattle (Kanchev *et al.* 1976, Dobson *et al.* 1987). Although these effects are likely to be due to the inhibitory action of these agents on the PGF2α synthesis by the uterine endometrium or/and on

the pituitary function (Dobson *et al.* 1987), it is possible that glucocorticoid also acts directly to modulate CL function. Glucocorticoid inhibits prostaglandin synthesis in rat ovary (Kol *et al.* 1998), and inactivation of glucocorticoid by 11HSD2 appears to be necessary for the regression of CL in the pregnant rat just prior to parturition (Waddell *et al.* 1996). Taken together, these reports suggest that cortisol may inhibit the CL development and regression directly. The expression of 11β-HSDs observed in the present study, therefore, may be a part of the regulatory system that facilitates CL formation and regression through protecting local prostaglandin synthesis from circulating cortisol.

The expression of 11β-HSDs, especially that of 11HSD2 was low in the stage III CL. The down regulation of 11HSD2 might be due to high levels of P4 produced by the CL. It is reported that P4 decreased 11HSD2 bioactivity in cultured human placenta (Sun *et al.* 1998). High levels of P4 may also increase local concentrations of free cortisol by displacing it from its binding proteins (Andersen 2002). If similar regulations are operating in bovine CL, the high levels of P4 may create favorable environment for cortisol action in the mid CL. It is also possible that cortisol, in turn, increases P4 production (Hsueh & Erickson 1978, Kawate *et al.* 1993).

In summary, the bovine follicle and CL express 11β-HSD isoforms. The expression of 11HSD1 and 11HSD2 is tissue and stage specific: the preovulatory follicle before LH-surge only

expresses 11HSD1 while CL expresses both 11HSD1 and 11HSD2. The bovine 11HSD1 is likely to be a dehydrogenase and may act as an inactivator of cortisol. It is suggested that these two 11β-HSD isozymes may modulate ovarian glucocorticoid levels to create favorable environment for follicular maturation, CL formation and regression.

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References

Acosta TJ, Yoshizawa N, Ohtani M, & Miyamoto A 2002 Local changes in blood flow within the early and midcycle corpus luteum after prostaglandin F2α injection in the cow. *Biology of Reproduction* **66** 651-658.

Andersen CV 2002 Possible new mechanism of cortisol action in female reproductive organs: physiological implications of the free hormone hypothesis. *Journal of Endocrinology* **173** 211-217.

Chomczynski P & Sacchi N 1987 Single step method of RNA isolation by acid guanidinium thiocyanate phenol chloroform extraction. *Annals of Biochemistry* **162** 156-159.

Dobson H, Alam MG & Kanchev LN 1987 Effect of betamethasone treatment on luteal lifespan and the LH response to GnRH in dairy cows. *Journal of Reproduction and Fertility* **80** 25-30.

Fateh M, BenRafael Z, Benadiva CA, Mastroianni L Jr. & Flickinger GL 1989 Cortisol levels in human follicular fluid. *Fertility and Sterility* **51** 538-541.

Fitzpatrick SL & Richards JS 1991 Regulation of cytochrome P450 aromatase messenger ribonucleic acid and activity by steroids and gonadotropins in rat granulosa cells. *Endocrinology* **129** 1452-1462.

Garverick HA, Erb RE, Niswender GD & Callahan CJ 1971 Reproductive steroids in the bovine. 3. Changes during the estrous cycle. *Journal of Animal Science* **32** 946-956.

Gaytan F, Morales C, Bellido C & Sanchez-Criado JE 2002 Selective apoptosis of luteal endothelial cells in dexamethasone-treated rats leads to ischemic necrosis of luteal tissue. *Biology of Reproduction* **66** 232-240.

Gomez-Sanchez EP, Ganjam V, Chen YJ, Cox DL, Zhou MY, Thanigaraj S & Gomez-Sanchez CE 1997 The sheep kidney contains a novel unidirectional, high affinity NADP(+)-dependent 11β-hydroxysteroid dehydrogenase (11β-HSD-3). *Steroids* **62** 444-450.

Harlow CR, Jenkins JM & Winston RML 1997 Increased follicular fluid total and free cortisol levels during the luteinizing hormone surge. *Fertility and Sterility* **68** 48-53.

Hillier SG & Tetsuka M 1998 An anti-inflammatory role for glucocorticoids in the ovaries? *Journal of Reproduction and Immunology* **39** 21-27.

Hsueh AJW & Erickson GF 1978 Glucocorticoid inhibition of FSH-induced estrogen production in cultured rat granulosa cells. *Steroids* **32** 639-648.

Ireland JJ, Murphee RL & Coulson PB 1980 Accuracy of predicting stages of bovine estrous cycle by gross appearance of the corpus luteum. *Journal of Dairy Science* **63** 155-60.

Kanchev LN, Dobson H, Ward WR & Fitzpatrick RJ 1976 Concentration of steroids in bovine peripheral plasma during the oestrous cycle and the effect of betamethasone treatment. *Journal of Reproduction and Fertility* **48** 341-345.

Kawate N, Inaba T & Mori J 1993 Effects of cortisol on the amounts of estradiol-17β and progesterone secreted and the number of luteinizing hormone receptors in cultured bovine granulosa cells. *Animal Reproduction Science* **32** 15-25.

Kobayashi S & Miyamoto A 2000 Prostaglandin F2α increases lipoprotein utilization for progesterone in bovine early corpora lutea in vitro. *Journal of Reproduction and Development* **46** 335-339.

Kol S, Ben-Shlomo I, Payne DW, Ando M, Rohan RM & Adashi EY 1998 Glucocorticoids suppress basal (but not interleukin-1-supported) ovarian phospholipase A2 activity: evidence for glucocorticoid receptor-mediated regulation. *Molecular Cellular Endocrinology* **137** 117-125.

Krozowski Z, Li KX, Koyama K, Smith RE, Obeyesekere VR, Stein-Oakley A, Sasano H, Coulter C, Cole T & Sheppard KE 1999 The type I and type II 11β-hydroxysteroid dehydrogenase enzymes. *Journal of Steroid Biochemistry and Molecular Biology* **69** 391-401.

Li PS & Wagner WC 1983 Effects of hyperadrenal states on luteinizing hormone in cattle. *Biology of Reproduction* **29** 11-24.

Luck MR 1988 Enhanced secretion of oxytocin from bovine granulosa cells treated with adrenal steroids. *Journal of Reproduction Fertility* **83** 901-907.

Lyimo ZC, Nielen M, Ouweltjes W, Kruip TMA & van Eerdenburg FJCM 2000 Relationship among estradiol, cortisol and intensity of estrous behavior in dairy cattle. *Theriogenology* **53** 1783-1795.

Michael AE, Collins TD, Norgate DP, Gregory L, Wood PJ & Cooke BA 1999 Relationship between ovarian cortisol:cortisone ratios and the clinical outcome of in vitro fertilization and embryo transfer (IVF-ET). *Clinical Endocrinology* **51** 535-540.

Milvae RA 1986 Role of luteal prostaglandins in the control of bovine corpus luteum functions. *Journal of Animal Science* **62 Suppl 2** 72-78.

Miyamoto A, Okuda K, Schweigert FJ & Schams D 1992 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*. *Journal of Endocrinology* **135** 103-114.

Roussel JD, Clement TJ, Aranas TJ & Seybt SH 1983 Changes in circulating plasma levels of cortisol in lactating and non-lactating dairy cattle during the estrous cycle. *Theriogenology* **19**

535-539.

Rozen S & Skaletsky HJ 2000 Primer3 on the WWW for general users and for biologist programmers. In *Bioinformatics Methods and Protocols: Methods in Molecular Biology*, pp 365-386. Eds S Krawetz & S Misener Totowa, NJ: Humana Press.

Spicer LJ & Chamberlain CS 1998 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* **9** 153-161.

Spicer LJ & Zinn SA 1987 Relationship between concentrations of cortisol in ovarian follicular fluid and various biochemical markers of follicular differentiation in cyclic and anovulatory cattle. *Journal of Reproduction Fertility* **81** 221-226.

Sun K, Yang K & Challis JR 1998 Regulation of 11β-hydroxysteroid dehydrogenase type 2 by progesterone, estrogen, and the cyclic adenosine 5'-monophosphate pathway in cultured human placental and chorionic trophoblast. *Biology of Reproduction* **58** 1379-1384.

Tetsuka M, Milne M, Simpson GE & Hillier SG 1999 Expression of 11β-hydroxysteroid dehydrogenase, glucocorticoid receptor and mineralocorticoid receptor genes in rat ovary. *Biology of Reproduction* **60** 330-335.

Tetsuka M, Thomas FJ, Thomas MJ, Anderson RA, Mason JI & Hillier SG 1997 Differential expression of messenger ribonucleic acids encoding 11β-hydroxysteroid dehydrogenase types 1 and 2 in human granulosa cells. *Journal of Clinical Endocrinology and Metabolism* **82** 2006-2009.

Urban RJ, Bodenburg YH, Nagamani M & Peirce J 1994 Dexamethasone potentiates IGF-I actions in porcine granulosa cells. *American Journal of Physiology, Endocrinology and Metabolism* **267** E115-E123.

Waddell BJ, Benediktsson R & Seckl JR 1996 11β-Hydroxysteroid dehydrogenase type 2 in the rat corpus luteum: Induction of messenger ribonucleic acid expression and bioactivity coincident with luteal regression. *Endocrinology* **137** 5386-5391.

Wijayagunawardane MP, Miyamoto A, Cerbito WA, Acosta TJ, Takagi M & Sato K 1998 Local distributions of oviductal estradiol, progesterone, prostaglandins, oxytocin and endothelin-1 in the cyclic cow. *Theriogenology* **49** 607-618.

Yang JG, Yu CC & Li PS 2002 Dexamethasone enhances follicle stimulating hormone-induced P450scc mRNA expression and progesterone production in pig granulosa cells. *Chinese Journal of Physiology* **44** 111-19..

Yang K, Langlois DA, Campbell LE, Challis JR, Krkosek M & Yu M 1997 Cellular localization and developmental regulation of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) gene expression in the ovine placenta. *Placenta* **18** 503-509.

Yding Andersen C, Morineau G, Fukuda M, Westergaard LG, Ingerslev HJ, Fiet J & Byskov AG 1999 Assessment of the follicular cortisol:cortisone ratio. *Human Reproduction* **14** 1562-1568.

Yong PY, Thong KJ, Andrew R, Walker BR & Hillier SG 2001 Development-Related Increase

in Cortisol Biosynthesis by Human Granulosa Cells. *Journal of Clinical Endocrinology and Metabolism* **85** 4728-4733.

Table 1 Primers for RT-PCR

Gene	Primer	Sequence	GenBank No. [1]	Position[2]
11βHSD type 1	sense	TGCGAATGAGGAATTCAGACCA	M10901	133-154
(598 bp)	anti-sense	GTCTGTGTCGATGAGGCCAAGA	M10901	709-730
11βHSD type 2	sense	CCTGACCAAGCCAGGAGACATT	S46827	547-568
(707 bp)	anti-sense	TCGGCACATAGGGACTGATGAA	S46827	1634-1655
βactin	sense	GTTCAACACTCCTGCCATGTAT	K00622	150-171
(277 bp)	anti-sense	GTAGCAGAGCTTCTCCTTGATG	K00622	405-426

1: GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide).

2: Nucleotide position in the reported sequence.

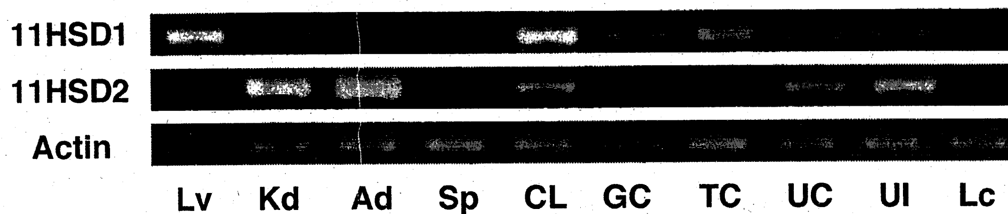


Figure 1 Detection of 11β-HSD type 1 (11HSD1) and type 2 (11HSD2) mRNAs in various organs by RT-PCR. The levels of 11HSD1 and 11HSD2 mRNA were quantified by a semi-quantitative RT-PCR using actin as an internal standard. A 598 bp band for 11HSD1 was detected in the liver (Lv: 100%), kidney (Kd: 3% of the liver), adrenal (Ad: 1%), spleen (Sp: 2%), CL (18%), granulosa cells (GC: 12%), thecal tissue (TC: 5%), uterine caruncle (UC: 6%) and inter-caruncular (UI: 3%) tissues, whereas a 707 bp band for 11HSD2 was detected in the Kd (100%), Ad (84% of the kidney), CL (11%), UC (22%) and UI (35%) with a weak signal also detected in the Sp (1%). No signal was detected in leukocytes (Lc). GC and TC were harvested from a preovulatory oestrogen active follicle (>10 mm) in the follicular phase, while a CL sample was collected from a stage II CL.

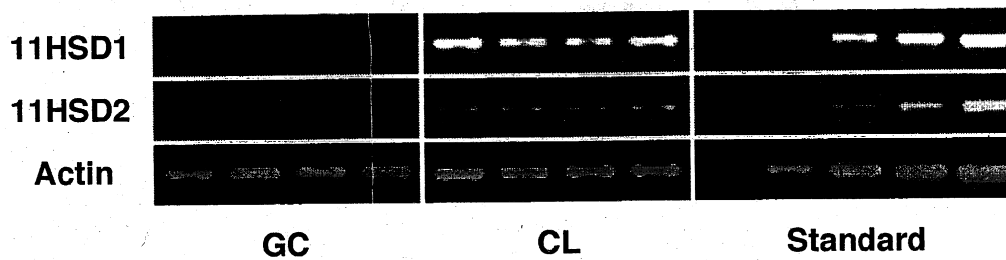
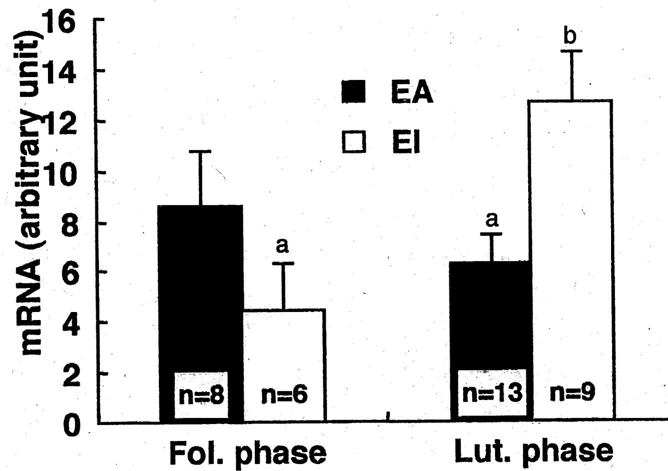


Figure 2 Expression of 11β-HSD type 1 (11HSD1) and type 2 (11HSD2) in the bovine granulosa cells and CL. Granulosa cells were harvested from preovulatory oestrogen active follicles (>10 mm) in the follicular phase, while luteal samples were collected from the stage II CL. The levels of 11HSD1 and 11HSD2 mRNA were quantified by a semi-quantitative RT-PCR. A set of serially diluted total RNA from the liver (11HSD1: 2, 8, 32, 125, 500ng), adrenal gland (11HSD2: 2, 8, 32, 125, 500ng) or CL (actin: 8, 32, 125, 500, 2000ng) was included to each RT-PCR trial to construct a standard curve. Representative samples are shown.



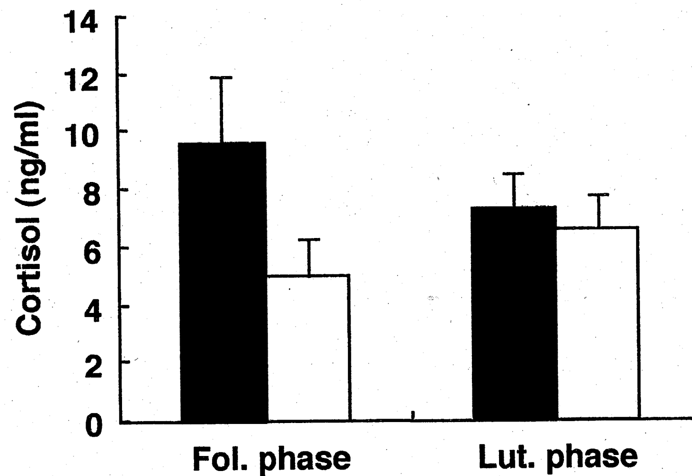


Figure 3 Expression of 11β-HSD type 1 (11HSD1) mRNA in granulosa cells and levels of cortisol in follicular fluid. Granulosa cells were harvested from follicles (>10 mm) in luteal and follicular phases. The follicles were classified into four groups according to their hormonal status (E2:P4 >1: oestrogen active: EA, E2:P4 <1: oestrogen inactive: EI) and stages of oestrous cycle. The levels of mRNA encoding 11HSD1 were quantified by a semi-quantitative RT-PCR (a), while concentrations of cortisol were measured by an EIA (b). Values are mean ± s.e.m. Significantly different from each other: a,b P<0.05.

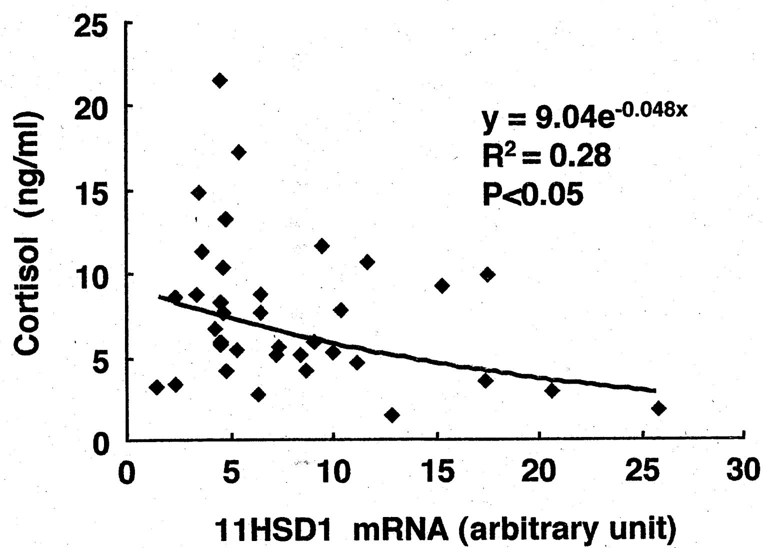
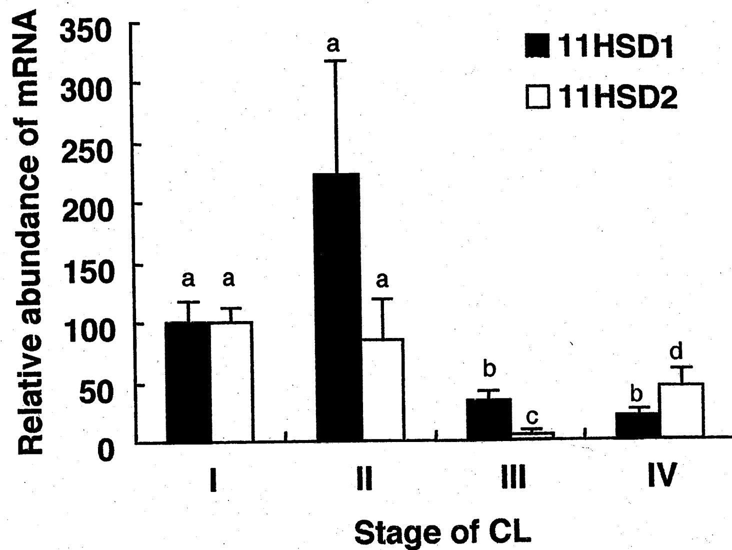


Figure 4 Relationship between levels of 11β-HSD type 1 (11HSD1) mRNA in granulosa cells and concentrations of cortisol in follicular fluid. When all data were combined, a significant negative correlation was found between levels of 11HSD1 mRNA and cortisol (R^2=0.18, $P<0.05$, n=36).



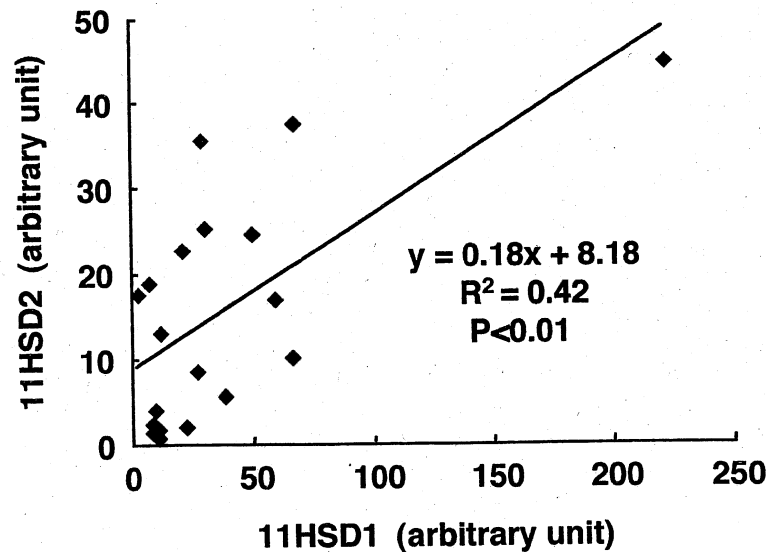


Figure 5 Expression of 11β-HSD type 1 (11HSD1) and type 2 (11HSD2) mRNA in the bovine CL. CL were collected from the stages I-IV CL (stage I: Day1-4, stage II: Day5-10, stage III: Day 11-17, stage IV: Day18-20). The levels of mRNA encoding 11HSD1 and 11HSD2 were quantified by a semi-quantitative RT-PCR (a). Values are expressed as percentage of the group I (mean ± s.e.m., n=5). Significantly different from each other within same isozyme: a,b and b,d $P<0.05$; a,c $P<0.01$. A significant positive correlation was found between levels of 11HSD and 11HSD2 mRNA (R^2=0.42, $P<0.01$, n=20).

Journal of Endocrinology

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19 March 2003

Dear Dr Tetsuka

JOE5183 Research Paper ESPERE 440
Expression of 11b-hydroxysteroid dehydrogenases in bovine follicle and corpus luteum

I am pleased to be able to tell you that the above paper is now quite acceptable for publication in the *Journal of Endocrinology*. It has been copy-edited and sent to the typesetters for page layout, so proofs should reach you in about four weeks.

Yours sincerely

pp Ailsa L Bailey
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