

## JSAR Outstanding Research Award

# Studies on the Regulation of Expression of Luteinizing Hormone Receptor in the Ovary and the Mechanism of Follicular Cyst Formation in Ruminants

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**Abstract.** In the series of studies, changes of expression and regulation of luteinizing hormone (LH) receptor in the ovary of domestic ruminants were examined. Furthermore, mechanisms of formation of follicular cysts in domestic ruminants, caused by stress and so on, were endocrinologically elucidated. Results of the studies provide the following conclusions. (1) The quantity of LH receptor in the bovine antral follicles increases rapidly in the latter stage of its development. (2) The quantity of LH receptor and its mRNA in the bovine and caprine corpus luteum increase during their developments. The increase of the receptor in the caprine luteal development is regulated by LH through the receptor mRNA level. (3) At least, three splice variants of LH receptor mRNA exist in the bovine luteal tissue and the variant receptors are expressed at different cellular sites according to its structure. (4) Intracellular consecutive cysteine residues of LH receptor are palmitoylated and thereby inhibit internalization of the receptor. (5) As a mechanism of the bovine follicular cyst caused by stress, it is suggested that increased secretions of progesterone and cortisol from the adrenal gland exert inhibitory effects on the hypothalamus and follicle, respectively, and subsequently LH and FSH surges are blocked, then finally ovulation is suppressed and the follicle becomes cystic.

**Key words:** Luteinizing hormone (LH) receptor, Follicle, Corpus luteum, Follicular cyst, Domestic ruminant

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It has been suggested, in domestic ruminants, that luteinizing hormone (LH) has a pivotal role in the regulation of ovarian functions [1-5]. The action of LH on the ovarian cell is mediated through its specific receptor located on the plasma membrane. Based upon the sequences of full-length cDNA encoding the LH receptor [6-10], it has been proposed that the LH receptor belongs to a family of guanine nucleotide-binding (G) protein-coupled receptors that consist of a long extracellular domain, seven transmembrane helices, and a short intracellular carboxyl-terminal domain. Upon binding to the LH receptor, the

hormone stimulates adenylyl cyclase activity, cyclic adenosine 3', 5'-monophosphate production, and consequently biological reactions such as steroidogenesis is enhanced [11]. Thus, the quality and quantity of LH receptor are important factors affecting the sensitivity to its ligand.

Adenohypophyseal and placental gonadotropins, which possess various degrees of LH activity, and gonadotropin-releasing hormone (GnRH), which stimulates endogenous LH release, have been used as drugs to treat ovarian dysfunctions and control ovaries artificially in cattle, sheep and goats [12-14]. Development and prevalence of estrus and ovulation synchronization for the artificial insemination and embryo transfer

of cows increase the opportunity of using the hormonal drugs [15–17]. The efficaciousness of the treatment with GnRH and gonadotropins possessing LH activity depends partly on the quality and quantity of LH receptor in the ovary.

The present series of studies were carried out to examine changes of expression and regulation of LH receptor at the levels of protein and mRNA in the ovary of domestic ruminants. Furthermore, mechanisms of formation of follicular cysts in domestic ruminants, caused by stress and so on, were elucidated endocrinologically, focusing on hypothalamus-pituitary-ovarian axis.

### Changes of Expression and Regulation of LH Receptor in the Ovary of Domestic Ruminants

#### *Changes of quantity of LH receptors in the development of antral follicles and in the cystic follicles*

In order to determine changes of quantity of LH receptors during the bovine follicular development and in the ovarian cysts, numbers of LH receptors in granulosa cells and theca interna and concentrations of steroids in follicular fluid in antral follicles at the developing stages and in cystic follicles were examined [18, 19]. As a result, the numbers (receptors/cell) of LH receptors in granulosa cells and theca interna increased rapidly in the latter stage of antral follicular development

concomitantly with augmentation of aromatase activity [18]. The results also showed that the number of LH receptor (receptors/cell) is reduced in follicular cysts as compared with that in the large antral follicles [18]. Total number of LH receptor per cyst in the theca interna of the luteinized cysts was comparable to that of the large antral follicles, while the total number was reduced for the follicular cysts [19]. The changes of LH receptor content and estradiol secretion during the development of the bovine antral follicle are shown schematically in Fig. 1.

We also demonstrated that the injections of low dose hCG after the treatment with an intravaginal progesterone release device promote follicular maturation and fertile estrus induction in the goats during the early postpartum nursing period [20]. In this study, it is plausible that the treatment with low dose hCG may also increase the LH receptor content thus allowing the follicles to ovulate and luteinize in response to the LH surge induced by the increment of estradiol-17 $\beta$  in the goat.

#### *Changes of quantity of LH receptors in the development of corpus luteum and its regulation*

The number of LH receptors in the bovine corpus luteum during the estrous cycle increased in the course of development and maintained high during the pregnancy [21]. Steady-state level of mRNA encoding the LH receptor in the bovine corpus luteum also increased during the luteal

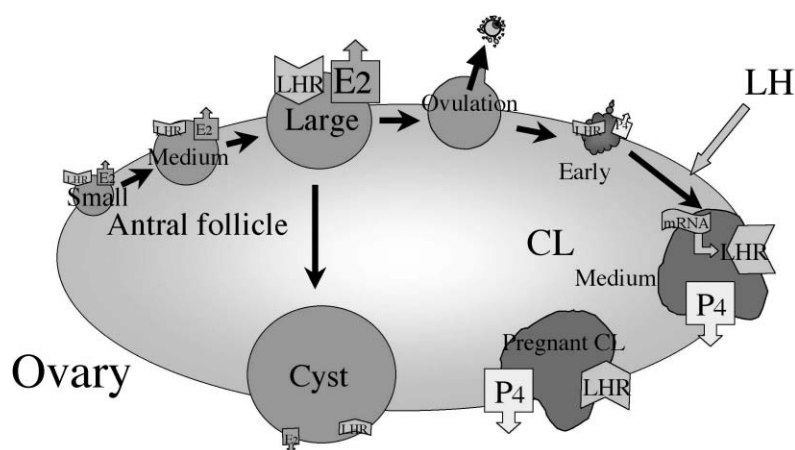


Fig. 1. Schematic diagram of changes of LH receptor content and estradiol-17 $\beta$  secretion during the development of the bovine antral follicle and in the cystic follicle, and changes and regulation of LH receptor content during the development of corpus luteum in cows and goats. LHR, LH receptor; E<sub>2</sub>, estradiol-17 $\beta$ ; P<sub>4</sub>, progesterone.

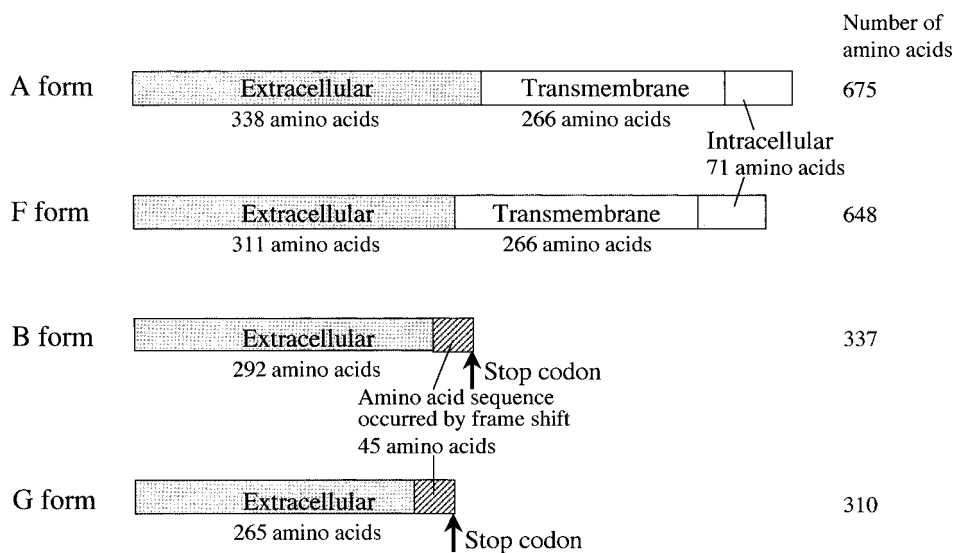
development [22]. The increments of LH receptor and its mRNA in the luteal development during the estrous cycle were also observed in the goat [23]. Such increments were partially inhibited by administration of GnRH antagonist, which potently suppresses the pulsatile LH release in peripheral blood [5], suggesting that LH up-regulates its receptor protein through augmentation of the amount of its transcript (Fig. 1)[23].

*Expression of splice variants for LH receptor mRNA and protein*

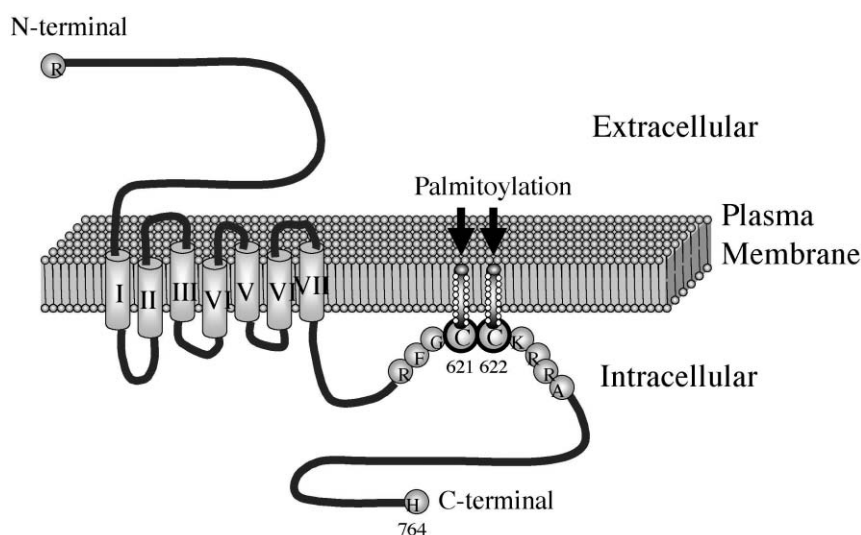
The presence of multiple transcripts encoding LH receptor in the corpus luteum was revealed by Northern blot analyses for rats [24], pig [7], sheep [25], horse [26] and human [27]. Upon sequencing of the mRNAs, some of multiple forms encoding the LH receptor were determined to be splice variants [7, 25–28]. In the present study, we characterized splice variants for LH receptor mRNA in the bovine corpus luteum and examined the levels of the variants during the luteal development. The study showed that at least three splicing variants of LH receptor mRNA including

F, B and G forms in addition to the full-length A form exist in the bovine corpus luteum [22]. The F form shows complete deletion of exon X and is spliced from the 3'-end of exon IX to the 5'-end of exon XI, in frame (Fig. 2). The B form is spliced from the 3'-end of exon X into the coding region of exon XI, and the splicing generates a frame shift and a premature stop codon (Fig. 2). The G form is spliced from the 3'-end of exon IX to the same acceptor site as the B form in the coding region of exon XI, and the splicing generates a frame shift and a premature stop codon (Fig. 2). Thus, the F form receptor has the complete transmembrane and intracellular domains while the B and G form receptors do not have the transmembrane and intracellular domains. Furthermore, all of the forms of LH receptor mRNA increased in a coordinated manner during the development of bovine corpus luteum [22].

Complete cDNA sequences of the three splice variants of bovine LH receptor were cloned in a mammalian expression vector and transfected into COS-7 cells [29, 30]. Scatchard analysis of the intact cells transfected with the A or F form of bovine LH



**Fig. 2.** Schematic diagram indicating numbers of amino acids of a full-length form (A form), and splice variants (F, B and G forms) of bovine LH receptor. A and F forms consist of an extracellular domain, a transmembrane domain and an intracellular domain. The extracellular domain of F form is shorter by 27 amino acids than that of A form because the exon X is completely deleted and spliced from the 3'-end of exon IX to the 5'-end of exon XI, in frame. B or G form is spliced from the 3'-end of exon X into the coding region of exon XI, and the splicing generates a frame shift. The frame shift causes a unique 45-amino acid sequence (bar with oblique lines) and a premature stop codon (arrow). The extracellular domain of G form is shorter by 27 amino acids than that of B form because of complete deletion of exon X as observed in the F form.



**Fig. 3.** Proposed diagram indicating palmitoylation of consecutive two cysteines just after the seventh transmembrane domain of LH receptor. The sites of palmitoylation at Cys 621 and 622 residues of rat LH receptor are indicated. I-VII, seven transmembrane domains.

receptor cDNA showed high affinity and low capacity of [ $^{125}$ I]-hCG binding sites on the cells. No binding activities were observed in the intact cells transfected with the B or G form of bovine LH receptor cDNA. However, Scatchard analysis of detergent-solubilized cells transfected with A, F, B or G form of bovine LH receptor cDNA showed binding activity with similar affinity among the forms. These results suggest that the F form of bovine LH receptor protein is expressed on the cell surface in the same way as the full-length A form. Besides, the data demonstrate that the B and G forms of the receptor protein are trapped intracellularly.

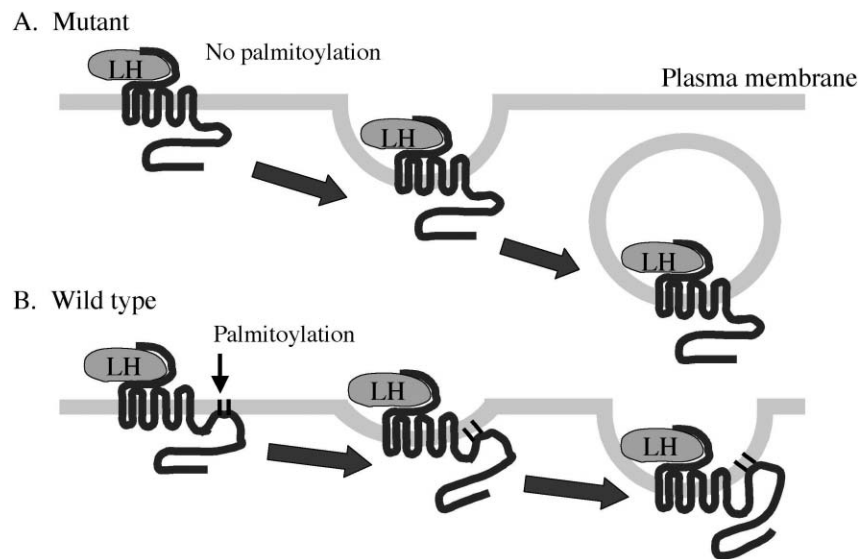
#### *Palmitoylation of LH receptor and its role*

It has been suggested that the LH receptor own not only transducing function of the signal from LH, but turning-off action of the agonist response in the face of continuous agonist exposure, namely, desensitization of the receptor [31]. The desensitization consists of uncoupling of the receptor from its cognate G proteins and internalization of the receptor-ligand complex. The intracellular domain of the LH receptor is supposed to be responsible for the uncoupling and internalization [31]. An intracellular cysteine residue present in the juxtamembrane region of the C-terminal tail of many G-protein coupled

receptors including rhodopsin and  $\beta_2$ -adrenergic receptor has been shown to be palmitoylated [32, 33]. The LH receptor has two adjacent cysteines in this position and these cysteines are conserved among species [6–10, 29]. Thus, we examined whether the two adjacent cysteines in the intracellular domain of the LH receptor are palmitoylated and any functions of the palmitoylation in regulation of the receptor [34, 35]. The results showed that both cysteine residues of the rat LH receptor (Cys 621 and 622) was palmitoylated creating a membrane-anchoring site at the putative cytoplasmic domain (Fig. 3) [34]. This palmitic acid-mediated anchoring decreased the ligand-induced receptor internalization thereby prolonging the retention of the ligand-bound receptor on the cell surface (Fig. 4) [34]. Besides, we demonstrated that palmitoylation inhibited ligand-induced receptor down-regulation [35].

#### **Mechanism of Follicular Cyst Formation in Ruminants**

Follicular cysts are a serious cause of reproductive failure in cattle because they occur frequently and prolong the intervals from postpartum to first estrus and conception [36]. The bovine follicular cysts have been reported since



**Fig. 4.** Schematic diagram of internalization of palmitoylation-deficient mutants (A) and a wild type of LH receptor (B). The rate of internalization of the mutant LH receptor is faster than that of the wild type receptor.

1831 [37]. However, causes and mechanisms of occurrence of the bovine follicular cysts have not been elucidated. We have been studying on the endocrinology of hypothalamus-pituitary-ovarian axis regarding formation mechanisms of follicular cysts in ruminants.

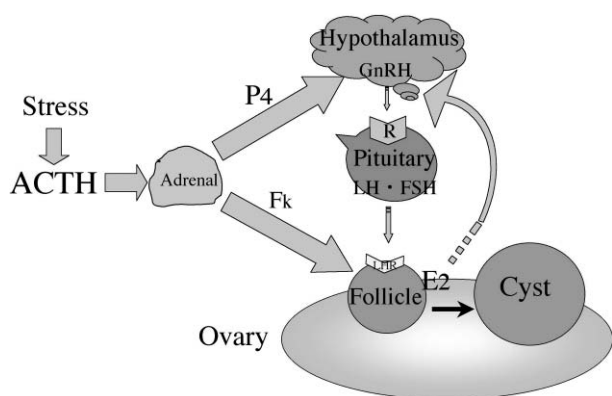
It has been suggested that stress is a possible cause for bovine follicular cysts since Garm reported that adrenal gland was enlarged in cows with ovarian cysts at 1949 [37]. Later, it was reported that the follicular cystic disease could be induced by treatment with adrenocorticotrophic hormone (ACTH) in cows by Liptrap *et al.* [38]. We demonstrated that, during the formation of follicular cysts induced by treatments with ACTH, the preovulatory release of LH and FSH is blocked and that the suppressed release of FSH and LH might be caused by increases in secretions of cortisol and progesterone, and by the decrease in secretion of estradiol-17 $\beta$  [39]. Furthermore, we examined direct effects of steroids, those are released from adrenal glands when animals are stressed, on hypothalamus, anterior pituitary or ovarian follicle. The results demonstrate that high concentration of progesterone act directly on the bovine pituitary stalk-median eminence of the hypothalamus to inhibit the release of GnRH [40]. Also, it is suggested that the direct inhibitory effect of cortisol on secretion of estradiol-17 $\beta$  and LH

receptor content in cultured bovine granulosa cells [41]. The series of these results suggest the following possible formation mechanism for the bovine follicular cyst caused by stress (Fig. 5) [42]. First, ACTH stimulates releases of progesterone and cortisol upon the cow is stressed. Then, increased secretion of progesterone inhibits release of GnRH from the hypothalamus. On the other hand, enhanced secretion of cortisol decreases the LH receptor contents and estradiol secretion of the antral follicle. Consequently, positive feedback action of estradiol to hypothalamus and pituitary is deteriorated and the LH surge is suppressed. Finally, the ovulation is blocked and the follicle becomes cystic (Fig. 5).

In naturally occurring cases of the bovine follicular cysts, we demonstrate that the function of the anterior pituitary is not abnormal [43, 44], suggesting that the disappearance of the LH surge is probably due to the abnormality in the hypothalamus [45]. We also showed in an aged goat with follicular cysts that lack of response to LH surges may be a cause for the cystic disease [46].

## Conclusions

The quantities of LH receptor in follicles and corpus luteum of the domestic ruminants including



**Fig. 5.** Schematic diagram of a possible mechanism for formation of bovine follicular cysts by stress. Increased secretion of progesterone ( $P_4$ ) from adrenal glands inhibits GnRH release from hypothalamus. Increased secretion of cortisol (Fk) from adrenal glands lower LH receptor (LHR) content and secretion of estradiol- $17\beta$  ( $E_2$ ) in antral follicles. Consequently, feedback action of estradiol- $17\beta$  to hypothalamus-pituitary is weakened and LH surge is diminished. Then the ovulation is blocked and the follicle becomes cystic.

cattle and goats increase during their developments. The increase of the receptor during the caprine luteal development is regulated by, at least, LH through the receptor mRNA level. Splice variants of LH receptor are expressed at different cellular sites according to its structure. The LH receptor is palmitoylated at its intracellular domain and thereby the receptor numbers on cell surface can be regulated. Stress can cause the bovine follicular cyst through a possible mechanism that

increased progesterone and cortisol from the adrenal gland inhibit functions of the hypothalamus and follicle, respectively.

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