RAPID NON-GENOMIC CONTROL BY MEMBRANE ESTRADIOL RECEPTOR, GPR30, FOR LUTEINIZING HORMONE SECRETION FROM BOVINE ANTERIOR PITUITARY

膜型エストロジェン受容体 **GPR30** による
迅速でノンゲノミックなウシ下垂体前葉からの **LH** 分泌の調節機構

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RAPID NON-GENOMIC CONTROL BY MEMBRANE ESTRADIOL RECEPTOR, GPR30, FOR LUTEINIZING HORMONE SECRETION FROM BOVINE ANTERIOR PITUITARY

A Dissertation

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Abstract

Estradiol secreted from the ovaries is the most important feedback regulator for both the hypothalamus and anterior pituitary (AP) in controlling the secretion of gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH) in animals. Therefore, abnormalities of the estradiol feedback induce various reproductive diseases. However, the details of the feedback mechanisms are remained to be clarified.

G protein-coupled receptor 30 (GPR30) is an estradiol receptor located on the plasma membrane, and it initiates several rapid, non-genomic signaling events. GPR30 has recently been identified in rat AP; however, little is known about the role of GPR30 in controlling LH secretion from gonadotropes in animals. To fill this research gap, we hypothesized that GPR30 is expressed in bovine AP and mediates estradiol inhibition of GnRH-induced LH release. We confirmed the expressions of GPR30 mRNA and protein by RT-PCR, western blotting, and immunohistochemistry.

In next, we cultured bovine AP cells (n=8) for 3 days in steroid-free conditions and then treated them with increasing concentrations (0.001nM, 0.01nM, 0.1nM, 1nM, and 10nM) of estradiol or a GPR30-specific agonist, G1, for 5min before GnRH stimulation. As expected, estradiol at 0.001-0.1nM inhibited the GnRH-stimulated LH secretion. However, we found also that G1 at 0.001nM was able to inhibit this secretion ($P<0.05$). In contrast, both estradiol and G1 at higher doses were less efficient in suppressing the GnRH-stimulated LH secretion. Neither estradiol nor G1 suppressed GnRH-stimulated follicle-stimulating hormone secretion.

In separate experiments, fluorescent immunohistochemistry and immunocytochemistry revealed that approximately 50% of GPR30-positive cells express

LH, and about 30% of LH-positive cells express GPR30. Therefore, GPR30 is expressed in bovine gonadotropes and other AP cells and may contribute to rapid negative estradiol feedback of GnRH-induced LH secretion.

Our data suggested that GPR30 is expressed in bovine gonadotropes and may contribute to the rapid, negative estradiol feedback effect observed in GnRH-induced LH secretion. However, bovine gonadotropes may have another undefined plasma membrane receptor that mediates. STX is an agonist for a recently characterized membrane estrogen receptor whose structure has not been identified. We evaluated whether STX suppresses GnRH-induced LH release from bovine AP cells. We cultured AP cells (n=12) for 3 days in steroid-free conditions, followed by increasing concentrations (0.001, 0.01, 0.1, 1 and 10 nM) of estradiol or STX for 5 min before GnRH stimulation until the end of the experiment. Estradiol (0.001 to 0.1 nM) significantly suppressed GnRH-stimulated LH secretion, whereas STX did not affect GnRH-stimulated LH secretion at any of the tested concentrations. Therefore, STX, unlike estradiol and G1, possesses no suppressive effect on GnRH-induced LH release from bovine AP cells.

The GPR30 is the only clearly identified membrane estradiol receptor in bovine AP cells. In order to verify whether GPR30 is the receptor mediating such effect, we conducted a study to evaluate the effect of GPR30-specific antagonist, G36, on the estradiol rapid suppression of GnRH-induced LH secretion. Pre-treatment for 5min with G36 inhibited the estradiol suppression of LH secretion from cultured AP cells. Therefore, GPR30 is the main important receptor for rapid estradiol suppression.

In order to verify the effect of short time treatment with estradiol is mediated by non-genomic mechanism, we evaluated the effect of estradiol on the gene expressions of

LHα, LHβ, or FSHβ subunits. The AP cells (n = 5) were cultured for 3 days in steroid-free conditions and then treated them with 0.01 nM estradiol for 5 min before GnRH stimulation. Real-time PCR analyses revealed that pre-treatment with estradiol ($P<0.05$) did not decrease the gene expressions of the LHα, LHβ, or FSHβ subunits in the AP cells. Therefore, the estradiol suppression is non-genomic mechanism.

Further studies were conducted to clarify the cytoplasmic pathway in the downstream of GPR30 in bovine AP cells using inhibitors. Pre-treatment with ERK1/2/5 inhibitor and PKA inhibitor inhibited the estradiol or G1 suppression of LH secretion from cultured AP cells. Therefore, both ERK1/2/5 pathway and PKA pathway are important pathways in the downstream of GPR30 to suppress LH secretion in the non-genomic mechanism.

Cyclic AMP is the key molecule in the cytoplasmic pathway to increase LH secretion from ovine gonadotrope cells. In order to clarify whether cAMP pathway is the cytoplasmic pathway in the downstream of GPR30 in bovine AP cells, another study was conducted utilizing cAMP measurement. cAMP measurements analyses revealed that pre-treatment with small amounts of estradiol significantly decreased cAMP in the AP cells rapidly. Therefore, cAMP is the central molecule in the cytoplasmic mechanism in the downstream of GPR30 to inhibit LH secretion non-genomically.

In the dissertation studies, we found that AP of heifers with quiescent ovary or cystic follicle show abnormal responses to the rapid effect of estradiol. Thus, the GPR30 on the cell surface and the cytoplasmic pathways may be the reason to induce the ovarian diseases.

In conclusion, estradiol binds to GPR30 on the surface of gonadotrope, decreased cAMP, activated PKA and ERK1/2/5 pathways to decrease LH secretion in a rapid, non-genomic mechanism.

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List of Abbreviations

ABI	: Applied biosystems
ANOVA	: Analysis of variance
AP	: Anterior pituitary
bFSH	: Bovine follicle stimulating hormone
BLASTN	: Nucleotide basic local alignment search tool
bLH	: Bovine luteinizing hormone
BSA	: Bovine serum albumin
cAMP	: Cyclic adenosine monophosphate
cDNA	: Complementary deoxyribonucleic acid
C	: Celsius
CCD	: Charge-coupled device
CHO	: Chinese hamster ovary cells
CO2	: Carbon dioxide
d	: Day(s)
DAB	: Diaminobenzidine
DAPI	: 4',6-diamidino-2-phenylindole
DMEM	: Dulbecco's modified eagle's medium
DMSO	: Dimethyl sulfoxide
E2-BSA	: Estradiol conjugated with bovine serum albumin
ECL-Primer	: Enhanced chemiluminescence-primer
ER	: Estrogen receptor
ERα	: Estrogen receptor α
ERβ	: Estrogen receptor β
ERK	: Extracellular signal-regulated kinase
FSH	: Follicle stimulating hormone
GPR30	: G protein-coupled receptor 30
GnRH	: Gonadotropin releasing hormone
h	: Hour(s)

HEK293 : Human embryonic kidney 293 cells

HEPES : 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HRP : Horseradish peroxidase

IgG : Immunoglobulin G

kg : Kilogram(s)

L : Liter(s)

LH : Luteinizing hormone

MAPK : Mitogen-activated protein kinase

MCF7 : Michigan cancer foundation-7 cells

min : Minute(s)

MIX : 3-isobutyl-1-methyl-xanthine

mL : Mililiter(s)

mM : Millimolar

mRNA : Messenger ribonucleic acid

n : Number

NCBI : National Center for Biotechnology Information

ng : Nanogram(s)

NIDDK : National Institute of Diabetes and Digestive and Kidney Diseases

nl : Nanoliter

nM : Nanomolar

oFSH : Ovine follicle stimulating hormone

oLH : Ovine luteinizing hormone

PBS : Phosphate buffered saline

PCR : Polymerase chain reaction

pg : Picogram

PKA : Protein kinase A

PLSD : Protected least significant difference

RIA : Radioimmunoassay

RNA : Ribonucleic acid

RT-PCR : Reverse transcription polymerase chain reaction

SD : Standard deviation

SEM	: Standard error of mean
TSH	: Thyroid stimulating hormone
µL	: Microliter
wk	: Week(s)

CHAPTER I

General Introduction

1.1. Background information

Estradiol secreted from the ovaries is the most important feedback regulator for both the hypothalamus and pituitary in controlling the secretion of gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH) in animals. During the luteal phase of the estrous cycle, blood concentrations of estradiol fluctuate between 0.004 nM (1 pg/mL) and 0.030 nM (8 pg/mL) in heifers and cows (Endo et al., 2012; Spicer and Echternkamp, 1986). Such low concentrations exert a negative feedback effect on GnRH secretion from the arcuate nucleus in the hypothalamus (Clarke, 1995; Evans et al., 1994; García-Galiano et al., 2012); and suppress LH mRNA expression in the anterior pituitary (AP) of ewes (Mercer et al., 1993). In contrast, increased estradiol secretion from the dominant follicle before ovulation leads to blood estradiol concentrations that exceed 0.037 nM (10 pg/mL) (Spicer and Echternkamp, 1986), inducing a pre-ovulatory GnRH surge from the preoptic area (Smith et al., 2009). The timing of this pre-ovulatory surge in GnRH, which is followed by a LH surge, requires the pituitary to exhibit increased sensitivity to GnRH (Nett et al., 1984). Abnormalities of this feedback induce various reproductive diseases, including follicular cysts in cows (Todoroki and Kaneko, 2006).

To induce these important feedback effects from ovary to hypothalamus and pituitary, estradiol binds to nuclear-localized estrogen receptors α or β (ERα or ERβ) and alters genomic gene transcription (Hewitt and Korach, 2003). The pivotal roles of ERα in particular have been well established in the mediation of estrogen action (Gieske et al., 2008; Sánchez-Criado et al., 2012).

In addition to the genomic feedback effects, recent studies have suggested that estradiol initiates a signaling cascade associated with an undefined plasma membrane

receptor in the pituitary. Arreguin-Arevalo and Nett (2005) reported that 60 min of pretreatment with 0.1 nM or higher concentration of estradiol or estradiol conjugated with BSA (E_2-BSA), an impermeable estradiol analog, suppresses GnRH-stimulated LH release in primary AP cell culture derived from ovariectomized ewes. These authors also reported that 10 nM and lower concentration of ERα- or ERβ-selective agonists have no significant effect on the GnRH-stimulated LH release from ovine AP cells. Therefore, their study suggests an important role for this undefined plasma membrane receptor. Furthermore, this receptor may control sensitivity of the AP against GnRH using a cytoplasmic signaling cascade, which includes early events within 15 min after estradiol injection in ewes (Iqbal et al., 2007). Therefore, this undefined plasma membrane receptor has an important role in the estradiol inhibition of GnRH-stimulated LH release from the ovine AP.

G protein–coupled receptor 30 (GPR30; also known as G protein-coupled estrogen receptor 1) is a 354–amino acid protein. GPR30 is a plasma membrane estradiol receptor that can bind estradiol-GPR30 to initiate several rapid, non-genomic signaling events in the cytoplasm (Maggiolini and Picard, 2010). Recently, GPR30 has been identified in the plasma membrane and cytoplasm of a variety of target tissues, including the rat brain and AP, using immunohistochemistry and *in situ* hybridization (Brailoiu et al., 2007; Hazell et al., 2009). However, the role of GPR30 in LH secretion from gonadotropes has not been investigated in any species, including ruminants.

Recently, a novel plasma membrane estradiol receptor involved in mediating non-genomic effects was identified, although its structure, or even its mRNA and amino acid sequences, have yet to be elucidated (Qiu et al., 2003). Since this receptor can be probed by a non-steroidal diphenylacrylamide compound called STX, it is referred to as the STX receptor. STX is the ligand that specifically binds to the plasma membrane estradiol

receptor, but not to ERα, ERβ, or GPR30 (Kenealy et al., 2011; Qiu et al., 2003; Tobias et al., 2006). STX receptors play important roles in inducing estradiol's rapid effects in GnRH-producing neurons (Kenealy et al., 2011; Zhang et al., 2010). To the best of our knowledge, the effects of STX on LH secretion from gonadotropes have not been clarified in any species.

Cytoplasmic mechanism for rapid estradiol suppression of LH secretion from AP cells is an important fundamental knowledge, although little is known also in other biological fields. Hsieh et al. (2007) used protein kinase A (PKA) inhibitor, H89, to clarify that PKA pathway is the main pathway for effect of estrogen in attenuating liver injury after trauma-hemorrhage. PKA pathway is the important pathway to control FSHβ gene expression in the gonadotrope-derived LβT2 cells (Thompson et al. 2013). Ishida et al. (2010) utilized H89 and ERK1/2/5 inhibitor, U0126, to report that both ERK1/2/5 pathway and PKA pathway are important pathways for genomic effect of estradiol in lactotroph. However, it is not clear whether PKA pathway and ERK1/2/5 pathway are the pathways in the downstream of GPR30 to suppress LH secretion in non-genomic mechanism.

Cyclic AMP is the key molecule in the cytoplasmic pathway to increase LH secretion from ovine gonadotrope cells, by modulating Ca2+-activated K+ channels (Adams et al., 1979; Sikdar et al., 1989). The cAMP production is attenuated by GPR30 on the plasma membrane of various cells including HEK293, CHO, and MCF7 cells ectopically expressing GPR30 (Broselid et al., 2014; Mo et al., 2013). Therefore, cAMP pathway may be the pathway for estradiol suppression of LH secretion in non-genomic manner. However, it is remained to clarify whether estradiol pre-treatment has any effect on the cAMP increment in AP cells.

Therefore, this study examined the hypothesis that GPR30 is expressed in the

bovine AP and mediates estradiol inhibition of GnRH-induced LH release by a non-genomic mechanism. The next study was planned to investigate the hypothesis that the STX receptor mediates the rapid, negative estradiol feedback effect on the AP by evaluating whether STX suppress GnRH-induced LH release from cultured bovine AP cells. After clarifying the important receptor to induce estradiol inhibition of GnRH-induced LH release, another study was conducted to verify absence of effect of the short time estradiol treatment on mRNA expressions of LH subunits genes. Furthermore, this dissertation study was conducted to clarify cytoplasmic pathway in the downstream of GPR30.

1.2. Objectives of the study

Based on the above background information, this dissertation study was conducted with the following main objectives:

i. To determine the expression of GPR30 in bovine AP.

ii. To determine the role of GPR30 in mediating estradiol inhibition of GnRH-induced LH release.

iii. To determine the possible role of STX in mediating estradiol inhibition of GnRH-induced LH release.

iv. To determine the effect of GPR30 antagonist, G36, in estradiol inhibition of GnRH-induced LH release.

v. To determine the effect of short-time estradiol treatment on the amount of mRNA of LHα, LHβ and FSHβ subunits.

vi. To determine the effect of U0126 and H89 on estradiol or G1 inhibition of GnRH-induced LH release.

vii. To determine the effect of estradiol on cAMP production.

viii. To report the abnormal response to estradiol in heifers with quiescent ovary or cystic follicle.

1.3. Contents of dissertation

This dissertation consists of ten chapters. Chapter I (General Introduction) deals with the background information and main objectives of the study. In Chapter II, we have reviewed the literatures directly or indirectly related to our study. Chapter III clarified the expression of GPR30 in bovine AP and the effects of estradiol and GPR30 agonist, G1, on GnRH-induced LH secretion. In Chapter IV, we examined the effects of STX, a novel estrogen membrane receptor agonist, on GnRH induced LH secretion from cultured bovine AP cells. In Chapter V, we examined the effects of GPR30 antagonist, G36, on estradiol suppression of GnRH induced LH secretion in bovine AP. Chapter VI evaluated the effects of estradiol on the amount of mRNA for LHα, LHβ and FSHβ subunits in cultured bovine AP. Chapter VII examined the effects of protein kinase inhibitors, U0126 and H89, on estradiol suppression of GnRH-induced LH release in cultured bovine AP. Chapter VIII clarified the effect of estradiol on cAMP production from cultured bovine AP. Chapter IX reported abnormal response of AP to estradiol in heifers with quiescent ovary and cystic follicle. Finally, Chapter X discussed the main findings of this dissertation study and their implications.

CHAPTER II

Review of Literature

2.1. Importance of LH in reproduction

LH is a glycoprotein hormone consist of two different subunits, α and β subunits (Pierce and Parsons, 1981). Within a species, α subunit is common among three glycoprotein hormones in the AP. They are LH, follicle-stimulating hormone (FSH) and thyroid-stimulating hormone (TSH), whereas the β subunit is diverse among the three hormones and provide the biological specificity of each hormone (Pierce and Parsons, 1981). It has been proposed that the constant levels of gonadotropin subunit messenger ribonucleic acids (mRNAs) play an important function in the biosynthesis of gonadotropin subunits, and are probably an important regulating site in the maintenance of gonadotropin homeostasis (Zmeili et al., 1986).

In the female, circulating levels of LH are essential for the production of steroid hormones that regulate the timing of ovulation and target tissue responses, as well as maintenance of the corpus luteum and therefore early pregnancy. LH stimulates the ovary to secrete estradiol, progesterone, and androgens in a cyclic manner and serves as the signal for ovulation. The LH surge induces multiple events such as the onset of oocyte meiotic maturation, granulosa cell luteinization, and corpus luteum establishment as well as the rupture of the dominant follicle conceding for the ovulation (Conti et al., 1998; Richards et al., 1998).

In the follicular phase, LH predominantly stimulates theca cells to produce androgens (Fortune and Armstrong, 1977). In the granulosa cells of the maturing ovarian follicle, cytochrome P_{450} aromatase converts androgens into estradiol (Hillier, 1994). At mid cycle, a dramatic spike in the release of LH triggers by estradiol has a positive feed-back effect on the hypothalamus (Ferin et al., 1974).

Besides of inducing ovulation, the LH surge also induces the formation of the corpus luteum. For this process, an adequate amplitude and duration of the LH surge are also important (Chandrasekher et al., 1994). During this phase, LH continues to stimulate the differentiated granulosa cells in the corpus luteum to produce estradiol (Sasano and Suzuki, 1997), progesterone (Richards et al., 1998) and inhibin A (McLachlan et al., 1989). These steroid hormones act upon the endometrium to make it receptive to embryo implantation.

LH is secreted in a pulsatile manner during the bovine estrous cycle; the frequency of the LH pulses decreases from the early- to the mid-luteal phase, and then increases up to the follicular phase (Rahe et al., 1980; Peters et al., 1994). LH is also released in large amounts for about 10 h in the preovulatory period; this is referred to as the "LH surge" and induces ovulation (Rahe et al., 1980; Kawate et al., 1996; Kawate et al., 1997). The variation in bovine LH content of the AP has been also examined during the estrous cycle (Desjardins and Hafs, 1968; Nett et al., 1987); it declines immediately after the LH surge, and rises from the mid-luteal to the follicular phase prior to the LH surge.

2.2. The declining of cattle reproduction and LH as possible cause

Recently, the declining of cattle reproduction in the world brings great emphasis focused on the reproductive endocrinology and physiology in cattle. This requires a comprehensive understanding of the endocrine and functional changes together with the reproductive functions of the animals.

In bovine reproduction, LH secretion is one of the important reproductive performance limiting factors, because LH plays an important role in control follicular maturation, ovulation, and development and maintenance of the corpus luteum (Fortune,

1994; Hansel and Blair, 1996). Several studies have linked LH with detrimental effects on reproductive function, such as irregular estrus cycles, anovulation, infertility, and abortus (Shoham et al., 1993).

Dairy cows have the low concentration of pulsatile LH secretion in early postpartum period (Kadokawa et al., 2000a, 2006). Such LH secretion is the one of important reason for the delayed resumption of reproductive function in postpartum (Kadokawa et al., 2000b).

A reduction in the LH preovulatory surge could conceivably lead to delayed ovulation (Siddiqui et al., 2010). Pulsatile LH secretion, which is important to stimulate estradiol secretion, is suppressed in dairy cows during summer (Gilad et al., 1993). This is one possible reason for reduced estrous behavior and extended estrous cycle (Wilson et al., 1998). Kanai et al. (1995) also reported a suppressed LH surge response to GnRH in female goats under heat stress. Recently, suppression of pulsatile LH release and the preovulatory LH surge reported previously in hot climates (Wise et al., 1988; Gilad et al., 1993; Chebel et al., 2004) has been also reported to occur in dairy cows in Hokkaido in northern Japan (Kadokawa, 2007).

2.3. The importance of estradiol for controlling LH secretion

Undoubtedly the gonadal steroid estradiol is the most critical factor to regulate the ovulatory cycle via both negative and positive feedback on the hypothalamic–pituitary axis in all animals studied to date. During the luteal phase of the estrous cycle, blood concentrations of estradiol fluctuate between 0.004 nM (1 pg/mL) and 0.030 nM (8 pg/mL) in heifers and cows (Endo et al., 2012; Spicer and Echternkamp, 1986). Such low concentrations exert a negative feedback effect on GnRH secretion from the arcuate

nucleus in the hypothalamus (Clarke, 1995; Evans et al., 1994; Garcia-Galiano et al., 2012); and suppress LH mRNA expression in the AP of ewes (Mercer et al., 1993). In contrast, increased estradiol secretion from the dominant follicle before ovulation leads to blood estradiol concentrations that exceed 0.037 nM (10 pg/mL) (Spicer and Echternkamp, 1986), inducing a pre-ovulatory GnRH surge from the preoptic area (Smith et al., 2009). The timing of this pre-ovulatory surge in GnRH, which is followed by a LH surge, requires the AP to exhibit increased sensitivity to GnRH (Nett et al., 1984). Abnormalities of estradiol feedback induce various reproductive diseases, including follicular cysts (Todoroki and Kaneko, 2006).To induce these important feedback effects from ovary to hypothalamus and AP, estradiol binds to nuclear-localized ERα or ERβ and alters genomic gene transcription (Hewitt and Korach, 2003). The pivotal roles of ERα in particular have been well established in the mediation of estrogen action (Gieske et al., 2008; Sanchez-Criado et al., 2012).

2.4. The cytoplasmic pathways for estradiol effect

Recent studies have suggested that estradiol initiates a signaling cascade associated with signaling pathways leading to calcium influx (Chaban et al., 2004), cAMP (Abraham et al., 2003), nitric oxide production, phospholipase C activation, or inositol phosphate generation (Le Mellay et al., 1997). The MAPK/ERK1/2/5 pathway can also be rapidly activated by estrogens in various cell types, such as endothelial (Chen et al., 2004), adipocyte (Dos Santos et al., 2002), neuroblastoma (Watters et al., 1997), or breast cancer cell lines (Migliaccio et al,. 1996). Membrane activation of these rapid signaling cascades will then modulate gene transcription (Vasudevan and Pfaff, 2007). Estradiol has a high affinity for ERβ and triggers a suppressive effect in JKT-1 cells, whereas E2-BSA, which

prevents membrane crossing, binds to non-classical membrane ERs and promotes cell proliferation by activating rapid cell signaling, including PKA pathway (Bouskine et al., 2008).

2.5. Estrogen receptors (ERs)

ERs have been identified as nuclear transcription factors by O'Malley and Means (1974). Estrogen signals through at least two receptors, which are the classical ERα and ERβ. Also GPR30 has discovered recently (Revankar et al., 2005; Thomas et al., 2005). Some groups also have proposed additional presence of an unidentified membrane ER referred to as the STX receptor that can be probed by a non-steroidal diphenylacrylamide compound called STX (Qiu et al., 2003).

2.6. GPR30

GPR30 used to be an orphan member of the 7-transmembrane receptor family, which was first identified in the late 1990's (O'Dowd et al., 1998; Carmeci et al., 1997; Takada et al., 1997; Owman et al., 1996). Unlike classical ERs, GPR30 has significantly different physical properties. GPR30 has been identified as a membrane associated receptor in 2005 (Thomas et al., 2005). GPR30 does not act directly as a transcription factor (Maggiolini et al., 2004; Albanito et al., 2007).

2.7. GPR30 dependent cellular functions

Filardo et al. (2000) investigated the functional role of GPR30 in the rapid activation of kinases by estrogen in breast cancer cells. They revealed that estrogen-mediated activation of ERK1/2/5 in ER-negative SKBr3 cells as well as GPR30-

transfected ER-negative MDA-MB-231cells. These cells displayed expression of GPR30 correlated with the functional response to estrogen. Another study of this group suggested that ER-negative cells could preserve sensitivity to estrogen via the expression of GPR30 (Filardo, 2002).

Evidence showed that GPR30 likely binds estrogen. Thomas et al. (2005) revealed that estrogen treatment of GPR30-transfected cell membranes also led to the reduced production of cAMP. However, it is possible that GPR30 and ERs work in concert for some estrogen effects, for example, estrogen-induced thymic atrophy (Wang et al., 2008a).

2.8. GPR30 selective ligand, G1 and G36

Bologa et al. (2006) screened a library of approximately 10,000 compounds for chemical similarity to estrogen and tested the top 100 compounds for GPR30 activity. They found one compound displayed activity against GPR30, serving as an agonist of the receptor and termed G1. G1 was inactive against classical ERs, thus, represented the first selective GPR30 ligand (Bologa et al., 2006).

The discovery of a set of specific agonists G1 and antagonists (G15 and G36) of the GPR30 stimulated research and resulted in the discovery of a number of interesting GPR30-related effects, especially in the vascular endothelium, the central nervous system, and the endocrine pancreas (Smiley and Khalil, 2009; Zhang et al., 2010; Nadal et al., 2011).

Studies shown that G-1 was able to elicit calcium mobilization as well as PI3K activation in cells expressing GPR30, but not in cells expressing either ERα or ERβ (Bologa et al., 2006). Albanito et al. (2007) demonstrated that G-1, through GPR30,

induced gene expression of c-*fos* in an ERE-independent manner; however ERK1/2/5 pathway is activated in ovarian cancer cells.

Also in the field of reproductive physiology, important roles of GPR30 has been documented using G1, for example, in the estrogen-mediated stimulation of primordial follicle formation in the hamster ovary (Wang et al., 2008b), where GPR30 is expressed in both granulosa and theca cells and its expression is regulated by gonadotropins.

In the field of neurobiology, important role of GPR30 has also been documented using G1 (Kuhn et al., 2008; Alyea et al., 2008).

G1 activity was absent in GPR30 knockout mice, verifying the physiological activity of G-1 through GPR30 (Haas et al., 2009; Wang et al., 2009). G1 can work in bovine muscle satellite cell cultures (Kamanga-Sollo et al., 2008). Therefore, G1 can be used for various bovine cells.

G15 is the first generation of GPR30 antagonist, but now it is well-known that G15 acts as a partial agonist of classical ERα (Dennis et al., 2011). Dennis et al. (2011) developed selective antagonist of GPR30, G36. Then, they reported that G36 inhibits estrogen and G-1-mediated calcium mobilization as well as ERK1/2/5 activation.

CHAPTER III

(Study I)

Expression of Estradiol Receptor, GPR30, in Bovine Anterior Pituitary (AP) and Effects of GPR30 Agonist on GnRH-induced LH Secretion

Abstract

GPR30 is an estradiol receptor located on the plasma membrane, and it initiates several rapid, non-genomic signaling events. GPR30 has recently been identified in rat AP; however, little is known about the role of GPR30 in controlling LH secretion from gonadotropes in animals. To fill this research gap, we hypothesized that GPR30 is expressed in bovine AP and mediates estradiol inhibition of GnRH-induced LH release. We confirmed the expressions of GPR30 mRNA and protein by RT -PCR, western blotting, and immunohistochemistry. We cultured bovine AP cells (n = 8) for 3 days in steroid-free conditions and then treated them with increasing concentrations (0.001 nM, 0.01 nM, 0.1 nM, 1 nM, and 10 nM) of estradiol or a GPR30-specific agonist, G1, for 5 min before GnRH stimulation. As expected, estradiol at 0.001–0.1 nM inhibited the GnRH-stimulated LH secretion. However, we found also that G1 at 0.001 nM was able to inhibit this secretion ($P < 0.05$). In contrast, both estradiol and G1 at higher doses were less efficient in suppressing the GnRH-stimulated LH secretion. Neither estradiol nor G1 suppressed GnRH-stimulated FSH secretion. In separate experiments, fluorescent immunohistochemistry and immunocytochemistry revealed that approximately 50% of GPR30-positive cells express LH, and about 30% of LH-positive cells express GPR30. In conclusion, GPR30 is expressed in bovine gonadotropes and other AP cells and may partially contribute to rapid negative estradiol feedback of GnRH induced LH secretion.

3.1. Introduction

The secretion of estradiol from the ovaries is a powerful feedback regulator for both the hypothalamus and pituitary in controlling the secretion of GnRH and LH in all domestic animals studied to date. During the luteal phase of the estrous cycle, blood concentrations of estradiol fluctuate between 0.004 nM (1 pg/mL) and 0.030 nM (8 pg/mL) in heifers and cows (Endo et al., 2012; Spicer and Echternkamp, 1986). Such low concentrations exert a negative feedback effect on GnRH secretion from the arcuate nucleus in the hypothalamus (Clarke, 1995; Evans et al., 1994; Garcia-Galiano et al., 2012); and suppress LH mRNA expression in the AP of ewes (Mercer et al., 1993). In contrast, increased estradiol secretion from the dominant follicle before ovulation leads to blood estradiol concentrations that exceed 0.037 nM (10 pg/mL) (Spicer and Echternkamp, 1986), inducing a pre-ovulatory GnRH surge from the preoptic area (Smith et al., 2009). The timing of this pre-ovulatory surge in GnRH, which is followed by a LH surge, requires the AP to exhibit increased sensitivity to GnRH (Nett et al., 1984). To induce these important feedback effects from ovary to hypothalamus and AP, estradiol binds to nuclear-localized ERα or ERβ and alters genomic gene transcription (Hewitt and Korach, 2003). The pivotal roles of ERα in particular have been well established in the mediation of estrogen action (Gieske et al., 2008; Sánchez-Criado et al., 2012).

In addition to the genomic feedback effects, recent studies have suggested that estradiol initiates a signaling cascade associated with an undefined plasma membrane receptor in the AP. Arreguin-Arevalo and Nett (2005) reported that 60 min of pretreatment with 0.1 nM or higher concentration of estradiol or E2-BSA, an impermeable estradiol analog, suppresses GnRH-stimulated LH release in primary AP cell culture derived from

ovariectomized ewes. These authors also reported that 10 nM and lower concentration of ERα- or ERβ-selective agonists have no significant effect on the GnRH-stimulated LH release from ovine AP cells. Therefore, their study suggests an important role for this undefined plasma membrane receptor. Furthermore, this receptor may control sensitivity of the AP against GnRH using a cytoplasmic signaling cascade, which includes early events within 15 min after estradiol injection in ewes (Iqbal et al., 2007). Therefore, this undefined plasma membrane receptor has an important role in the estradiol inhibition of GnRH-stimulated LH release from the ovine AP.

GPR30 is a plasma membrane estradiol receptor that can bind estradiol-GPR30 to initiate several rapid, non-genomic signaling events in the cytoplasm (Maggiolini and Picard, 2010). Recently, GPR30 has been identified in the plasma membrane and cytoplasm of a variety of target tissues, including the rat brain and AP, using immunohistochemistry and *in situ* hybridization (Brailoiu et al., 2007; Hazell et al., 2009). However, the role of GPR30 in LH secretion from gonadotropes has not been investigated in any species, including ruminants. Therefore, this study examined the hypothesis that GPR30 is expressed in the bovine AP and mediates estradiol inhibition of GnRH-induced LH release by a non-genomic mechanism.

3.2. Materials and methods

All experiments were performed according to the Guiding Principles for the Care and Use of Experimental Animals in the Field of Physiological Sciences (Physiological Society of Japan) and approved by the Committee on Animal Experiments of the School of Veterinary Medicine, Yamaguchi University.

3.2.1. RT-PCR, sequencing of amplified products, and homology search in gene databases

Anterior pituitaries were collected from multiparous Japanese Black cows (n = 3) and treated with RNA later [Applied Biosystems (ABI), Foster City, CA, USA]. Total RNA was extracted from the AP using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). After digestion by DNase-I (Qiagen, Valencia, CA, USA), first-strand cDNA was synthesized by the transcription of 1 ng of total RNA using ReverTra Ace qPCR RT Master Mix (Toyobo Co. Ltd., Osaka, Japan) according to the manufacturer's protocol.

In order to determine the expression of GPR30 mRNAin the AP, PCR was performed using 1 of 2 pairs of primers designed by Primer Express Software v3.0 (ABI) based on reference sequences of bovine GPR30 [National Center for Biotechnology Information (NCBI) reference sequences of bovine GPR30 are XM 606236.3 and XM 002698169.1]. The expected PCR-product size of GPR30 using the first primer pair is 436 bp (nucleotides 206–641; forward primer: 5′-AGATGACCATCCCTGACCTG- 3′; reverse primer: 5′-GAGTAGCAGAGGCCGATGAC-3′). The expected PCR-product size of GPR30 using the second primer pair is 300 bp (nucleotides 195–494; forward primer: 5′-CTTCCGGGAGAAGATGACCAT-3′; reverse primer: 5′-

ACGGAGGCCATCCAGATGA-3′). Using a Veriti 96-well Thermal Cycler (ABI), PCR was performed using 50 ng of cDNA and polymerase with proofreading activity (KOD-plus-Neo, Toyobo Co. Ltd.) under the following conditions: 94 °C for 2 min for pre-denaturing, 5 cycles of 98 °C for 10 s and 74 °C for 30 s, 5 cycles of 98 °C for 10 s and 72 °C for 30 s, 5 cycles of 98 °C for 10 s and 70 °C for 30 s, and 35 cycles of 98 °C for 10 s and 68 °C for 30 s. PCR products were separated on 1.5% agarose gel by electrophoresis with a molecular marker (Gene Ladder 100 (0.1–2 kbp), Nippon Gene, Tokyo, Japan), stained with ethidium bromide, and observed using a transilluminator. The PCR products were purified with the NucleoSpin Extract II kit (Takara Bio Inc., Shiga, Japan) and then sequenced with a sequencer (ABI3130, ABI) using one of the PCR primers and the Dye Terminator v3.1 Cycle Sequencing Kit (ABI). The obtained sequences were used as query terms with which to search the homology sequence in the DDBJ/GenBankTM/EBI Data Bank using the nucleotide basic local alignment search tool (BLASTN) optimized for highly similar sequences (MEGABLAST), which is available on the NCBI website, and were compared with the most significant alignment.

3.2.2. Western blotting for GPR30

Anterior pituitaries were collected from multiparous Japanese Black cows (n = 3). The isolated tissues (100 mg) were frozen in liquid nitrogen, ground, and homogenized using the tissue protein extraction reagent (T-PER, Thermoscientific, Rockford, IL, USA) containing protease inhibitors (Halt protease inhibitor cocktail, Thermoscientific). Total protein content of each tissue homogenate was estimated using the bicinchoninic acid kit (Thermoscientific). Tissue samples (1 μg of total protein) were then loaded onto polyacrylamide gels with whole-cell lysate of MCF-7 mammary adenocarcinoma cells (sc-

2206, Santa Cruz, Heidelberg, Germany) as positive controls. Molecular weight markers ranging from 10 kDa to 170 kDa (PageRuler prestained protein ladder, 26616, Thermoscientific) were used to identify the bands for GPR30. The proteins were electrophoresed through preformed sodium dodecyl sulfate polyacrylamide gels (Criterion TGX precast gel, Bio-Rad, Hercules, CA). The gels were run at 200 V for 30 min. The proteins were then transferred to polyvinylidene fluoride membranes using the Transblot turbo transfer system (Bio-rad). Immunoblotting was performed with anti-GPR30 rabbit antibody (sc-48525-R, Santa Cruz; 1:1000 dilution) after treatment with blocking buffer containing 0.1% Tween 20 and 5% non-fat dry milk. Epitope mapping of the antigens (near the N-terminus of human origin) reveals that the region is conserved across species, and hence the antibody we used can detect GPR30 in the majority of animal species, including humans, mice, rats, dogs, pigs, and cattle. The anti-GPR30 rabbit antibody has been used for immunostaining MCF-7 breast cancer cells (Madeo and Maggiolini, 2010). Incubation with the antibody was done overnight at 4 °C. Following washes with 10 mM Tris–HCl (pH 7.6) containing 150 mM NaCl and 0.1% Tween 20, horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (KPL Inc., Gaithersburg, MD, USA; 1:50000 dilution) was added and incubated at 25 °C for 1 h. Protein bands were visualized using an ECL-Prime chemiluminescence kit (GE Healthcare, Amersham, UK) and a charge-coupled device (CCD) imaging system (LAS-3000 Mini, Fujifilm, Tokyo, Japan).

3.2.3. Immunohistochemistry of bovine pituitary using anti-GPR30 antibody

Multiparous Japanese Black cows (n = 3) were stunned using a captive bolt pistol, and were then exsanguinated by a throat cut. Within 5 min of slaughter, the heads were placed on ice. Within 15 min of slaughter, pituitaries had been dissected at the midline, and

the APs were separated from the posterior pituitaries, cut into small blocks (<0.8 cm thick), and fixed in 4% paraformaldehyde at 4 °C for 16 h. After fixation, the blocks were placed in 30% sucrose diluted in PBS until infiltrated with sucrose. The blocks were then frozen in an embedding medium (Tissue-Tek OCT compound, Sakura Finetechnical Co. Ltd., Tokyo, Japan) and maintained at −80 °C until sectioning into 15-μm sagittal sections using a cryostat (CM1900, Leica Microsystems Pty Ltd., Wetzlar, Germany) and mounting on microscope slides (MAS coat Superfrost, Matsunami-Glass, Osaka, Japan). After treatment with 0.3% hydrogen peroxide in PBS for 10 min to inactivate endogenous peroxidase, followed by rinsing and treatment with 0.3% Triton X-100 in PBS for 15 min, the sections were incubated with 0.5 mL of PBS containing 10% normal goat serum for blocking for 1 h. The sections were then incubated overnight at 4 °C in PBS containing the same anti-GPR30 rabbit antibody as described above (1:1000 dilution) and 0.5% normal goat serum. After overnight incubation with the primary antibody, the sections were washed thoroughly and processed for 3,3′-diaminobenzidine (DAB) staining using a commercial kit (EnVision+ DualLink System-HRP, K4063, DakoCytomation, Carpinteria, CA, USA). Briefly, sections were incubated with 1 drop of goat anti-rabbit IgG conjugated to HRP-labeled polymer for 1 h, followed by washing and a final incubation with 1 mL of DAB chromogen substrate solution (Liquid DAB+ substrate chromogen system, K3467, DakoCytomation) for 20 min. The stained sections mounted on microscope slides were dehydrated in ethanol, cleared in xylene, and covered with a slip using a mountant (DPX, 360294H, BDH Laboratory Supplies, Poole, UK) for microscopic observation (Eclipse E800, Nikon, Tokyo, Japan) attached to a CCD camera (Pixera600ES, Pixera Japan, Kawasaki, Japan) and its controller (Studio3.0.1, Pixera Japan). To verify the specificity of the signals, we included several negative control sections in which the primary antibody

had been omitted or preabsorbed with 5 nM GPR30 protein (sc-2206, Santa Cruz) or in which negative control rabbit IgG (Wako Pure Chemicals, Osaka, Japan) had been used instead of the primary antibody.

3.2.4. Pituitary cell culture and analysis of the effects of estradiol and GPR30-specific agonist, G1, on GnRH-induced LH and FSH secretion

G*Power 3 for windows (Faul et al., 2007) was used to estimate the required number of APs with an error probability of 0.05 and a statistical power of 0.95. Nett et al. (1987) reported that the amounts of LH and GnRH receptors in AP were higher during the luteal phase than during the immediate post-estrus period in heifers. Therefore, post-pubertal Japanese Black heifers in the middle of the luteal phase (n = 8, 26 months old) were stunned using a captive bolt pistol and then exsanguinated by cutting of the throat. The heads were placed on ice-cold saline. Then, APs were obtained, stored in ice-cold 25 mM HEPES buffer (pH 7.2) containing 10 mM glucose and transported on ice to the laboratory. The experiment was repeated 8 times with each of the 8 different pituitary glands, using 4 wells per treatment. Each experiment began with enzymatic dispersal of AP cells using a method previously described (Hashizume et al., 1994) and confirmation of cell viability of greater than 90% by Trypan blue exclusion. Total cell yield was $19.8 \times 10^6 \pm 0.8 \times 10^6$ cells per pituitary gland. The dispersed cells were then suspended in phenol red – free Dulbecco's Modified Eagle's Medium (DMEM; 21063-029, Gibco, Grand Island, NY, USA) containing 1% nonessential amino acids (100×; Gibco), 100 IU/mL penicillin, 50 μg/mL streptomycin, 10% horse serum (Gibco), and 2.5% fetal bovine serum (Gibco). Both the horse serum and the fetal bovine serum had been previously treated with dextran coated charcoal to remove steroid hormones. After the cells (2.5×10^5 cells/mL, total 0.5

mL) had been plated in 24- well culture plates (MS-80240, Sumitomo Bakelite, Tokyo, Japan), they were maintained at 37°C in a humidified atmosphere of 5% CO_2 for 82 h. The wells were washed twice with PBS and then incubated with 490 µL DMEM containing 0.1% BSA for 2 h. Pretreatment was performed by adding 5 µL of DMEM alone or 5 µL of DMEM containing various concentrations (from 0.1 nM to 1000 nM) of estradiol (052-04041, Wako Pure Chemicals) or a GPR30-specific agonist (Bologa et al., 2006), G1 (G6798, Sigma–Aldrich, Saint Louis, MI, USA). The cells were incubated while gently shaking for 5 min, and then, cells were treated with 5 µL of 100 nM GnRH (Peptide Institute Inc., Osaka, Japan) dissolved in DMEM for 2 h in order to stimulate LH secretion. The pretreatment plus the GnRH treatment yielded a final concentration of 0.01 nM, 0.1 nM, 1 nM, or 10 nM estradiol or G1 in the wells that had received 1 nM, 10 nM, 100 nM, or 1000 nM of estradiol or G1, respectively, and a final concentration of 1 nM of GnRH. In the preliminary study, LH secretion was stimulated by increasing amounts of GnRH, with a peak at 1 nM GnRH, and reducing secretion at GnRH concentrations higher than 1 nM. Therefore, we used 1 nM of GnRH in this study. The "control" wells contained 5 µL of DMEM that had not undergone pretreatment with estradiol or G1 for 5 min or incubation with GnRH for 2 h. The "GnRH" wells contained 5 µL of DMEM that had not undergone pretreatment with estradiol or G1 for 5 min but had been incubated with GnRH for 2 h. After incubation with GnRH, the medium was collected for radioimmunoassay (RIA) of LH.

3.2.5. Radioimmunoassay to measure gonadotropin concentration in culture media

LH concentrations in the culture media were assayed in duplicate by double antibody RIA using ^{125}I-labeled bLH and anti-oLH-antiserum (AFP11743B and

AFP192279, National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), Bethesda, CA, USA). The limit of detection was 0.40 ng/mL. At 2.04 ng/mL, the intra- and inter-assay coefficients of variation were 3.6% and 6.2%, respectively. Follicle-stimulating hormone (FSH) concentrations in the culture media were assayed in duplicate by double antibody RIA using ^{125}I-labeled bFSH and anti-oFSH antiserum (AFP5332B and AFPC5288113, NIDDK). The limit of detection was 0.20 ng/mL. At 4.00 ng/mL, the intra- and inter-assay coefficients of variation were 4.3% and 7.1%, respectively.

3.2.6. Dual fluorescent immunohistochemistry and confocal microscopic observation

In order to evaluate co-localization between GPR30 and LH in AP tissues, APs were collected from multiparous Japanese Black cows (n = 3), and tissues were prepared using the immunohistochemistry protocol described above. Incubation in a cocktail of primary antibodies (anti-GPR30 rabbit antibody, 1:1000; and anti-LH mouse monoclonal antibody (Matteri et al., 1987), 1:1000; 12 h, 4°C) was followed by a cocktail of fluorochrome conjugated secondary antibodies (4 μg/mL Alexa Fluor 488 Goat Anti-rabbit IgG (Invitrogen) and 4 μg/mL Alexa Fluor 546 Goat Anti-Mouse IgG (Invitrogen) and 1 μg/mL of 4′,6′-diamino-2-phenylindole (DAPI; Wako Pure Chemicals) for 2 h at room temperature. The anti-LH mouse monoclonal antibody recognizes the LHα subunit and does not cross-react with other pituitary hormones (Iqbal et al., 2009). The stained sections were mounted on microscope slides and covered with a slip using a mountant (Vectashield hard set mounting medium, Vector Laboratories, Burlingame, CA, USA) for confocal microscopic observation (LSM710, Carl Zeiss, Gottingen, Germany). The fluorescent microscopic images were scanned with × 63 oil immersion objectives and recorded by a

CCD camera to prepare confocal images (ZEN2010, Carl Zeiss). The overlap between GPR30 and LH immunoreactivities was assessed in confocal images of dual-immunofluorescent specimens. The percentages of single-labeled and double labeled GPR30-immunoreactive and LH-immunoreactive cells were also determined quantitatively from the specimens using 8 representative confocal images per pituitary.

3.2.7. Imaging cytometry analysis to evaluate co-localization between GPR30 and LH in the cultured AP cells

Anterior pituitary cells derived from multiparous Japanese Black cows (n = 4) were cultured using the protocol described above for 3 days in a 24-well plate. The cells were treated with 0.3% hydrogen peroxide in PBS for 10 min and 0.3% Triton X-100 in PBS for 15 min. The treated cells were incubated with the primary antibody cocktail described above for 12 h at 4 ^{o}C and then incubated with the same cocktail of fluorochrome-conjugated secondary antibodies and DAPI for 2 h at room temperature. Image acquisition was performed on an IN Cell Analyzer 2000 microscope (GE Healthcare). Images from DAPI and Alexa Fluor 488 and 546 staining were monitored through a ×20 objective and acquired by a large CCD camera (2048 × 2048 pixel, 7.4 μm/pixel). Analysis of co-localization of GPR30 and LH was performed using Developer toolbox ver.1.6 software (GE Healthcare). A total of more than 1800 cells per field were typically analyzed, and up to 9 fields (5.198 mm^2 as total area of the 9 fields) per well were captured in experiments performed in duplicate, i.e., in each experiment, data were derived from 20519.4 ± 1302.2 individual cells per pituitary gland.

3.2.8. Data analysis

LH concentrations in the control samples for each pituitary were averaged, and the mean value was set at 100%. LH concentrations in the treated samples for each pituitary were averaged, and the mean LH values were expressed as a percentage of the control value. FSH concentrations in the control samples for each pituitary were averaged, and the mean value was set at 100%. FSH concentrations in the treated samples for each pituitary were averaged, and the mean FSH values were expressed as a percentage of the control value. Data were analyzed using Statview version 5.0 for Windows (SAS Institute, Inc., Cary, NC, USA). The statistical significance of differences in LH or FSH concentration were analyzed by one-factor analysis of variance (ANOVA) followed by post hoc comparisons using Fisher's protected least significant difference (PLSD) test. The level of significance was set at $P < 0.05$. Data are expressed as mean ± standard error of the mean (SEM).

3.3. Results

3.3.1. Expression of GPR30 mRNA and protein in bovine AP

The expected sizes of PCR product (436 bp and 300 bp) were observed after agarose gel electrophoresis (**Fig. 3.1a**). Homology searches in the gene databases for the obtained sequence of amplified products using the first primer pair revealed that the best match alignment was bovine GPR30 (XM 002698169.1), which had a query coverage of 100%, an e-value of 0.0, and a maximum alignment identity of 99%. Homology searches using the second primer pair revealed that the best match alignment was also bovine GPR30 (XM 606236.3), which had a query coverage of 100%, an e-value of 1e-118, and a maximum alignment identity of 97%. No other bovine gene was found to have homology for the obtained sequences of both amplified products, leading to the conclusion that the sequences of the amplified products were identical to the sequence of bovine GPR30. The presence of GPR30 in bovine AP was verified by western blot analysis (**Fig. 3.1b**). The molecular weight of GPR30 was found to be 39.5 kDa, which is similar to the expected molecular weight. In peroxidase-based immunohistochemistry, positive stained cells were observed in the sections that had been stained with anti-GPR30 antibody (**Fig. 3.2a**) but not with anti-GPR30 antibody pre-absorbed with GPR30 protein (**Fig. 3.2b**).

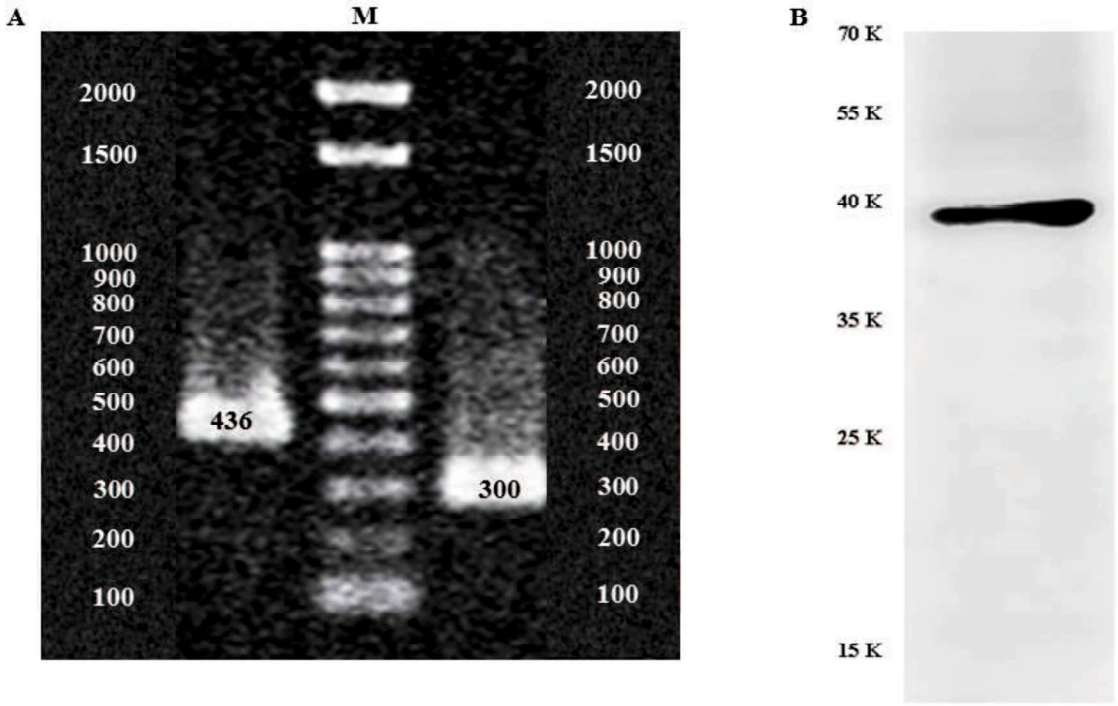

Fig. 3.1. Expression of GPR30 mRNA detected by RT-PCR (A) and GPR30 protein detected by western blotting with anti-GPR30 antibody (B). (A) Electrophoresis of PCR-amplified DNA products using 1 of 2 pairs of primers for bovine GPR30 and cDNA derived from bovine anterior pituitary (AP). Left and right lanes demonstrate that the DNA products obtained were of the size that had been expected—436 bp and 300 bp, respectively. The central lane (M) is the DNA marker. (B) Result of western blotting using anti-GPR30 antibody and extract from female bovine AP, indicating protein expression of GPR30 at 39.5 kD.

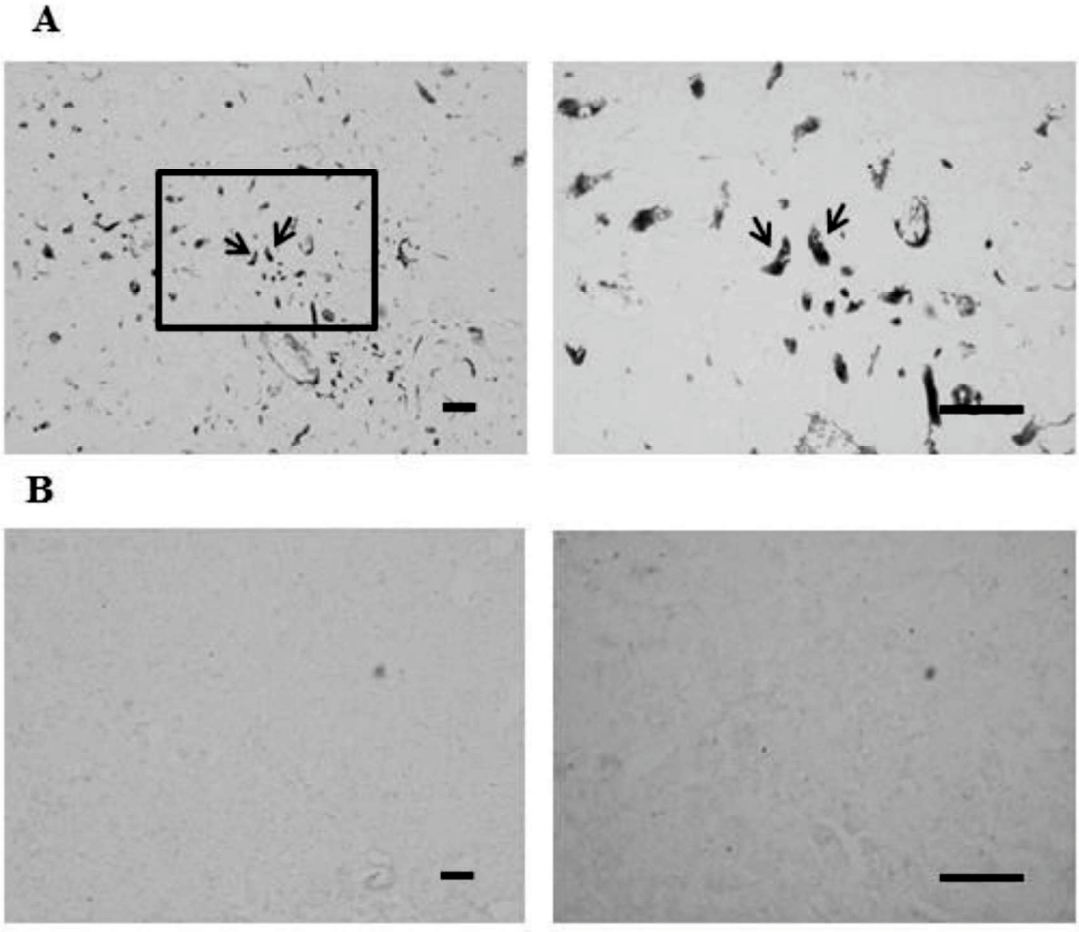

Fig. 3.2. Immunohistochemistry of cells stained with anti-GPR30 antibody (A), or anti-GPR30 antibody pre-absorbed with GPR30 protein (B). Left panels show stained cells as indicated by arrows in lower magnification, and right panels show stained cells in higher magnification. Scale bars are 100 μm.

3.3.2. Effects of estradiol and G1 on LH and FSH secretion from cultured AP cells

Fig. 3.3 shows the effect of various concentrations of estradiol and G1 on GnRH-stimulated LH secretion from the cultured AP cells. The ANOVA had sufficient statistical power (0.96) to show a significant effect of different additives on GnRH-stimulated LH secretion ($P < 0.05$). The LH concentration in the medium of GnRH wells (32.1 ± 2.7 ng/mL) was higher than in the control wells (18.7 ± 2.5 ng/mL). Estradiol at 0.001–0.1 nM inhibited the GnRH-stimulated LH secretion. Also that G1 at 0.001 nM was able to inhibit this secretion ($P < 0.05$). In contrast, both estradiol and G1 increased at higher doses were less efficient to suppress the GnRH-stimulated LH secretion.

Fig. 3.4 shows the effect of various concentrations of estradiol and G1 on GnRH-stimulated FSH secretion from cultured AP cells. The effect of different additives was not significant.

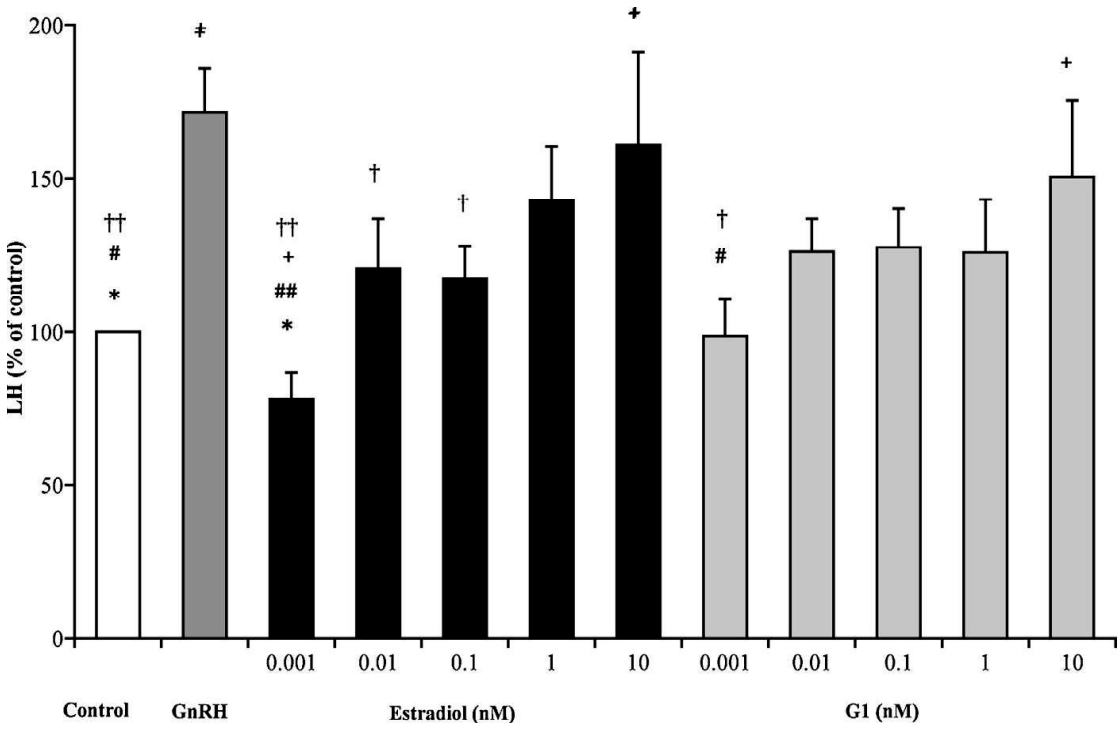

Fig. 3.3. Comparison of the effect of various concentrations of estradiol (black bars) or G1 (gray bars) in media containing 1 nM GnRH on luteinizing hormone (LH) secretion from cultured bovine AP cells. LH concentrations in control cells (cultured in medium alone) were averaged and the mean value set at 100%. LH concentrations of the treated groups are expressed as a percentage of the control. Each value represents the mean concentration ± standard error of the mean (SEM).

+$P < 0.05$: significant differences compared to the control.

$^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$: significant difference compared to GnRH.

$^{\#}P < 0.05$, $^{\#\#}P < 0.01$: significant differences compared to 10 nM estradiol.

*$P < 0.05$: significant differences compared to 10 nM G1.

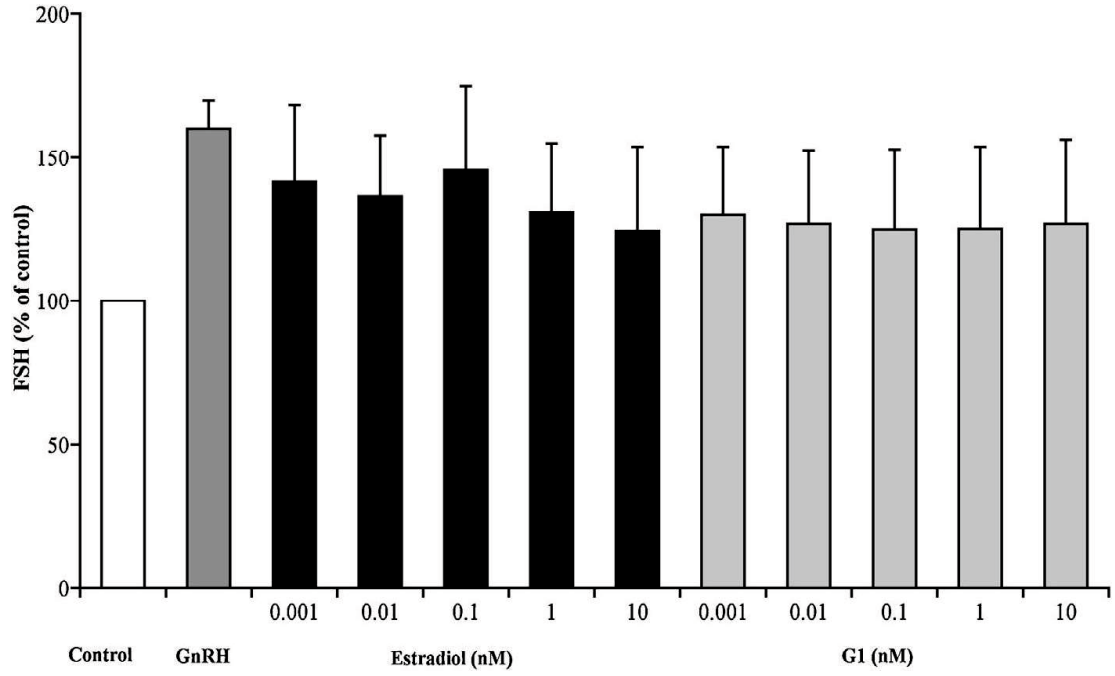

Fig. 3.4. Comparison of the effect of various concentrations of estradiol (black bars) or G1 (gray bars) in media containing 1 nM GnRH on follicle-stimulating hormone (FSH) secretion from cultured bovine AP cells. FSH concentrations in control cells (cultured in medium alone) were averaged and the mean value set at 100%. FSH concentrations of the treated groups are expressed as a percentage of the control. Each value represents the mean concentration ± SEM.

3.3.3. GPR30 and LH localization in the AP

Using dual-fluorescent immunohistochemistry, confocal microscopy showed GPR30-positive cells (green) and LH-positive cells (red) in bovine AP (**Fig. 3.5**). We found both no co-localization (**Fig. 3.5a**) and co-localization (**Fig. 3.5b**) between GPR30 and LH. In the analyses using 8 photos per pituitary gland, we observed 36.0 ± 4.0 GPR30-positive cells, 58.7 ± 6.8 LH-positive cells, and 16.7 ± 1.2 dual-positive cells. We observed that 28.7 ± 0.7% of the LH positive cells were also GPR30-positive, and 47.8 ± 4.3% of the GPR30-positive cells were also LH-positive in the AP specimens.

We further examined the localization of GPR30 and LH using cultured AP cells. Among the 20519.4 ± 1302.2 cultured AP cells per pituitary, the imaging cytometer detected 3414.1 ± 471.6 GPR30-positive cells, 6471.8 ± 1352.4 LH positive ells, and 1587.4 ± 227.2 dual-positive cells. The imaging cytometer revealed that 29.5 ± 4.7% of the LH positive cells were also GPR30-positive, and 52.5 ± 8.6% of the GPR30-positive cells were also LH-positive.

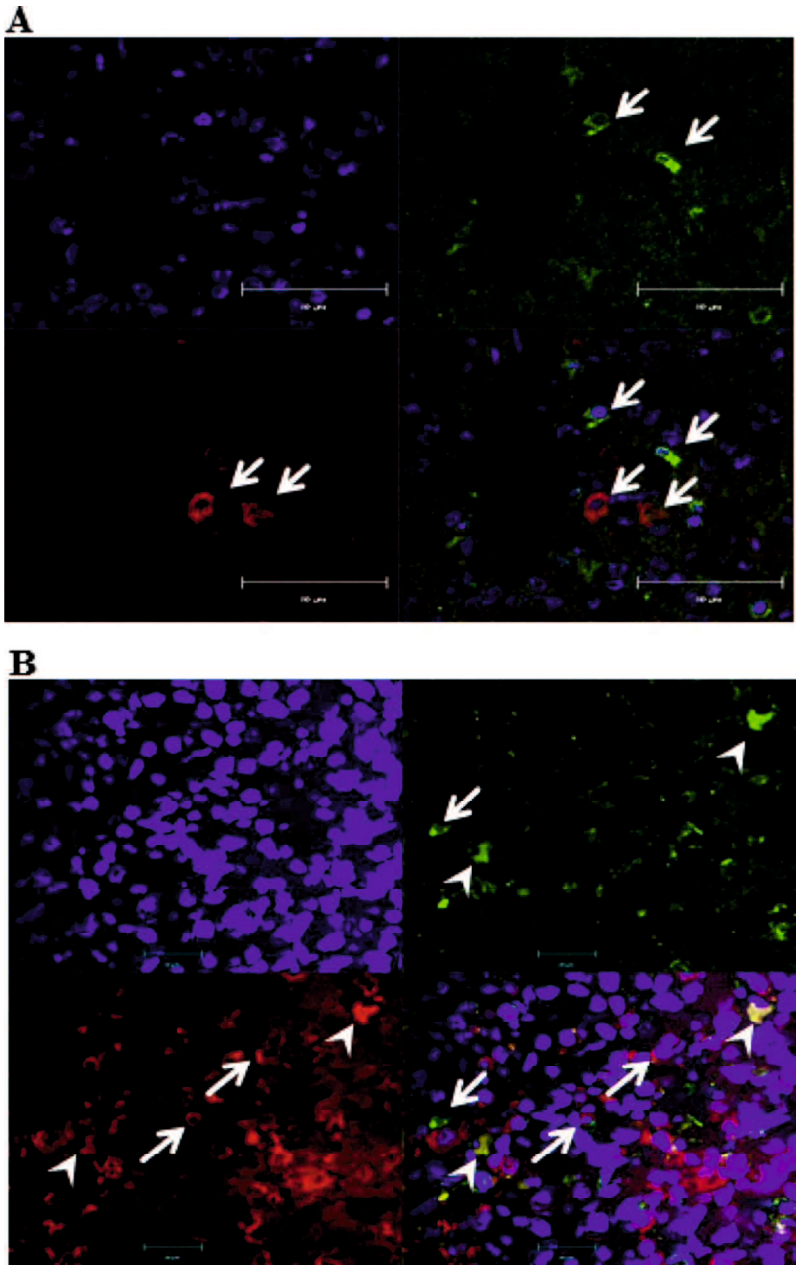

Fig. 3.5. Images of dual-fluorescent immunohistochemistry captured by a laser confocal microscope for GPR30 (green) and LH (red) with counter-staining by DAPI (blue) in bovine AP. Arrows show positively stained cells. Image A shows no co-localization between GPR30 and LH, whereas image B shows both co-localization (yellow), indicated by the arrowheads, and no co-localization, indicated by arrows between GPR30 and LH. Scale bars are 100 µm in the image A, and 50 µm in the image B.

3.4. Discussion

We observed the expression of GPR30 mRNA and protein in bovine AP, and showed by fluorescent immunohistochemistry that about 50% of GPR30-positive cells express LH, and about 30% of LH positive cells express GPR30 in bovine AP. These data suggested that estradiol might bind with GPR30 to induce rapid, non-genomic effects on both gonadotropes and other cell types in bovine AP. In this study, the 5-min pre-treatment with low concentrations of estradiol and G1 inhibited the GnRH stimulated LH secretion. In contrast, higher concentrations of both estradiol and G1 were less efficient in suppressing GnRH-stimulated LH secretion. Therefore, we need to discuss the dose-dependent non-genomic effect of estradiol, as well as the contribution of GPR30 in mediating this effect.

In the primary culture of individual bovine AP cells, 0.001–0.1 nM of estradiol significantly suppressed the GnRH-induced LH secretion. In contrast, although 0.001 nM of G1 significantly suppressed the GnRH-induced LH secretion, 0.01 nM and 0.1 nM of G1 showed only weak inhibition. Blood concentrations of estradiol fluctuate between 0.004 nM and 0.030 nM during the luteal phase of the estrous cycle in heifers and cows (Endo et al., 2012; Spicer and Echternkamp, 1986). Therefore, our data suggest only a small contribution of GPR30 to induce low estradiol suppression of LH secretion during the luteal phase. In this study, the fluorescent immunohistochemistry revealed that about one-third of the LH-positive cells expressed GPR30. Therefore, our data suggest that bovine AP may have another undefined, non-GPR30 plasma membrane receptor to induce low estradiol suppression of LH secretion.

This study is the first report to evaluate the rapid, non-genomic effect of estradiol on LH secretion in bovine. Previous studies have used 0.01–100 nM estradiol for 15 min or

60 min (Arreguin-Arevalo and Nett, 2005), or 1.0 nM estradiol for 5 min (Iqbal et al., 2007) in primary pituitary cell cultures derived from multiple ewes, which represents another ruminant species with seasonal reproduction. Our results were in conflict with these previous studies reporting that 1.0 nM and 10 nM of estradiol inhibited GnRH-stimulated LH release from cultured ovine pituitary cells. E2-BSA, as well as estradiol, increases GnRH analog binding to ovine gonadotropes through a nonclassical signaling mechanism (Davis et al., 2011). In this study, higher concentrations of both estradiol and G1 were less efficient in suppressing GnRH-stimulated LH secretion. One explanation is that compared to ovine gonadotropes, bovine gonadotropes might require a lower concentration of estradiol and a shorter time to increase GnRH analog binding. Another possible reason is that a third factor might be present in the primary pituitary culture, which might have an important role in estradiol suppression of GnRH-stimulated LH release. Both GnRH and estradiol are proximal regulators of LH secretion from gonadotropes; however, recent studies have revealed that various peptides (e.g., pituitary acting polypeptide (Winters and Moore, 2011), and kisspeptin (Richard et al., 2009; Suzuki et al., 2008) produced in the AP exert paracrine and autocrine functions to modulate LH secretion. Therefore, there might be many differences in the culture condition used in the present study and previous studies to induce different paracrine or autocrine functions of these peptides and to show the different responses in the GnRH-induced LH release against estradiol.

Our study did not show any rapid effect of estradiol or G1 on GnRH-stimulated FSH release. Arreguin-Arevalo and Nett (2006) reported that neither estradiol nor E2-BSA infusion for less than 4 h suppressed GnRH-stimulated mechanism. Further studies on gonadotropes are required to clarify the intracellular molecular mechanisms

downstream of GPR30 binding to estradiol that can inhibit LH secretion without changing FSH secretion.

Fluorescent immunohistochemistry and immunocytochemistry revealed that about half of GPR30-positive cells do not express LH. Such non-gonadotrope in the AP may be lactotropic, because recent studies reported that estradiol and E2-BSA have rapid effects on lactotropes to increase prolactin secretion (Christensen et al., 2011; Sosa et al., 2012). Another recent study reported that lactotropes in goldfish secrete the novel, recently discovered hormone, secretoneurin, to stimulate LH secretion from gonadotropes (Trudeau et al., 2012). Therefore, further studies are required to clarify the role of GPR30 for lactotropes in domestic animals.

In conclusion, GPR30 is expressed in both gonadotropes and other AP cells, and GPR30 may contribute to rapid negative estradiol feedback GnRH-induced LH secretion.

CHAPTER IV

(Study II)

Effects of STX, a Novel Estrogen Membrane Receptor Agonist, on GnRH-Induced LH Secretion from Cultured Bovine AP Cells

Abstract

STX is an agonist for a recently characterized membrane estrogen receptor whose structure has not been identified. We evaluated whether STX suppresses GnRH–induced LH release from bovine AP cells. We cultured AP cells (n =12) for 3 days in steroid-free conditions, followed by increasing concentrations (0.001, 0.01, 0.1, 1 and 10 nM) of estradiol or STX for 5 min before GnRH stimulation until the end of the experiment. Estradiol (0.001 to 0.1 nM) significantly suppressed GnRH-stimulated LH secretion, whereas STX did not affect GnRH-stimulated LH secretion at any of the tested concentrations. In conclusion, STX, unlike estradiol, possesses no suppressive effect on GnRH induced LH release from bovine AP cells.

4.1. Introduction

The study in chapter III suggested that GPR30 is expressed in bovine gonadotropes and may partially contribute to the rapid, negative estradiol feedback effect observed in GnRH-induced LH secretion. However, bovine gonadotropes may have another undefined plasma membrane receptor that mediates.

Recently, a novel plasma membrane estradiol receptor involved in mediating non-genomic effects was identified, although its structure, or even its mRNA and amino acid sequence, have yet to be elucidated (Qiu et al., 2003). Since this receptor can be probed by a non-steroidal diphenylacrylamide compound called STX, it is referred to as the STX receptor. STX is the ligand that specifically binds to the plasma membrane estradiol receptor, but not to ERα, ERβ, or GPR30 (Kenealy et al., 2011; Qiu et al., 2003; Tobias et al., 2006). STX receptors play important roles in inducing estradiol's rapid effects in GnRH-producing neurons (Kenealy et al., 2011; Zhang et al., 2010).

To the best of our knowledge, the effects of STX on LH secretion from gonadotropes have not been clarified in any species. Therefore, the present study was planned to investigate the hypothesis that the STX receptor mediates the rapid, negative estradiol feedback effect on the AP by evaluating whether STX suppresses GnRH-induced LH release from cultured bovine AP cells.

4.2. Materials and methods

4.2.1. Pituitary cell culture and analysis of the effects of STX on GnRH-induced LH secretion

Anterior pituitaries were obtained from post-pubertal Japanese Black heifers in the middle of the luteal phase (n = 12, 26 months old). The experiment was repeated 12 times (i.e., 12 different pituitary glands) using 4 wells per treatment. Enzymatic dispersal of AP cells and culture were performed using the method described in the chapter III. Cells were plated in the 24-well culture plates and maintained at 37 °C in a humidified atmosphere of 5% CO2 for 82 hr. The wells were washed twice with PBS and then incubated with 490 µl DMEM containing 0.1% BSA for 2 hr. Pretreatment was performed by adding 5 µl of either DMEM alone or DMEM containing various concentrations (ranging from 0.1 nM to 1,000 nM) of estradiol (052- 04041; Wako Pure Chemicals) or STX (donated by Prof. Martin Kelly, Oregon Health and Science University, OR, USA). The cells were incubated while gently shaking for 5 min and subsequently treated with 5 µl of DMEM containing 100 nM GnRH (Peptide Institute Inc.) for 2 h to stimulate LH secretion. Thus, the total treatment time with estradiol or STX was 2 h and 5 min. The pretreatment plus the GnRH treatment yielded a final concentration of 0.001, 0.01, 0.1, 1 or 10 nM estradiol or STX in the wells that had received 0.1, 1, 10, 100 or 1,000 nM of estradiol or STX, respectively, and a final concentration of 1 nM of GnRH. The “control” wells contained 5 µl of DMEM that had not undergone pretreatment with estradiol, STX or GnRH. The “GnRH” wells contained 5 µl of DMEM that had not undergone pretreatment with estradiol or STX, but had been incubated with GnRH for 2 h. After incubation with GnRH, the medium was collected for the immunoassay of LH.

4.2.2. Data analysis

The LH concentrations in the control samples for each pituitary were averaged, and the mean value was set to 100%. The LH concentrations from the 4 replicate wells for each pituitary were averaged, and the mean LH value was expressed as a percentage of the control value. The statistical significance of differences of the LH concentration was analyzed by one-factor ANOVA followed by post hoc comparisons using Fisher's PLSD test. The level of significance was set at $P < 0.05$. Data are expressed as mean ± SEM.

4.3. Results

Figure 4.1 shows the effects of various concentrations of estradiol and STX on GnRH-stimulated LH secretion from the cultured AP cells. The mean LH concentration in the medium of GnRH wells (15.3 ± 0.8 ng/ml) was significantly greater ($P < 0.01$) than that of the control wells (10.3 ± 0.7 ng/ml). The wells with 1 nM and 10 nM estradiol displayed significantly greater ($P < 0.05$ and $P < 0.01$, respectively) mean LH concentration than the control wells. The wells with 0.001 to 0.01 nM ($P < 0.01$) and 0.1 nM ($P < 0.05$) of estradiol displayed lower mean LH concentrations than the GnRH wells. The wells with 0.001 and 0.01 nM of estradiol displayed significantly lower ($P < 0.01$) mean LH concentration compared to wells with 10 nM estradiol. None of the wells containing STX displayed significant different mean LH concentrations compared to control or GnRH wells.

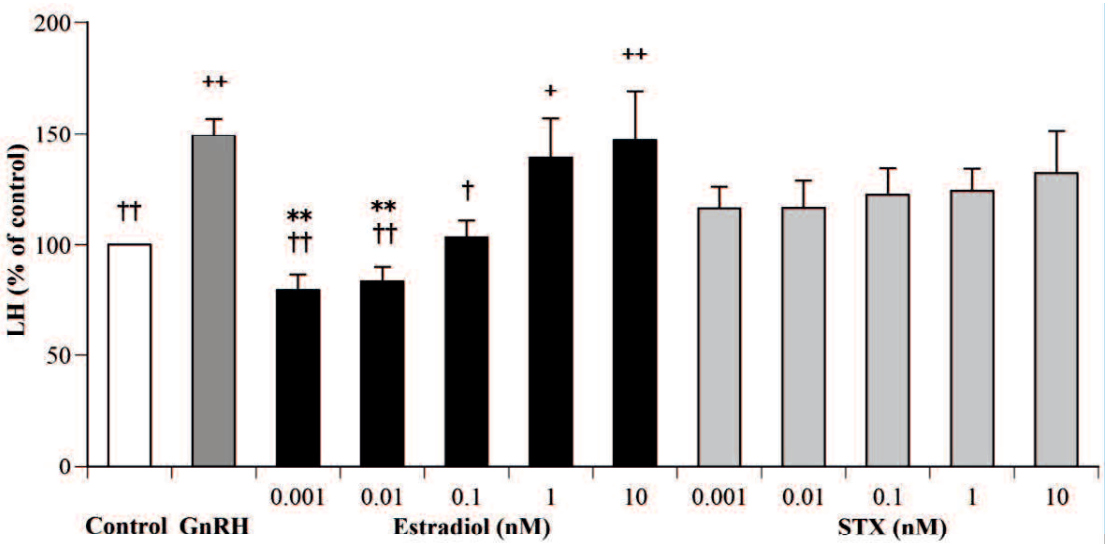

Fig. 4.1. Effects of various concentrations of estradiol (black bars) or STX (gray bars) in DMEM containing 1 nM GnRH on LH secretion from cultured bovine AP cells. The LH concentrations in control cells (cultured medium only) were averaged with the mean value set at 100%. The LH concentrations of the treated groups are expressed as a percentage of the control. Each value represents the mean concentration ± SEM (n =12)

+ $P < 0.05$, ++ $P < 0.01$: significant differences compared with the control

† $P < 0.05$, ††$P < 0.01$: significant difference compared with GnRH

** $P < 0.01$: significant differences compared with 10 nM estradiol

4.4. Discussion

This is the first report evaluating the non-genomic effects of STX on LH secretion from AP, although previous studies have reported on the role of the STX receptor in mediating the rapid, negative estradiol feedback effect on various hypothalamic neurons (Kenealy et al., 2011; Qiu et al., 2003; Zhang et al., 2010). In chapter III, we have previously reported that low concentrations of estradiol or the GPR30 agonist, G1, suppressed GnRH-induced LH secretion in bovine AP cells within a 5-min period. Thus, the present results revealed that STX, unlike estradiol or G1, possesses no suppressive effect on GnRH-stimulated LH secretion in the bovine AP, suggesting that GPR30 may play a more important role in LH secretion from bovine gonadotropes than the STX receptor. Because the STX receptor mediates GnRH secretion from primate neurons by non-genomic mechanism (Kenealy et al., 2011), differences in the non-genomic mechanism may exist between gonadotropes and GnRH neurons. Therefore, further studies on the STX receptor in ruminants are required.

The mRNA and amino acid sequences of STX-receptor have not yet been published, and thus far, no specific antibody against the STX receptor has been developed. Therefore, it was impossible to measure STX expression levels or the cellular localization of the STX receptor in AP cells in any species. Additional tools, including antibodies and antagonists, need to be developed before further characterization of this receptor.

In this study, 10 nM estradiol was found to be less efficient in suppressing GnRH-stimulated LH secretion than the physiological concentrations, 0.01 and 0.1 nM, of estradiol. Further studies are required to clarify the molecular mechanism by which estradiol suppresses LH secretion.

In conclusion, the present results revealed that STX, unlike estradiol and G1, possesses no suppressive effect on GnRH-stimulated LH secretion in the bovine AP.

CHAPTER V

(Study III)

Effects of GPR30 Antagonist, G36, on Estradiol Suppression of GnRH-induced LH Secretion in Cultured Bovine AP

Abstract

Picomolar concentrations of estradiol produce rapid suppression of GnRH-induced LH secretion from the AP of cattle. In order to clarify whether GPR30 is the receptor mediating such effect, we conducted experiment to evaluate effect of GPR30-specific antagonist, G36, on the estradiol rapid suppression of GnRH-induced LH secretion. The AP cells (n = 10) were cultured for 3 days in steroid-free conditions. The AP cells were treated with 0.01 nM G36 for 5 min, then, also with 0.01 nM estradiol for 5 min before GnRH stimulation. Pre-treatment with G36 inhibited the estradiol suppression of LH secretion from cultured AP cells. The pretreatment with 0.01 nM G36 without 0.01 nM estradiol had no effect on the GnRH-induced LH secretion. Therefore, we concluded that GPR30 is the receptor for the rapid estradiol suppression of the GnRH-induced LH secretion from bovine AP cells.

5.1. Introduction

Recent studies have suggested that estradiol binds with a plasma membrane receptor in the AP, thereby suppressing LH, but not FSH, in a rapid, non-genomic mechanism (Arreguin-Arevalo and Nett, 2005; Iqbal et al., 2007). A GPR30 is a plasma membrane estradiol receptor that can bind estradiol-GPR30, thus initiating several rapid, non-genomic signaling events in the cytoplasm (Maggiolini and Picard, 2010). It is present in the plasma membrane of rat AP cells (Brailoiu et al., 2007; Hazell et al., 2009) and in cattle gonadotropes, where it may contribute to rapid negative estradiol feedback regulation of GnRH-induced LH secretion as shown in chapter III. Selective agonists of ERα and ERβ at concentrations below 10 nM have no effect on GnRH-stimulated LH release from AP cells of sheep (Arreguin-Arevalo and Nett, 2005). STX is an agonist for STX receptor. However, it has no effect on LH secretion from the AP cells of cattle. Therefore the GPR30 is the only clearly identified membrane estradiol receptor, and its study is important for understanding reproduction in ruminants

G36 is a GPR30-specific antagonist that was recently developed for human cancer research (Dennis et al., 2011; Méndez-Luna et al., 2015; Scaling et al., 2014). This antagonist binds to receptors of bovine satellite cells (Kamanga-Sollo et al., 2014). Our previous studies did not report the effects of G36 on estradiol induced GnRH suppression on LH secretion in cultured AP cells. Therefore, in the present study, G36 was used to test the hypothesis that estradiol suppresses GnRH-induced LH release from the AP of cattle via GPR30 in a rapid mechanism.

5.2. Materials and methods

5.2.1. Analysis of the effects of G36 on estradiol suppression of GnRH-induced LH secretion

Anterior pituitaries were obtained from post-pubertal Japanese Black heifers (n = 10, 26 months of age). The experiment was repeated ten times with each of the ten different pituitary glands, using four wells per treatment. The AP cells were cultured at 37 °C in 5% CO_2 for 82 h. After washing with PBS, the cells were incubated with 485 µL of DMEM containing 0.1% BSA for 2 h. Cells were pre-treated with 5 µL of DMEM alone or with 5 µL of DMEM containing 1 nM G36 (Azano Biotech, Albuquerque, NM, USA). After 5 min of gentle shaking, either 5 µL of DMEM alone or 5 µL of DMEM containing 1 nM estradiol was added to each culture well. The cells were incubated while gently shaking for 5 min, after which they were incubated for 2 h with 5 µL of 100 nM GnRH dissolved in DMEM in order to stimulate LH secretion. There were five treatment conditions as follows: (1) “control” wells were treated with 5 µL of DMEM, but were not treated with G36 or incubated with GnRH; (2) “GnRH” wells were treated with 5 µL of DMEM, but were not treated with G36, and were incubated with GnRH (final concentration, 1 nM); (3) “estradiol” wells were pre-treated with 5 µL of DMEM and were then treated with estradiol (final concentration, 0.01 nM) and incubated with GnRH (final concentration, 1 nM); (4) “estradiol and G36” wells were pre-treated with G36 (final concentration, 0.01 nM) and were then treated with estradiol (final concentration, 0.01 nM) and incubated with GnRH (final concentration, 1 nM); (5) “G36” wells were pre-treated with G36 (final concentration, 0.01 nM) and were then treated with 5 µL of DMEM and

incubated with GnRH (final concentration 1 nM). After 2 h of incubation, the medium was collected for the immunoassay of LH.

5.2.2. Data analysis

The concentrations of LH in the control samples for each of the pituitary glands were averaged, and the mean value was set at 100%. The concentrations of LH in the treated samples for each of the pituitary glands were averaged, and the mean LH values were expressed as percentages of the control value. The statistical significance of differences in LH concentration were analyzed by using one-factor ANOVA with concentrations of LH as the dependent variable and treatment as the independent variable, followed by *post-hoc* comparisons by using Fisher's PLSD test. The level of significance was set at $P < 0.05$. Data were expressed as mean ±SEM.

5.3. Results

5.3.1. Effects of G36 on estradiol suppression of LH secretion

Figure 5.1. depicts the effects of G36 on estradiol suppression of GnRH-stimulated LH secretion from cultured AP cells. The LH concentration in the medium of GnRH wells was greater than that in control wells. Pre-treatment with 0.01 nM estradiol in the absence of G36 treatment suppressed GnRH-stimulated LH secretion. In contrast, pre-treatment with 0.01 nM estradiol in the presence of G36 treatment had no suppressive effect on GnRH-stimulated LH secretion. Treatment with G36 alone had no effect on GnRH-stimulated LH secretion.

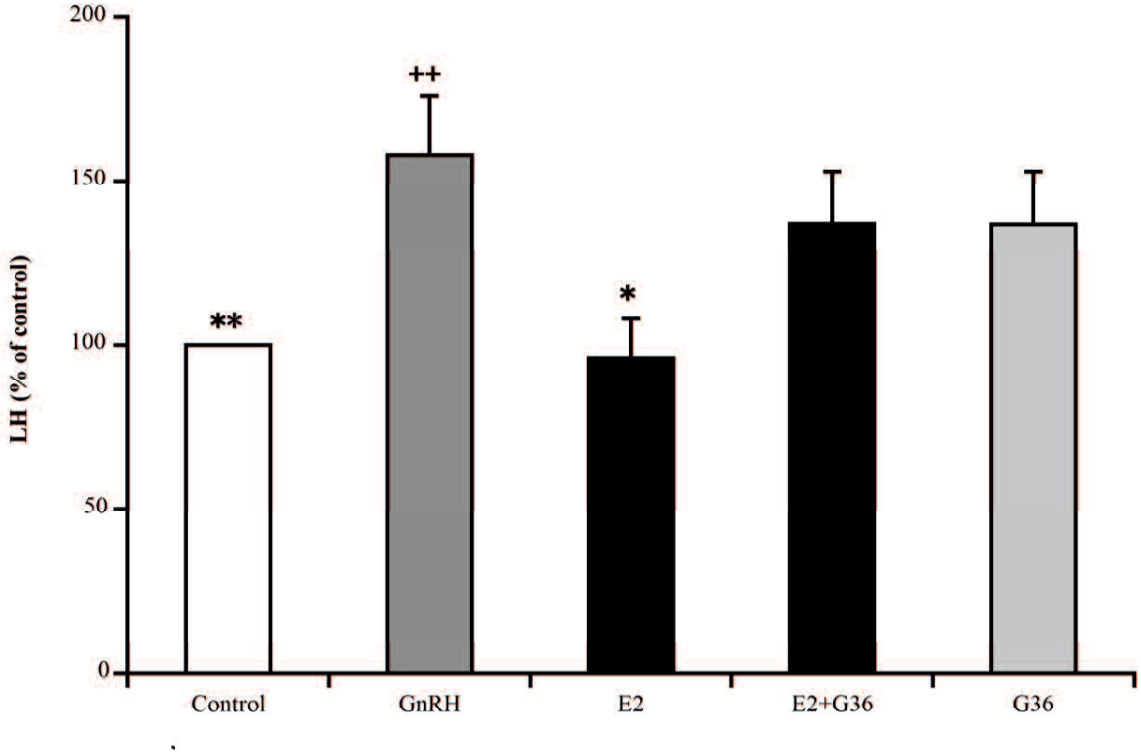

Fig 5.1. Comparison of the effects of the GPR30 antagonist, G36, on estradiol- (black bars) suppression of GnRH-induced LH secretion from cultured bovine AP cells. The final concentrations of G36, estradiol and GnRH were 0.01 nM, 0.01 nM, and 1 nM, respectively. LH concentrations in control cells (cultured medium only) were averaged with the mean value set at 100%. The LH concentrations of the treated group are expressed as a percentage of the control. Each value represents the mean concentrations ± SEM (n =10).

++P < 0.01; indicates differences compared to the control;

*P < 0.05, **P < 0.01; indicates differences compared to GnRH alone

5.4. Discussion

In the present study, pre-treatment with the GPR30 antagonist inhibited estradiol suppression of LH secretion. This suggests that GPR30 is the receptor to which estradiol binds, thereby mediating the suppressive effect of estradiol on LH secretion.

Previous investigations of the feedback mechanisms of estradiol on GnRH-induced LH release in primary AP cell cultures derived from multiple ovariectomized ewes used 0.01–100 nM estradiol for 15 min, 30 min, or 60 min (Arreguin-Arevalo and Nett, 2005) or 1.0 nM estradiol for 5 min (Iqbal et al., 2009). Present results are not consistent with the findings in these previous studies, which reported that 1.0 nM and 10 nM estradiol blocked GnRH-induced LH release from cultured ovine pituitary cells. Possible reasons for these inconsistencies have been described previously in the previous chapter. Cupp et al. (1995) reported that the amplitude of LH pulses was greater in ovariectomized cows than in intact cows. Therefore, additional possible explanations for the observed discrepancies are (1) the use of pituitary glands derived from intact heifers or from ovariectomized ewes, (2) the presence or absence of steroid hormones and phenol red in the culture media, and (3) the timing of GnRH stimulation after pre-treatment.

G15 is the first generation of GPR30 antagonist, but now it is well-known that G15 acts as a partial agonist of ERα as described by Dennis et al. (2011). Also in our preliminary study, G15 did not show any antagonistic role against LH secretion (data not shown). Therefore, this dissertation study used G36.

In conclusion, GPR30 is the receptor for the rapid estradiol suppression of the GnRH-induced LH secretion from bovine AP cells.

CHAPTER VI

(Study IV)

Effects of Short-Time Estradiol Treatment

on the Amount of mRNA of LHα, LHβ and FSHβ Subunits

Abstract

Picomolar concentrations of estradiol produce the rapid suppression of GnRH-induced LH secretion from the AP of cattle via GPR30. In order to verify the effect of short time treatment with estradiol is mediated by non-genomic mechanism, we evaluated the effect of estradiol on the gene expressions of LHα, LHβ and FSHβ subunits. The AP cells (n = 5) were cultured for 3 days in steroid-free conditions and then treated them with 0.01 nM estradiol for 5 min before GnRH stimulation. Quantitative real-time-PCR analyses revealed that pre-treatment with estradiol have no significant effect (P>005) on the gene expressions of LHα, LHβ, or FSHβ subunits in the AP cells. Therefore, the rapid effect of estradiol on the GnRH-induced LH secretion was non-genomic.

6.1. Introduction

During the luteal phase of the estrous cycle, picomollar concentration of estradiol exert a negative feedback effect on GnRH secretion from the arcuate nucleus in the hypothalamus (Clarke, 1995; Evans et al., 1994; García-Galiano et al., 2012) and suppress amounts of LH mRNA in the AP of ewes (Mercer et al., 1993). To induce these important feedback effects from the ovary to the hypothalamus and the pituitary, estradiol binds to nuclear-localized ERα or ERβ and alters gene transcription (Gieske et al., 2008; Hewitt and Korach, 2003; Sánchez-Criado et al., 2012).

GPR30 is present in the plasma membrane of rat AP cells (Brailoiu et al., 2007; Hazell et al., 2009) and in cattle gonadotropes as shown in the chapter III, where it contributes to rapid negative estradiol feedback regulation of GnRH-induced LH secretion as shown in the previous chapters. However, previous chapters did not evaluate the effects of short-time estradiol treatment on the of mRNA expression of LHα subunit (LHα), LHβ subunit (LHβ), and FSHβ subunit (FSHβ) subunit in cultured AP cells.

Therefore, in order to verify the effect of short time treatment with estradiol is mediated by non-genomic mechanism, mRNA for LHα, LHβ and FSHβ subunits was measured in cultured AP cells in the presence and absence of the estradiol treatment.

6.2. Materials and methods

6.2.1. AP cell culture and real-time PCRs to measure mRNAs of LHα, LHβ, and FSHβ subunits

Anterior pituitaries were obtained from post-pubertal Japanese Black heifers (n = 5, 26 months of age). The experiment was repeated five times with each of the five different pituitary glands, using two wells per treatment. After the enzymatic dispersal of AP cells, the AP cells were cultured for 82 h. After washing with PBS, the cells were incubated with 490 μL of DMEM containing 0.1% BSA for 2 h. Cells were pre-treated with 5 μL of DMEM alone or with 5 μL of DMEM containing 1 nM estradiol (final concentration, 0.01 nM). After gently shaking for 5 min, cells were treated for 2 h with 5 μL of 100 nM GnRH (final concentration, 1 nM, except the "control") dissolved in DMEM. There were three treatment conditions as follows: (1) "control" wells were treated with 5 μL of DMEM, but were not incubated with GnRH; (2) "GnRH" wells were treated with 5 μL of DMEM and were incubated with GnRH; (3) "estradiol" wells were pre-treated with estradiol and were incubated with GnRH. After the 2 h of incubation, total RNA was extracted from the wells by using the RNAiso Plus (Takara Bio Inc.) according to the manufacturer's protocol but with a slight modification, namely, the addition of 3 μL of high molecular weight acrylamide polymer solution (Ethachinmate, Nippon Gene) as a carrier solution for isopropyl alcohol precipitation of RNA. Possible contaminated genomic DNA was digested by using DNase I (Toyobo). The concentration and purity of each RNA sample were evaluated by using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA) to ensure that the A260:A280 nm ratio was in the acceptable range of 1.8–2.1. Electrophoresis of total RNA followed by staining with

ethidium bromide was performed to verify the mRNA quality of all samples and to ensure that the 28S:18S ratios were 2:1. The cDNA was synthesized from 2 μg of RNA from each sample in 20-μL reactions with random hexamer primers, by using a ReverTra Ace qPCR RT Master Mix (Toyobo).

Conventional PCR amplification with Tks Gflex DNA polymerase (Takara Bio Inc.), 20 ng of DNase-treated reverse-transcribed RNA, and primers was performed to prepare external standards for amplified fragments of cDNA products containing target sequences for real-time PCR of LHα (NCBI reference sequences of bovine *CGA*; NM_173901.3), LHβ (*LHB*; NM_173930.1), FSHβ (*FSHB*; NM_174060.1), and two housekeeping genes, namely, chromosome 2 open reading frame 29 (*C2orf29*; XM_002691150.2) and suppressor of zeste 12 (*SUZ12*; NM_001205587.1). The primers for the conventional PCRs were designed by using Primer Express Software V3.0 (ABI) based on the reference sequences (**Table 6.1**). The two housekeeping genes were used to normalize the real-time PCR results, because they are identified by GeNorm (Biogazelle, Zwijnaarde, Belgium) and Normfinder (Aarhus University Hospital, Aarhus, Denmark) programs as the most stable and reliable housekeeping genes in bovine endometrium and corpus luteum (Rekawiecki et al., 2012; Walker et al., 2009).

The presence of a single product was confirmed by using electrophoresis on a 2% (w/v) agarose gel. The PCR-amplified products from the cDNA were purified by using NucleoSpin Extract II columns (Takara Bio Inc.), firstly to prepare external standards and secondly to verify the DNA sequences by using a sequencer (ABI3130; ABI) with one of the PCR primers and the Dye Terminator V3.1 Cycle Sequencing Kit (ABI). The obtained sequences were used as query terms for homology searches in the DDBJ–GenBank–EBI

Data Bank, using the basic nucleotide local alignment search tool optimized for highly similar sequences (available from the NCBI website).

Table 6.2 shows the primers designed for the real-time PCRs by using Primer Express Software V3.0 (ABI) based on the reference sequences. The amounts of gene expression were measured in duplicate by using real-time PCR analyses, with 20 ng of cDNA, the CFX96 Real-Time PCR System (Bio-Rad) and the Power SYBR Green PCR Master Mix (ABI), together with a 5-point relative standard curve, non-template control, and no reverse transcription control. Series of ten-fold dilution standards were prepared by using the purified amplified fragments of DNA products. Temperature conditions for all genes were as follows: 95 °C for 10 min for pre-denaturation; 5 cycles each of 95 °C for 15 s and 66 °C for 30 s; and 40 cycles each of 95 °C for 15 s and 60 °C for 60 s. Melting curve analyses were performed at 95 °C for each amplicon and each annealing temperature, to ensure the absence of smaller non-specific products such as dimers. The concentrations of the PCR products were calculated by comparing the CT values of the unknown samples with the standard curve, using appropriate software (CFX manager V3.1, Bio-Rad). The amounts of gene expressions of LHα, LHβ, and FSHβ were normalized to the geometric means of C2orf29 and SUZ12 expression; thus, the amounts of these genes were divided by the geometric mean of C2orf29 and SUZ12 in each sample.

6.2.2. Data analysis

The amounts of gene expressions of LHα, LHβ, or FSHβ in the treated samples for each of the pituitary glands were averaged, and the mean gene expressions were expressed as percentages of the control value. The statistical significance of differences in gene expression were analyzed by using one-factor ANOVA, followed by post-hoc comparisons

with Fisher's PLSD test. The level of significance was set at $P < 0.05$. Data were expressed as mean ± SEM.

Table 6.1. Sequences and exonic localizations of the primers used to prepare external standards for conventional PCR

Gene	Primer	Sequence 5′-3′	Amplicon (bp)	Exon
LHα	Forward Reverse	5′-GGACGAAGAGCCATGGATTACT-3′ 5′-CCATCACTGTGGCCTTGGTA-3′	300	1st 4th
LHβ	Forward Reverse	5′-GGACTGCTGCTGTGGCTGCT-3′ 5′-GAGGAAGAGGATGTCTGGGAGC-3′	408	2nd 3rd
FSHβ	Forward Reverse	5′-AGAAGCTGCGAGCTGACCAA-3′ 5′-GCATCCGCTGCTCTTTATTCT-3′	350	2nd 3rd
C2orf29	Forward Reverse	5′-AAGTTTTTTCTTTCCCAGCTCATG-3′ 5′-CAGGAAGTTTGGCTGGAGTGA-3′	562	2nd 5th
SUZ12	Forward Reverse	5′-GGAAGAGACTGCCTCCATTTGA-3′ 5′-CCCTGAGACACCATCTGTTTCC-3′	1169	10th 16th

Table 6.2. Sequences and exonic localizations of the primers used for real-time PCRs

Gene	Primer	Sequence 5′-3′	Amplicon (bp)	Exon
LHα	Forward Reverse	5′-TCACCTCGGAAGCTACATGCT-3′ 5′-CCATCACTGTGGCCTTGGTA-3′	57	3rd 4th
LHβ	Forward Reverse	5′-GCCCTGTCTGTATCACTTTCAC-3′ 5′-AGGCAGCACCCGCTTCATG-3′	71	2nd 3rd
FSHβ	Forward Reverse	5′-GGAATGTGGCTTCTGCATAAGC-3′ 5′-TCTGGATATTGGGCCTTGCT-3′	101	2nd 3rd
C2orf29	Forward Reverse	5′-TCAGTGGACCAAAGCCACCTA-3′ 5′-CTCCACACCGGTGCTGTTCT-3′	170	3rd 4th
SUZ12	Forward Reverse	5′-CATCCAAAAGGTGCTAGGATAGATG-3′ 5′-TTGGCCTGCACACAAGAATG-3′	160	13th 14th

6.3. Results

6.3.1. Effects of estradiol on expressions of LHα, LHβ, and FSHβ genes

Figure 6.1 depicts the effects of estradiol on the mRNA expressions of LHα, LHβ, and FSHβ genes in cultured AP cells. There was no difference in the gene expressions among the investigated groups.

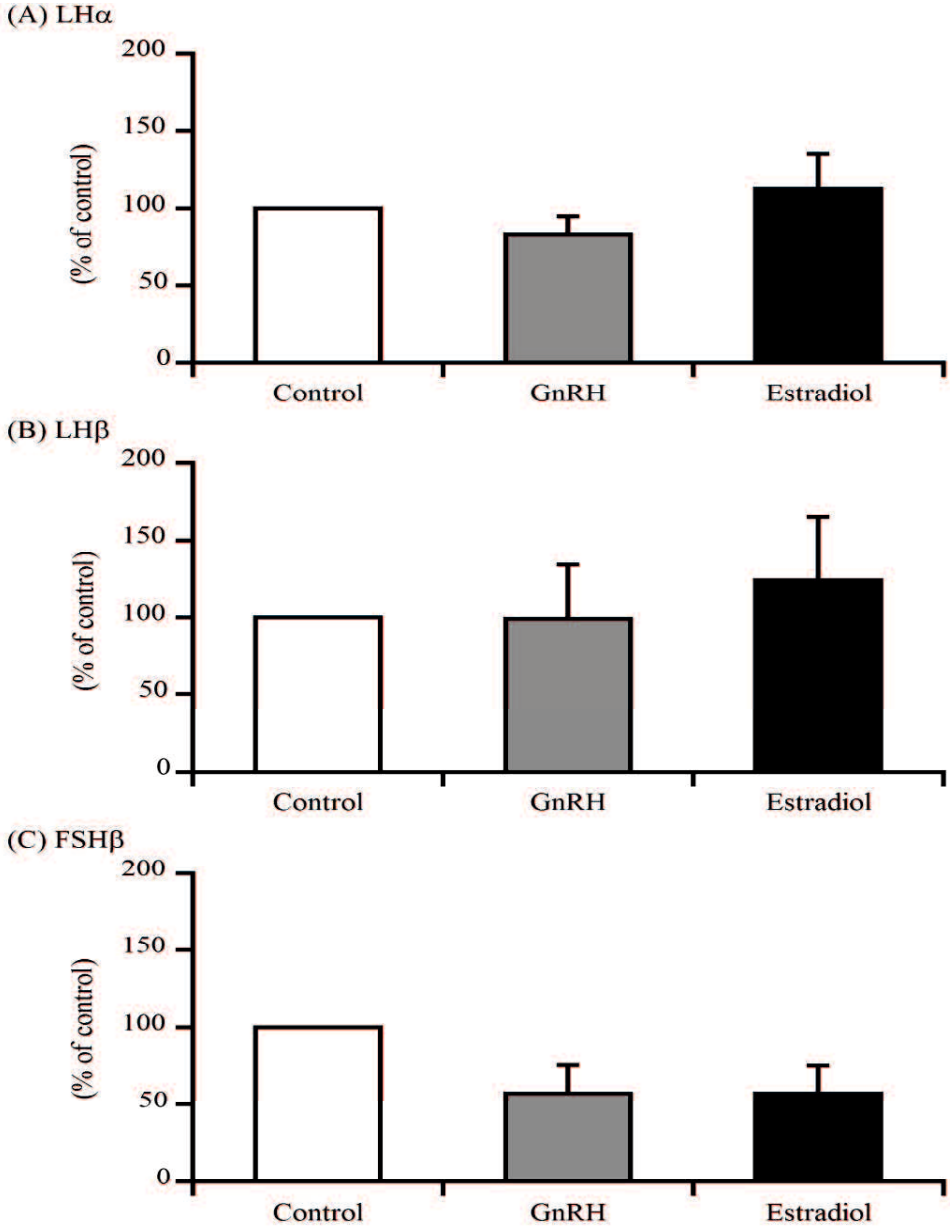

Fig. 6.1. Comparison of the effects of 0.01 nM estradiol (black bar) on gene expressions of the *LHα* subunit (A), *LHβ* subunit (B), and *FSHβ* subunit (C) in cultured bovine AP cells treated with 1 nM GnRH. Gene expressions were normalized to the geometric means of two housekeeping genes, *C2orf29* and *SUZ12*. Gene expressions in control cells (cultured in medium alone) were averaged, and the mean of gene expressions of treated groups are expressed as percentages of that value.

6.4. Discussion

In the present study, pre-treatment with small amounts of estradiol did not decrease the relative amounts of mRNA for LHα, LHβ or FSHβ subunits in the AP cells. Findings in the present study imply that estradiol suppress LH secretion from cultured bovine AP cells through non-genomic pathways.

CHAPTER VII

(Study V)

Effects of ERK1/2/5 Pathway Inhibitor and PKA Pathway Inhibitor on Estradiol or G1 Suppression of GnRH-Induced LH Secretion from Bovine AP Cells

Abstract

GPR30 is acknowledged as a membrane receptor for picomolar concentrations of estradiol, which cause the rapid, non-genomic suppression of GnRH-induced LH secretion from the bovine AP. A few studies have recently clarified that extracellular signal regulated kinase 1/2/5 (ERK1/2/5) and protein kinase A (PKA) might be involved in cytoplasmic signaling pathways of GPR30 in others cells. Therefore, this study was conducted to clarify whether ERK1/2/5 pathway or PKA pathway is the part of non-genomic mechanism of estradiol suppression. Bovine AP cells (n = 8) were cultured for 3 days in steroid-free conditions. The AP cells were treated with either 1 μM of ERK1/2/5 inhibitor (U1026) or 5 μM of PKA inhibitor (H89), or combination of U0126 and H89 for 30 min. Then, the AP cells were treated with 0.01 nM estradiol or G1 for 5 min before GnRH stimulation. Estradiol or G1 treatment without inhibitor pretreatment suppressed GnRH-induced LH secretion significantly ($P<0.01$). In contrast, pre-treatment with the inhibitors inhibited estradiol or G1 suppression of LH secretion from cultured AP cells. Therefore, both ERK1/2/5 pathway and PKA pathway are important for the rapid non-genomic suppression of estradiol on the GnRH-induced LH secretion from bovine AP cells.

7.1. Introduction

GPR30 binds estradiol to suppress LH secretion from bovine AP cells in the rapid, non-genomic manner. Cytoplasmic mechanism for the rapid, non-genomic estradiol suppression of LH secretion from AP cells is an important fundamental knowledge. However, little is known also for GPR30 in other biological fields. Dennis et al. (2011) reported that G36 selectively inhibits estrogen-mediated activation by GPR30, and G36 also inhibits estrogen and G1-mediated ERK1/2/5 activation. Therefore, ERK1/2/5 pathway may be important pathway for GPR30 to suppress LH secretion non-genomically. Hsieh et al. (2007) used protein kinase A (PKA) inhibitor, H89, and clarified that PKA pathway is the main pathway for effect of estrogen in attenuating liver injury after trauma-hemorrhage. PKA pathway is the important pathway to control FSHβ gene expression in the gonadotrope-derived LβT2 cells (Thompson et al. 2013). Ishida et al. (2010) utilized H89 and ERK1/2/5 inhibitor, U0126, to report that both ERK1/2/5 pathway and PKA pathway are important pathways for genomic effect of estradiol in lactotroph. However, it is not clear whether PKA pathway and ERK1/2/5 pathway are the pathways in the downstream of GPR30 to suppress LH secretion in non-genomic mechanism. Therefore, in the present study, U0126 and H89 were used to test the hypothesis that ERK1/2/5 and PKA are the important pathways for the rapid, non-genomic suppression by estradiol or G1 mediated by GPR30 on the GnRH-induced LH release from the bovine AP cells.

7.2. Materials and methods

7.2.1. Analysis of the effects of U0126, H89 and the combination of both inhibitors on estradiol suppression of GnRH-induced LH secretion

Anterior pituitaries were obtained from post-pubertal Japanese Black heifers (n = 8, 26 months of age). The experiment was repeated eight times with each of the eight different pituitary glands, using four wells per treatment. The AP cells were cultured at 37 °C in 5% CO_2 for 82 h. After washing with PBS, the cells were incubated with 485 µL of DMEM containing 0.1% BSA for 2 h. Cells were pre-treated with 5 µL of DMEM alone or with 5 µL of DMEM containing 100 µM of U1026 (Enzo Biochem Inc., New York, USA) and/or 500 µM of H89 (Lkt Laboratories Inc., St. Paul, MN, USA). After 30 min of incubation, either 5 µL of DMEM alone or 5 µL of DMEM containing 1 nM estradiol or 1 nM G1 was added to each culture well. The cells were incubated while gently shaking for 5 min, after which they were incubated for 2 h with 5 µL of 100 nM GnRH dissolved in DMEM in order to stimulate LH secretion. There were four treatment conditions as follows: (1) “control” wells were treated with 5 µL of DMEM, but were not treated with inhibitors or incubated with GnRH; (2) “GnRH” wells were treated with 5 µL of DMEM, but were not treated with inhibitors, and were incubated with GnRH (final concentration, 1 nM); (3) “estradiol or G1” wells were pre-treated with 5 µL of DMEM and were then treated with estradiol or G1 (final concentration, 0.01 nM) and incubated with GnRH (final concentration, 1 nM); (4) “estradiol or G1 and inhibitor” wells were pre-treated with either U0126 (final concentration, 1 µM), H89 (final concentration, 5 µM) or both and were then treated with estradiol (final concentration, 0.01 nM) and incubated with GnRH (final concentration, 1 nM). After 2 h of incubation, the medium was collected for immunoassay

of LH. The concentrations of U0126 and H89 used in the present study were identical to those used in a previous investigation of pathway in cultured lactotroph cells (Ishida et al., 2010).

7.2.2. Data analysis

The concentrations of LH in the control samples for each of the pituitary glands were averaged, and the mean value was set at 100%. The concentrations of LH in the treated samples for each of the pituitary glands were averaged, and the mean LH values were expressed as percentages of the control value. The statistical significance of differences in LH concentration were analyzed by using one-factor ANOVA with concentrations of LH as the dependent variable and treatment as the independent variable, followed by *post-hoc* comparisons by using Fisher's PLSD test. The level of significance was set at $P < 0.05$. Data were expressed as mean ± SEM.

7.3. Results

7.3.1. Effects of U0126 and H89 on GnRH-stimulated LH secretion

Figure 7.1 depicts the effects of U0126 alone, H89 alone or the combination of both inhibitors in the absence of estradiol or G1 on the GnRH-stimulated LH secretion from cultured AP cells. Treatment with U0126 alone, with H89 alone, or with both inhibitors had no effect on the GnRH-stimulated LH secretion.

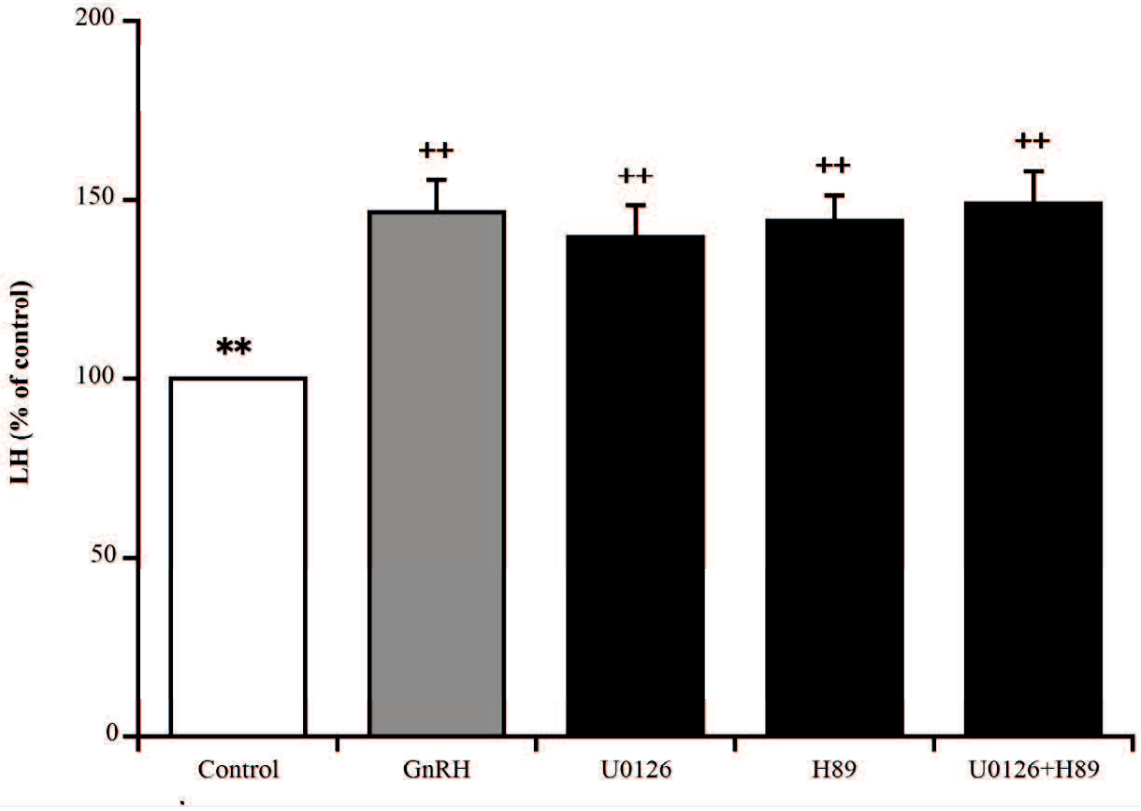

Fig 7.1. Comparison of the effects of U0126, H89 or combination of both inhibitors on the GnRH-induced LH secretion from cultured bovine AP cells. The final concentrations of U0126, H89, and GnRH were 1 μM, 5 μM, and 1 nM, respectively. LH concentrations in control cells (cultured in medium alone) were averaged, and the mean LH concentrations of treated groups are expressed as percentages of that value.

++$P < 0.01$: significant differences compared to the control.

**$P < 0.01$: significant differences compared to GnRH alone.

7.3.2. Effects of U0126 and H89 on estradiol suppression of LH secretion

Figure 7.2 depicts the effects of U0126 alone, H89 alone or combination of both inhibitors on estradiol suppression of GnRH-stimulated LH secretion from cultured AP cells. The treatment with 0.01 nM estradiol in the absence of inhibitors suppressed GnRH-stimulated LH secretion. In contrast, pre-treatment with 0.01 nM estradiol in the presence of U0126 alone, H89 alone, or both inhibitors inhibited the suppressive effect of estradiol on GnRH-stimulated LH secretion.

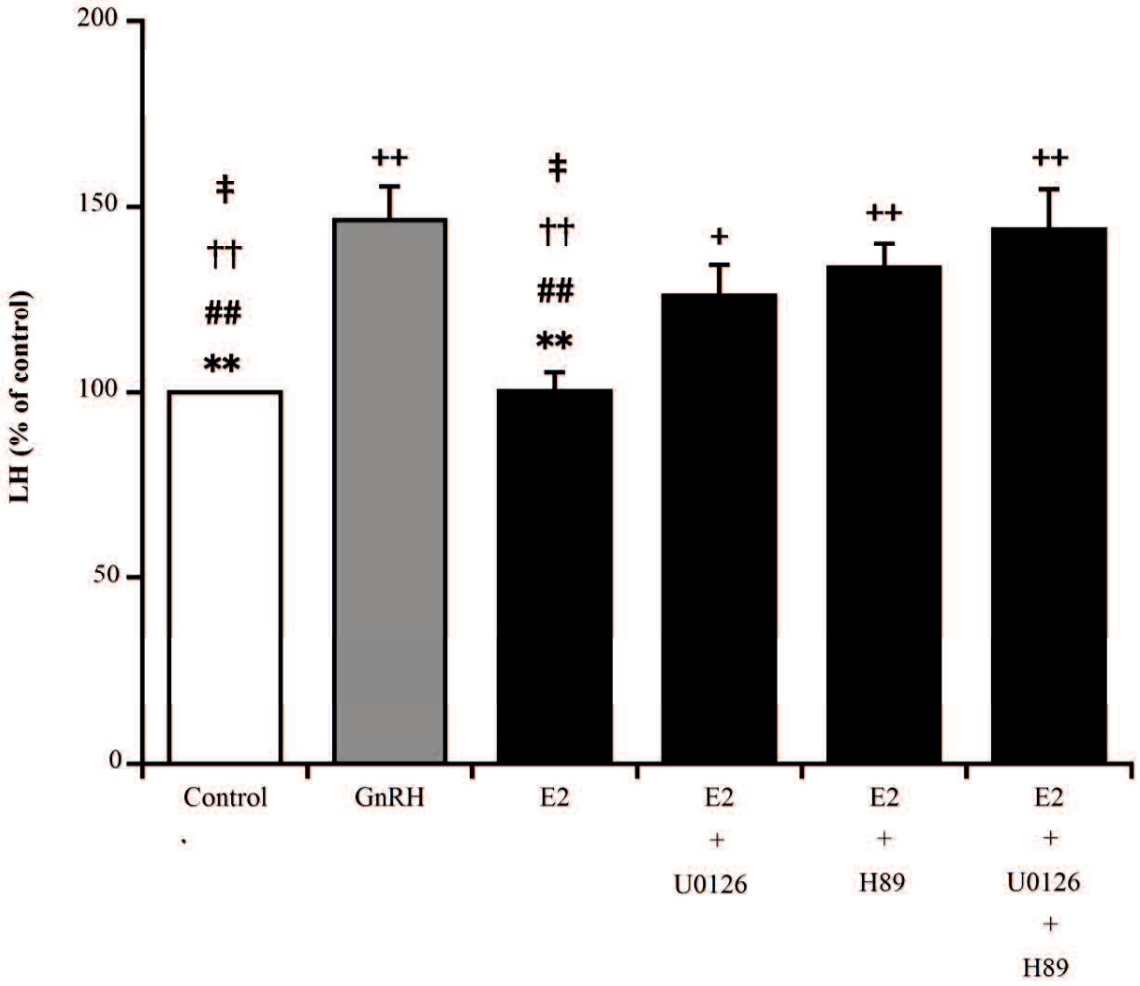

Fig 7.2. Comparison of the effects of U0126 alone, H89 alone or combination of both inhibitors on the estradiol-suppression of GnRH-induced LH secretion from cultured bovine AP cells. LH concentrations in control cells (cultured in medium alone) were averaged, and the mean LH concentrations of treated groups are expressed as percentages of that value.

+P < 0.05, ++P < 0.01: significant differences compared to the control.

**P < 0.01: significant differences compared to GnRH alone.

##P < 0.01: significant differences compared to E2+U0126+H89.

††P < 0.01: significant differences compared to E2+ H89.

‡P < 0.05: significant differences compared to E2+U0126.

7.3.3. Effects of U0126 and H89 on G1 suppression of LH secretion

Figure 7.3 depicts the effects of U0126 and H89 on G1 suppression of GnRH-stimulated LH secretion from cultured AP cells. Pre-treatment with 0.01 nM G1 in the absence of inhibitors treatment suppressed GnRH-stimulated LH secretion. In contrast, pre-treatment with 0.01 nM G1 in the presence of U0126 alone, H89 alone, or both inhibitors had no suppressive effect on GnRH-stimulated LH secretion. There was significant difference between U0126 alone pretreatment and both inhibitor pretreatment.

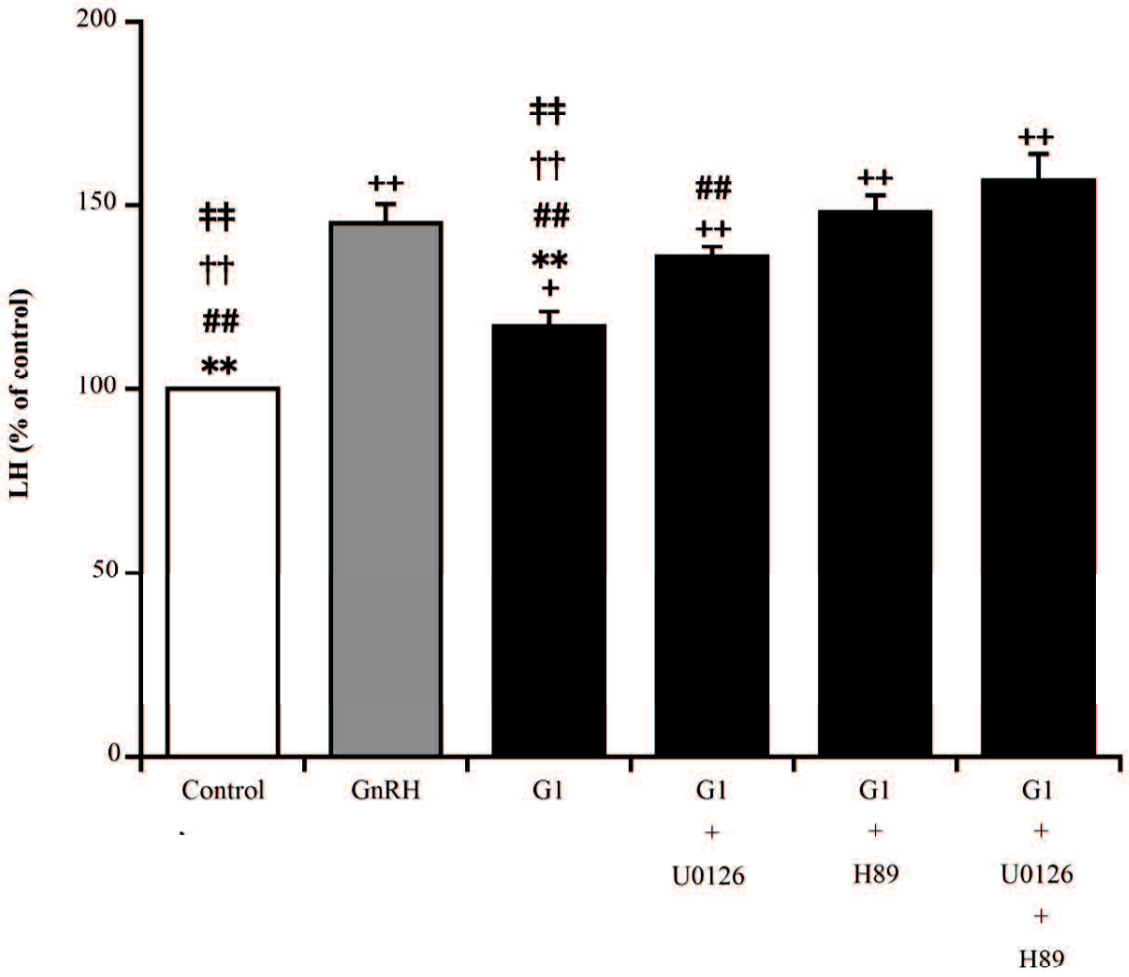

Fig 7.3. Comparison of the effects of U0126 and H89 on G1 suppression of the GnRH-induced LH secretion from cultured bovine AP cells. LH concentrations in control cells (cultured in medium alone) were averaged, and the mean LH concentrations of treated groups are expressed as percentages of that value.

+P < 0.05, ++*P* < 0.01: significant differences compared to the control.

**P < 0.01: significant differences compared to GnRH alone.

##P < 0.01: significant differences compared to G1+ U0126 + H89.

††P < 0.01: significant differences compared to G1+ H89.

‡‡P < 0.01: significant differences compared to G1+ U0126.

7.4. Discussion

In the present study, pre-treatment with ERK1/2/5 or PKA pathway inhibitors inhibited estradiol or G1 suppression of LH secretion. These pathways are important for genomic effect of estradiol in attenuating liver injury after trauma-hemorrhage (Hsieh et al. 2007), in controlling FSHβ gene expression in LβT2 cells (Thompson et al. 2013), and in controlling prolactin gene expression in lactotroph (Ishida et al. 2010). However, PKA pathway may not important also for estradiol genomic control of LHβ gene expression in LβT2 cells (Thompson et al. 2013). Ishida et al. (2010) reported that ERα is the receptor for controlling prolactin gene expression in lactotroph. Hsieh et al. (2007) reported that the non-genomic salutary effect of E2 in reducing hepatic injury after trauma-hemorrhage is mediated through the PKA-dependent pathway via GPR30 but not ERα. Therefore, these data revealed for first time in all species that both ERK1/2/5 and PKA pathways are important pathways for GPR30 to suppress LH secretion in gonadotropes in the non-genomic manner.

The U0126-alone pretreatment was weaker effect to recover than the both inhibitors pretreatment before G1 treatment, but not estradiol treatment. In contrast, there was no significant difference between H89-alone pretreatment and both inhibitors pretreatment before G1 or estradiol treatment. Therefore, PKA pathway may be more important than ERK1/2/5 pathway for GPR30.

In conclusion, these data supported the hypothesis that ERK1/2/5 and PKA pathways are the important pathway in the non-genomic mechanism in the downstream of GPR30 to suppress LH secretion from bovine gonadotrope.

CHAPTER VIII

(Study VI)

Effects of Estradiol on cAMP Production in Cultured Bovine AP

Abstract

Picomolar concentrations of estradiol produce rapid suppression of GnRH-induced LH secretion from the AP of cattle via GPR30. Cyclic AMP (cAMP) may be a central player in cytoplasm for the estradiol non-genomic suppression of LH secretion. Therefore this chapter study was conducted to measure cAMP in the AP cells after estradiol treatment. The AP cells (n = 5) were cultured for 3 days in steroid-free conditions and then treated them with 0.01 nM estradiol for 5 min before GnRH stimulation. Cyclic AMP measurements analyses revealed that pre-treatment with the small amounts of estradiol ($P < 0.05$) decreased cAMP production in the AP cells.

8.1. Introduction

Estradiol binds with GPR30 in the AP to suppress LH secretion in the rapid, non-genomic manner. Cyclic AMP is the key molecule in the cytoplasmic pathway to increase LH secretion from ovine gonadotropes, by modulating Ca^{2+}-activated K^{+} channels (Adams et al., 1979; Sikdar et al., 1989). The cAMP production is attenuated by GPR30 on the plasma membrane of various cells including HEK293, CHO, and MCF7 cells ectopically expressing GPR30 (Broselid et al., 2014; Mo et al., 2013). However, it is remained to clarify whether estradiol pre-treatment has any effect on the cAMP increment in AP cells. Therefore, in the present study, the effect of estradiol on cAMP production was measured in cultured AP cells.

8.2. Materials and methods

8.2.1. AP cell culture to evaluate effects of estradiol on the cAMP increment.

Anterior pituitaries were obtained from post-pubertal Japanese Black heifers (n = 5, 26 months of age) for culture at 37 °C, 5% CO_2 for 82 h in the 24-well culture plates. After washing with PBS, the cells were incubated with 490 µL of DMEM containing 0.1% BSA for 2 h. Cells were pre-treated with 5 µL of DMEM alone or with 5 µL of DMEM containing 1 nM estradiol (final concentration, 0.01 nM). After gently shaking for 5 min, cells were treated for 2 h with 5 µL of 100 nM GnRH (final concentration, 1 nM, except the "control") dissolved in DMEM containing dopamine (0.5 µM, Nacalai Tesque) and phosphodiesterase inhibitor [0.5 mM 3-isobutyl-1-methyl-xanthine (MIX; Sigma-Aldrich)]. Dopamine and MIX are required to measure cAMP in cultured gonadotropes from heterogeneous AP cells because the amount of cAMP in lactotrophs fluctuates; moreover, phosphodiesterase decreases the amount of cAMP to below the detection limits (Adams et al., 1979). The concentrations of dopamine and MIX used in the present study were identical to those used in a previous investigation of cAMP in cultured ovine AP cells (Adams et al., 1979). There were three treatment conditions as follows: (1) "control" wells were treated with 5 µL of DMEM, but were not incubated with GnRH; (2) "GnRH" wells were treated with 5 µL of DMEM and were incubated with GnRH; (3) "estradiol" wells were pre-treated with estradiol and were incubated with GnRH.

8.2.2. cAMP measurement

After the 2 h of treatment, the wells were washed twice with PBS and were then used for cAMP extraction with a cAMP Select EIA Kit (Cayman Chemical, Ann Arbor,

MI, USA) according to the manufacturer's protocol and following Mo et al. (2013). Briefly, the washed plates were frozen and thawed three times. Each well was supplemented with 150 µL of assay buffer from the cAMP EIA kit and was scraped by using a cell scraper to remove cells. The plate was then vortexed for 30 s and centrifuged at 400 *g* for 1 min. After two further cycles of vortexing and centrifugation, the supernatant was harvested into a microtube. The supernatant was then centrifuged at 1000 *g* for 10 min. The 50-µL duplicates of the obtained supernatant were assayed by using the cAMP EIA kit according to the manufacturer's protocol. After 90 min of color development, the plates were read at a wavelength 405 nm by using an iMark microplate reader (Bio-Rad). The limit of detection was 0.09 pmol/mL. At 22.2 pmol/mL, the intra- and inter-assay CV were 6.0% and 7.3%, respectively. The specificities of the cAMP EIA kit evaluated by the company were 100% for cAMP and <0.01% for cGMP, AMP, ATP, adenosine, and dibutyryl cAMP.

8.2.3. Data analysis

The concentrations of cAMP in the control samples for each of the pituitary glands were averaged, and the mean value was set at 100%. The concentrations of cAMP in the treated samples for each of the pituitary glands were averaged, and the mean values were expressed as percentages of the control value. The statistical significance of differences in cAMP concentration were analyzed by using one-factor ANOVA with concentrations of cAMP as the dependent variable and treatment as the independent variable, followed by *post-hoc* comparisons by using Fisher's PLSD test. The level of significance was set at $P < 0.05$. Data were expressed as mean ± SEM.

8.3. Results

8.3.1. Effects of estradiol on the cAMP increment

Figure 8.1 depicts the effects of estradiol on the cAMP increment in cultured AP cells. The cAMP increment in the GnRH wells was greater than that in the control wells. Pre-treatment with 0.01 nM estradiol suppressed cAMP accumulation ($P < 0.05$).

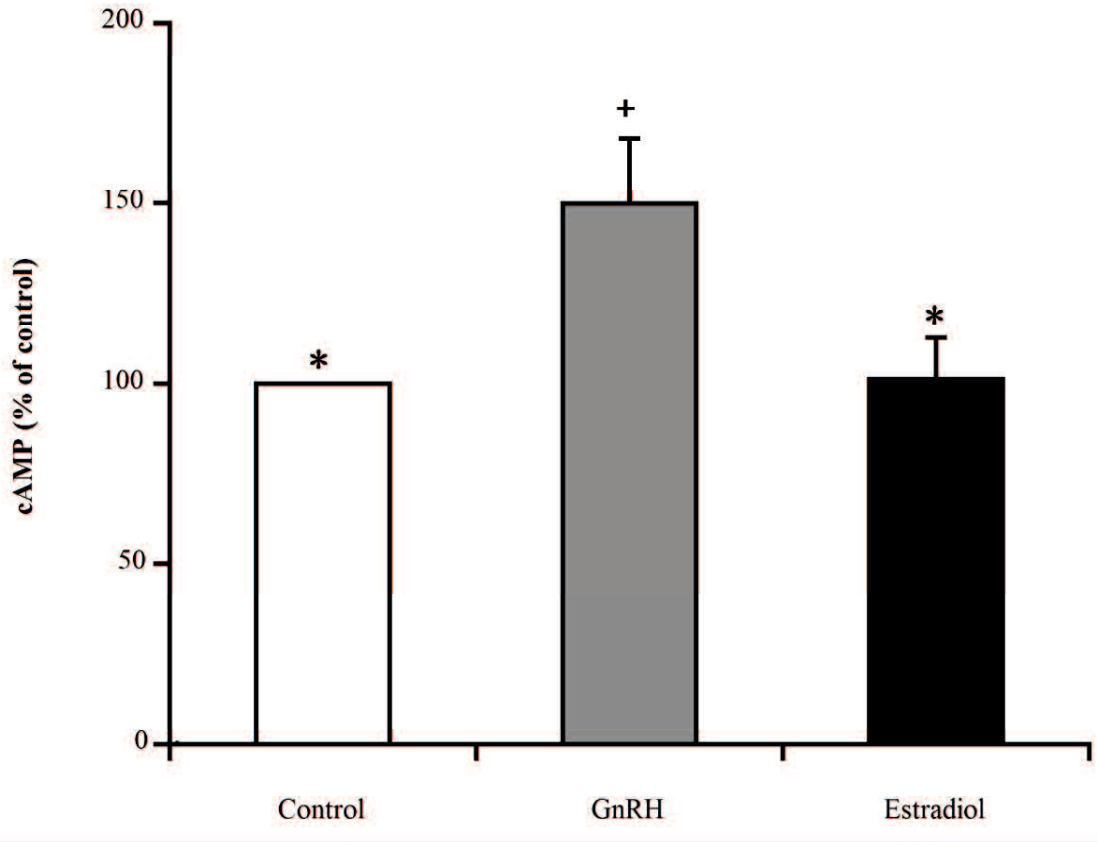

Fig 8.1. Comparison of the effects of 0.01 nM estradiol (black bar) on the cAMP increment in cultured bovine AP cells treated with 1 nM GnRH. cAMP concentrations in control cells (cultured in medium alone) were averaged, and the mean cAMP concentrations of treated groups are expressed as percentages of that value.

+P < 0.05: significant differences compared to the control.

*P < 0.05: significant differences compared to GnRH alone.

8.4. Discussion

Cyclic AMP is the key molecule in the cytoplasmic pathway to increase LH secretion from ovine gonadotropes, by acting as an intracellular second messenger that rapidly modulates Ca^{2+}-activated K^{+} channels (Adams et al., 1979; Sikdar et al., 1989). Furthermore, GPR30 forms a plasma membrane complex with membrane-associated guanylate kinase, discs, large homolog 4 (DLG4; or postsynaptic density protein 95), and protein kinase A-anchoring protein 5 (AKAP5); this complex inhibits cAMP production in HEK293 cells and CHO cells ectopically expressing GPR30 (Broselid et al., 2014). In the present study, pre-treatment with small amounts of estradiol decreased the cAMP increment in cultured AP cells. In addition, the AKAP5 (NM_174236) and DLG4 (NM_001191307) genes were amplified from the cDNA of bovine AP cells by using PCR. Results from the present study suggest that the GPR30-induced decrease in the cAMP increment has an important role in inhibiting LH secretion. Terasawa and Kenealy (2012) reported that estradiol affects various pathways in GnRH neurons, to induce cross-talk between cell surface receptors and nuclear receptors. Iqbal et al. (2007) showed that estradiol induced a rapid increase in the phosphorylation of second messenger proteins in ovine AP cells. Iqbal et al. (2009) subsequently proposed that estradiol inhibited the GnRH-induced increase in cytoplasmic calcium in ovine AP cells, in a dose-dependent manner. Hence, data from the present study imply that GPR30 and cAMP have important roles in the estradiol-induced suppression of LH secretion, but those ERs and other cytoplasmic pathways, PKA pathway and ERK1/2/5/ pathway as shown in chapter VII, are involved in inducing the rapid suppression of LH secretion by estradiol.

CHAPTER IX

(Study VII)

Abnormal Response to Estradiol

in AP of Heifers with Quiescent Ovary or Cystic Follicle

Abstract

Picomolar concentrations of estradiol produce rapid suppression of GnRH-induced LH secretion from the AP of cattle via GPR30. However, during the dissertation studies, we found AP of heifers with quiescent ovary or cystic follicle show abnormal responses to the rapid effect of estradiol. Thus, the GPR30 on the cell surface and the cytoplasmic pathways may be a reason to induce the ovarian diseases.

9.1. Introduction

Picomolar concentrations of estradiol produce rapid suppression of GnRH-induced LH secretion from the AP of cattle via GPR30. However, during the dissertation studies, we found AP of heifers with quiescent ovary and cystic follicle show abnormal responses to the rapid effect of estradiol. This chapter reports such abnormalities.

9.2. Materials and methods

Anterior pituitaries were obtained from Japanese Black heifers with quiescent ovary (n = 1, 26 months of age) or cystic follicle (n = 1, 26 months of age). The AP cells were cultured at 37 °C in 5% CO2 for 82 h. After washing with PBS, the cells were incubated with 485 μL of DMEM containing 0.1% BSA for 2 h. Either 5 μL of DMEM alone or 5 μL of DMEM containing 1 nM estradiol was added to each culture well. The cells were incubated while gently shaking for 5 min, after which they were incubated for 2 h with 5 μL of 100 nM GnRH dissolved in DMEM in order to stimulate LH secretion.

9.3. Results

Figure 9.1 depicts the effects of estradiol on the LH secretion in cultured AP cells of quiescent ovary female. The LH response to GnRH stimulation was small, compared to normal heifers shown in the previous chapters. There was no difference among the three treatments.

Figure 9.2 depicts the effects of estradiol on the LH secretion in cultured AP cells of cystic follicle female. The LH response to GnRH stimulation was large, and estradiol did not suppress the GnRH-induced LH secretion.

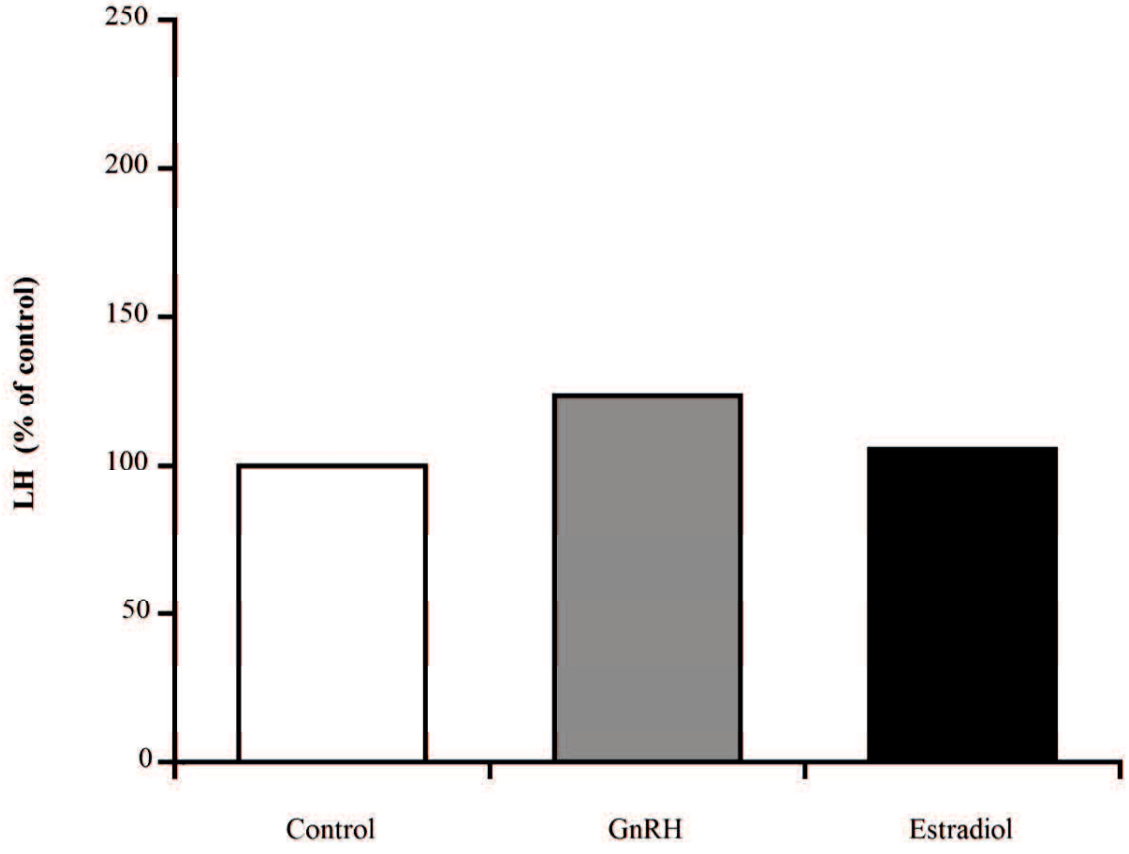

Fig 9.1. Comparison of the effects of 0.01 nM estradiol (black bar) on LH secretion in cultured bovine AP cells of quiescent ovary female treated with 1 nM GnRH. LH concentrations in control cells (cultured in medium alone) and LH concentrations of the treated groups are expressed as a percentage of the control.

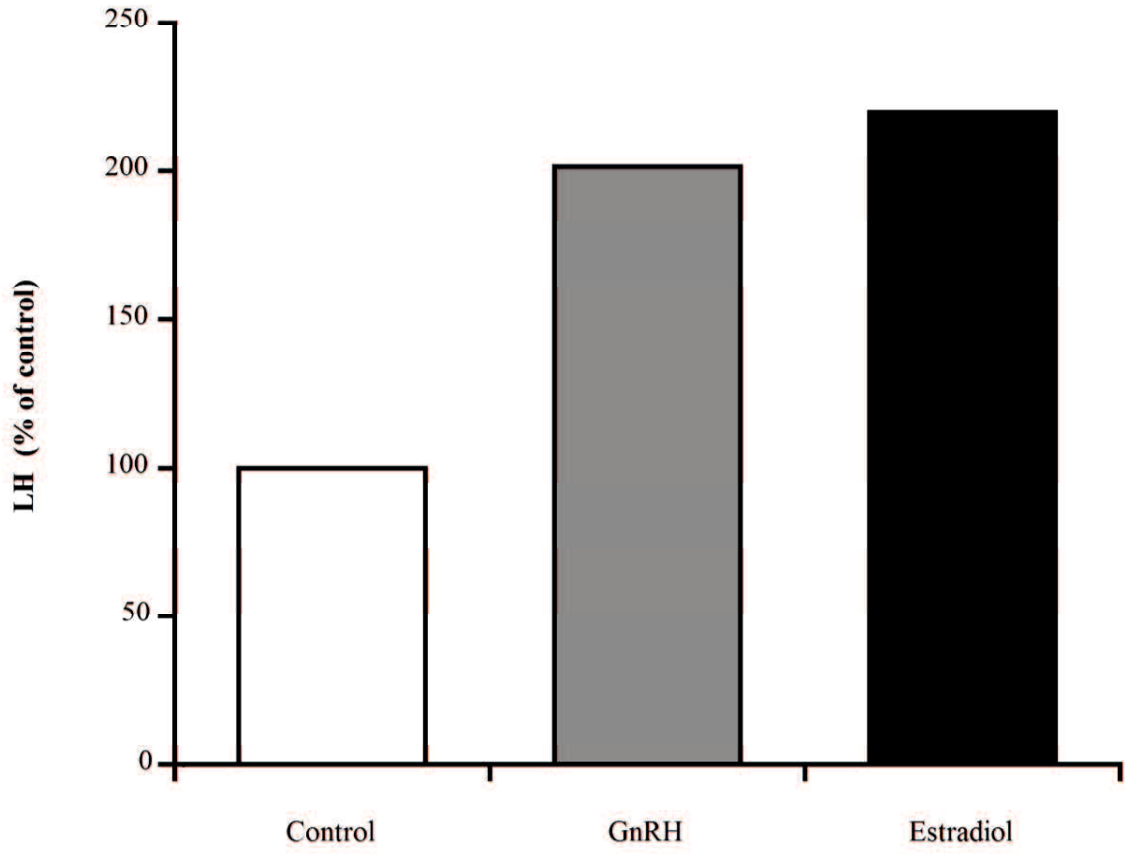

Fig 9.2. Comparison of the effects of 0.01 nM estradiol (black bar) on LH secretion in cultured bovine AP cells of cystic follicle female treated with 1 nM GnRH. LH concentrations in control cells (cultured in medium alone) and LH concentrations of the treated groups are expressed as a percentage of the control.

9.4. Discussion

These data suggested that the GPR30 on the cell surface and the cytoplasmic pathways may be the reason to induce the ovarian diseases. Kaneko et al. (2002) reported that the initial factor to induce follicular cyst is the suppression of LH surge because of the dysfunction in estradiol's positive feedback for LH secretion. Furthermore, they found that active pulsatile LH secretion in the lack of estradiol's negative feedback promotes continued growth of dominant follicle to make follicular cyst (Todoroki and Kaneko., 2006). Therefore, in order to cure the follicular cysts, it is important to restore the response of AP to estradiol's negative feedback to decrease LH secretion. Therefore, further studies were required to clarify pathophysiological mechanism in molecular level of APs of such abnormal females.

CHAPTER X

General Discussion and Conclusion

10.1. General discussion

In the study of Chapter III, we observed the expression of GPR30 mRNA and protein in bovine AP, and we showed using fluorescent immunohistochemistry that about 50% of GPR30-positive cells express LH, and about 30% of LH positive cells express GPR30 in bovine AP. These data suggested that estradiol might bind with GPR30 to induce rapid, non-genomic effects on both gonadotropes and other cell types in bovine AP. The study revealed that 5-min pre-treatment with low concentrations of estradiol and G1 inhibited the GnRH stimulated LH secretion. In contrast, higher concentrations of both estradiol and G1 were less efficient in suppressing GnRH-stimulated LH secretion.

The fluorescent immunohistochemistry revealed that about one-third of the LH-positive cells expressed GPR30. Therefore, the data suggest that bovine AP may have another undefined, non-GPR30 plasma membrane receptor to induce low estradiol suppression of LH secretion. To answer that question, we conducted another experiment in Chapter IV. Then, we revealed that STX, unlike estradiol or G1, possesses no suppressive effect on GnRH-stimulated LH secretion in the bovine pituitary gland, suggesting that GPR30 may play a more important role in LH secretion from bovine gonadotropes than the STX receptor.

To verify the importance of GPR30 for LH secretion from bovine AP cells, we conducted another experiment using the GPR30 specific antagonist, G36, in Chapter V. This study showed that pre-treatment with G36 inhibited estradiol suppression of LH secretion. Therefore, we concluded that GPR30 is the receptor to mediate the suppressive effect of estradiol on LH secretion.

In Chapter VI, we verified that the short time estradiol treatment has no effect on mRNA expressions of LHα, LHβ and FSHβ subunits in cultured AP cells. Therefore, estradiol suppresses LH secretion from cultured bovine AP cells through non-genomic manner.

In Chapter VII, pre-treatment with ERK1/2/5 and PKA inhibitors inhibited estradiol suppression of LH secretion. Dennis et al. (2011) reported that G36 selectively inhibits estrogen-mediated activation by GPR30, and G36 also inhibits estrogen- and G1-mediated calcium mobilization as well as ERK1/2/5 activation. On the other hand Zucchetti et al. (2014) reported that GPR30-AC-PKA pathway prevented estradiol-glucuronide induce cholestasis in rat liver. Therefore, ERK1/2/5 and PKA pathways may be important pathway for GPR30 to suppress LH secretion non-genomically.

In Chapter VIII, pre-treatment with estradiol decreased the cAMP increment in cultured AP cells. Recently, Broselid et al. (2014) reported that GPR30 forms a plasma membrane complex with the DLG4 kinase and PKA-anchoring protein 5 to inhibit cAMP production in the HEK293 cells and CHO cells models. Zucchetti et al. (2014) also reported that GPR30, PKA, and cAMP form a pathway for estradiol-glucuronide induced cholestasis in rat hepatocytes. Therefore, also in bovine gonadotrope, GPR30 may form the similar complex on cell membrane and form the pathway to decrease cytoplasmic cAMP, and to reduce LH secretion.

In Chapter IX, we showed the abnormal response of AP in heifers with quiescent ovary or cystic follicle against the rapid effect of estradiol. Kaneko et al. (2002) reported that the initial factor to induce follicular cyst is the suppression of LH surge because of the dysfunction in estradiol's positive feedback for LH secretion. They also found that active pulsatile LH secretion in the lack of estradiol's negative feedback promotes continued

growth of dominant follicle to make follicular cyst (Todoroki and Kaneko, 2006). Thus, any abnormality of GPR30 on the cell surface or the cytoplasmic pathways may be the reason to induce the ovarian diseases.

There is evidence that the plasma membrane is not a random sea of lipids. Lipid rafts are common and distinct relatively insoluble regions that have lower density and are less fluid than the surrounding membrane (Simons and Tooter, 2000; Head et al., 2014). These are liquid-ordered phase islands dispersed throughout the lipid bilayer matrix (Simons and Tooter, 2000; Head et al., 2014). These lipid rafts are thought to facilitate signaling by promoting the colocalization of membrane receptors and their downstream signaling components (Simons and Tooter, 2000; Head et al., 2014). Lipid rafts have been recognized as an important target in human medicine (George and Wu, 2012). The clonal murine gonadotrope cell line LβT2 expresses insulin receptor (Navratil et al., 2009) and glucocorticoid receptor (Wehmeyer et al., 2014) within lipid rafts containing GnRH receptor (GnRHR), and such rafts might provide a mechanism to integrate neuropeptides and energy homeostatic signals to modulate reproductive function. Furthermore, our group recently reported that GnRHRs were aggregated on a limited area of the cell surface of gonadotropes, possibly localized to lipid rafts (Kadokawa et al., 2014). Therefore, further study is required whether GPR30 and GnRHR are fellow passengers in the same lipid raft to affect each other.

There are various estrogen-like chemicals in environment surrounding domestic animals. Our group recently reported that zeranol, a non-steroidal mycoestrogen produced by Fusarium, suppress LH secretion from the AP of cattle via GPR30 in a rapid, non-genomic manner (Nakamura et al. 2015). Thomas and Dong (2006) reported that bisphenol A and nonylphenol, both well-known environmental estrogens, could bind with

GPR30. Taken together with these previous findings, the results of this dissertation study indicate the need for further studies to clarify the effects of estrogen-like chemicals on the rapid suppression of LH secretion in domestic animals. It is also important to identify other natural agonists of GPR30 that suppress LH secretion in a rapid mechanism in domestic animals.

10.2. Conclusion

Estradiol binds to GPR30, on the surface of bovine gonadotrope, decreased cAMP, activated PKA and ERK1/2/5 pathways to decrease LH secretion in a rapid, non-genomic mechanism. Any abnormality of GPR30 on the AP cell surface or the cytoplasmic pathways may induce the reproductive diseases.

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