

A novel intercellular communication by annexin A5:
Gonadotropin-releasing hormone control of blebbing and annexin A5
containing-ectosome formation of gonadotropes

Numfa Fungbun

2017

アネキシン A5 による新規の細胞間コミュニケーション: ギナドトロ
ピン放出ホルモンによるギナドトロフの小胞形成とアネキシン A5
含有エクソソーム形成の調節

Numfa Fungbun

平成 29 年度

Table of contents

| | |
|--|----|
| Chapter 1 Introduction | 8 |
| Chapter 2 Function of Annexin A5 in the pituitary gonadotropes | 11 |
| Introduction | 11 |
| Materials and methods | 13 |
| 1. Animals | 13 |
| 2. Primary culture of anterior pituitary cells | 13 |
| 3. Gonadotrope derived cell line, L β T2 cell culture | 15 |
| 4. Hormone assay | 16 |
| 5. Administration of rat recombinant ANXA5 to L β T2 cells | 18 |
| 6. Administration of rat recombinant ANXA5 with GnRH agonist (GnRH α) | 19 |
| 7. Knockdown of ANXA5 by siRNA | 19 |
| 8. Expression vector of ANXA5 | 20 |
| 9. Cell count | 21 |
| 10. DNA ladder | 22 |
| 11. Hemi-pituitary organ culture | 24 |
| 12. Immunohistochemistry for ANXA5 | 24 |
| 13. SDS-PAGE and Western blotting for ANXA5 | 26 |
| 14. Statistical analysis | 31 |
| Results | 32 |
| 1. Stimulatory effect of rat recombinant ANXA5 on LH release of L β T2 cells | 32 |
| 2. Stimulatory effect of rat recombinant ANXA5 on GnRH α action on LH release in primary culture of pituitary cells | 32 |
| 3. Inhibitory effect of ANXA5 knockdown by siRNA on GnRH α action on LH release in primary culture of pituitary cells | 32 |

| | |
|--|----|
| 4. Effect of ANXA5 expression on GnRHa action on LH release in primary culture of pituitary cells | 33 |
| 5. GnRHa suppression of L β T2 cell growth | 33 |
| 6. Apoptosis induction by GnRHa suppression of L β T2 cells growth | 33 |
| 7. GnRHa suppression of L β T2 cells growth and stimulation of LH release in a longer period of incubation | 33 |
| 8. Effect of GnRHa and high potassium on localization of ANXA5 in pituitary glands of rats | 34 |
| 9. Translocation of ANXA5 to outer-surface of plasma membrane | 34 |
| Discussion | 35 |
| Figures | 38 |
| Chapter 3 GnRH stimulation of ANXA5 containing extracellular vesicle (EV) formation of gonadotropes | 47 |
| Introduction | 47 |
| Materials and methods | 49 |
| 1. Animals | 49 |
| 2. Primary culture of anterior pituitary cells and L β T2 cell culture | 49 |
| 3. Poly-L-lysine coated cover slip preparation | 49 |
| 4. Immunocytochemistry of L β T2 cell culture for ANXA5 | 50 |
| 5. Double staining immunocytochemistry for ANXA5 and LH β | 52 |
| 6. Transmission electron microscopic (TEM) analysis of hemi-pituitary organ culture | 53 |
| 7. Isolation of extracellular vesicles by sequential centrifugation | 54 |
| 8. Negative staining of extracellular vesicles | 55 |
| 9. SDS-PAGE and Western blotting for ANXA5 | 55 |

| | |
|--|----|
| 10. Hormone assay | 57 |
| 11. Experimental designs | 57 |
| 12. Statistical analysis | 60 |
| Results | 61 |
| 1. GnRHa induces the formation of blebs containing ANXA5 from Gonadotropes | 61 |
| 2. Changes in ultrastructure after GnRHa treatment of hemi-pituitary organ culture | 61 |
| 3. Characterization of extracellular vesicles of L β T2 after GnRHa treatment | 61 |
| 4. Changes in ANXA5 content of extracellular vesicles after GnRHa treatment | 62 |
| 5. GnRH stimulation of ANXA5 containing extracellular vesicles is beneficial for LH release | 62 |
| 6. Enhancement of LH release by GnRHa treated 20,000 xg pellet fraction in a dose dependent manner | 63 |
| 7. ANXA5 profoundly detected in the 20,000 xg pellet fraction of plasma of 1 week ovariectomized rats | 63 |
| 8. Immunocytochemistry observation of inhibition of GnRHa stimulated extracellular vesicles containing ANXA5 in L β T2 | 63 |
| 9. Western blotting analysis of inhibition of GnRHa stimulated extracellular vesicles containing ANXA5 in L β T2 | 64 |
| Discussion | 65 |
| Figures | 69 |
| Chapter 4 GnRH stimulation of annexin A1 (ANXA1) expression | 82 |
| Introduction | 82 |
| Materials and methods | 84 |
| 1. Animals | 84 |
| 2. Primary culture of anterior pituitary cells and L β T2 cell culture | 84 |

| | |
|---|-----|
| 3. Immunocytochemistry of L β T2 cell culture for ANXA1 | 84 |
| 4. SDS-PAGE and Western blotting | 85 |
| 5. Double staining immunocytochemistry for ANXA1 and LH β | 87 |
| 6. Immunohistochemistry of pituitary after 2 weeks of ovariectomy for ANXA1 | 89 |
| 7. Sample preparation | 90 |
| 8. Statistical analysis | 92 |
| Results | 93 |
| 1. ANXA1 in the blebs after GnRHa treatment | 93 |
| 2. Content of ANXA1 of the 20,000 xg particulate fraction | 93 |
| 3. GnRHa augments ANXA1 protein expression in L β T2 cells | 93 |
| 4. Increase of ANXA1 protein expression in gonadotrope by GnRHa | 94 |
| 5. Change in ANXA1 distribution in the pituitary tissue of ovariectomized rats | 94 |
| 6. Increase of ANXA1 protein expression in pituitary gland of ovariectomized rats | 94 |
| Discussion | 95 |
| Figures | 98 |
| Chapter 5 Summary | 104 |
| Figure | 108 |
| Acknowledgements | 109 |
| References | 111 |

Chapter 1 Introduction

Intercellular communication is a prerequisite mechanism for a synchronized response and function performed by multicellular organisms. Mammals have developed various mechanisms for cell communication. They are called with different names, e.g. endocrine, juxtacrine, autocrine, paracrine and so on. Secretion of bioactive substances and existence of their receptors in a target cell are necessary to communicate among multiple cells. So, neurotransmitter, cytokine and growth factor would be categorized all in same on this point. Intercellular communication is attained also by gap junction between cells as seen in cardiomyocyte. Recently, a novel communication mechanism via extracellular vesicles that is probably evolutionarily old is attracting attention.

In this thesis, a relationship between one of phylogenetically very old hormones and also very old bioactive protein will be described. They are gonadotropin releasing hormone (GnRH) and annexin A5 (ANXA5). Both have been shown to play a significant role not only in reproduction but also in other cellular processes [28, 31].

GnRH is, a decapeptide hormone and a major regulator of reproduction [48]. It is produced by specialized neurons of the hypothalamus. The sequence of GnRH is same among mammals except for guinea pig, so far reported [48]. GnRH was reported also in phylogenetically very old organisms, e.g. octopus and protochordate [48]. So, it is thought that GnRH evolved from a general cell-cell communication tool to a hypothalamic neuro-hormone. At the median eminence of hypothalamus, GnRH is released in synchronized pulses from the nerve ending into the hypophyseal portal system [48]. Three distinct forms of GnRH, GnRH I, II and III, are known [10]. GnRH I and II have been demonstrated in most vertebrate classes while GnRH III has been found only in teleost fish [10]. In vertebrates, GnRH I in the hypothalamus regulates the production and release of gonadotropin at the anterior pituitary

gland [10]. GnRH II has been discovered initially as chicken GnRH II [49] and it was reported that GnRH II in the midbrain is involved in sexual behavior and food intake [10]. GnRH I and II mRNA have been reported to express in extra-hypothalamic region such as breast, in both normal and cancerous breast tissues [9]. There are two receptors for GnRH, GnRHR I and II, but II is a pseudogene in human [48]. So, in this thesis major GnRH, GnRH I, and the active receptor, GnRHR I, are studied.

Annexins are a family of structurally related calcium dependent phospholipid binding protein [41]. Annexins have been identified with twelve members (ANXA1-A11 and A13) in vertebrates [51]. Two principal domains are included in each annexins, namely C-terminus (core domain) and N-terminus. Annexins share similar structure of core domain with four repeats (eight repeats for annexin A6) of about 70 amino acids highly conserved sequence. The 3D model of annexins shows slightly curved disc. The convex phase harbors calcium ion and membrane binding sites. The concave phase points away from the membrane, thereby is available for other types of interaction [67]. N-terminus is diverse in sequence and length ranging from 12-169 residues among annexins [41]. None of annexins contain signal sequence in their structure but annexins are observed extracellular space. Transportation of annexins is proposed by Golgi/ER independent secretory pathway [67].

ANXA1 was first demonstrated to mediate anti-inflammatory action of cortisol by suppressing phospholipase A₂ [12]. ANXA7 was reported to play a role in exocytosis of chromaffin cells [8]. Other annexins also have been shown to be involved in different processes. However, there is no consistent idea for annexin function yet. ANXA5 was first found in the anterior pituitary tissues and later it was shown to augment gonadotropin secretion [30].

The relationship between GnRH and ANXA5 has been demonstrated by this laboratory [30, 34, 36]. GnRH stimulates the expression of ANXA5 in the pituitary gonadotropes [30]. GnRH utilizes ANXA5 to facilitate gonadotropin secretion at gonadotropes

[30]. However, a molecular mechanism for ANXA5 and its site of action on LH release are not known. Recently we found that the expression of ANXA1 among other annexins was also stimulated by GnRH [22].

In the present study, a mechanism for the augmentation of gonadotropin secretion by ANXA5, an effect of GnRH on ANXA5 in the gonadotropes and the relationship between another annexin member ANXA1 and GnRH were studied. Intercellular communication via extracellular vesicles will be discussed through these research projects.

Chapter 2 Function of Annexin A5 in the pituitary gonadotropes

Introduction

Relationship between gonadotropin releasing hormone (GnRH) and annexin A5 (ANXA5) has been well studied [34, 36, 87]. ANXA5 was first demonstrated in the pituitary tissues by immunohistochemistry [31, 35, 37]. ANXA5 distributes to most of anterior pituitary cells and it is characterized by staining of nuclear envelope, plasma membrane and occasionally in cytoplasm [31, 32, 35]. The existence of ANXA5 in the gonadotropes was shown by three-week ovariectomy in which the size of gonadotrope is increased and it is so called the castration cell. Castration cells were demonstrated to contain abundant ANXA5 in the nucleus and on plasma membranes [31]. Later, augmentation of ANXA5 mRNA expression in anterior pituitary gland was confirmed after ovariectomy [30, 37]. Increase of ANXA5 expression in the gonadotrope after ovariectomy was the result of enhanced GnRH secretion. GnRH agonist (GnRHa) administration increased ANXA5 expression [34, 36]. The direct relationship between GnRH and ANXA5 mRNA expression was demonstrated by the gonadotrope cell line, L β T2 [36]. Continuous GnRHa stimulation of ANXA5 mRNA expression in cultured L β T2 cells was remained high for at least 24 hrs unlike LH β mRNA expression that was downregulated even 6 hrs [36]. Administration of GnRHa daily for seven days induced ANXA5 mRNA expression in the pituitary tissue of the hypogonadal (*hpg*) GnRH deficient mice [87].

GnRH promotes the synthesis of ANXA5 in gonadotropes [30, 34] and L β T2 through mitogen activated protein kinase (MAPK) signaling [36]. Stimulatory effect of GnRHa on ANXA5 mRNA expression has been demonstrated both *in vitro* [30, 34, 36] and *in vivo* [30]. Augmentation of ANXA5 expression in the pituitary gland was observed by GnRHa administration. Lower expression of ANXA5 mRNA in the pituitary gland was demonstrated in

hpg mice [87]. These data clearly reveal that the synthesis of ANXA5 in the gonadotropes is physiologically under the control of GnRH.

ANXA5 exists not only in the pituitary gland but also in various peripheral tissues and GnRH stimulates the expression of ANXA5 also in those tissues [31]. So, ANXA5 is useful at least to utilize as a biomarker of GnRH action especially in peripheral tissues in which output of GnRH action is not known.

Administration of rat recombinant ANXA5 to primary culture of pituitary cells have been demonstrated to stimulate LH and FSH release in a dose dependent manner and to augment the effect of GnRH action on gonadotropin secretion [30]. This report was the first to clarify the stimulatory effect of ANXA5 on gonadotropin secretion [30]. Instead of accumulation of evidence for the stimulatory action of ANXA5 on gonadotropin secretion, it is not known so far a site and a mechanism by which ANXA5 functions.

This chapter aims to confirm the effect of ANXA5 on LH release and involvement of ANXA5 in GnRH stimulation of LH release in the gonadotrope. Relationship between the effect on LH release and cell proliferation of gonadotropes was also studied. The higher concentration of GnRHa stimulated LH release in a short period of treatment but rather suppressed LH release and inhibited cell growth in later period. Finally, the results of this chapter revealed that the stimulation of LH release and suppression of cell growth by GnRHa treatment were associated with translocation of ANXA5 to the periphery of cells. Externalization of ANXA5 was also demonstrated.

Materials and methods

1. Animals

Adult female Wistar Imamichi rats bred in our laboratory were maintained in light (5:00-19:00 hr) and temperature ($23\pm 3^{\circ}\text{C}$) controlled room. Laboratory chow and tap water were fed *ad libitum*. All procedures were done according to the guideline for animal treatment of Kitasato University and the experimental plan was approved by the committee (#16-089, 16-090).

2. Primary culture of anterior pituitary cells

a. Reagents, solutions and equipment

Culture medium

- Dulbecco's Modified Eagle Medium (DMEM; low glucose powder, L-glutamine, sodium pyruvate, pyridoxine hydrochloride, Gibco Life Technologies, Grand Island, NY, USA)

Non-essential amino acid solution (Gibco Life Technologies)

Antibiotic-antimycotic solution (Gibco Life Technologies)

Fetal bovine serum (Gibco Life Technologies)

HEPES buffer solution (Gibco Life Technologies)

Vacuum-driven filter (Stericup and Steritop, EMD Millipore Corporation, Billerica, MA, USA)

DMEM preparation

DMEM powder was dissolved in 900 ml of Milli-Q with gentle stirring followed by adding 3.7 g of NaHCO_3 . The solution was stirred until completely dissolved. HCl was added to the medium for adjusting pH to 7.2. The final volume was adjusted to 1,000 ml. Filtration of

the medium was done using vacuum-driven filter (Stericup and Steritop, EMD Millipore Corporation) with negative pressure.

Plain medium

Plain medium was prepared by adding 2% HEPES buffer solution.

Culture medium (low glucose)

The culture medium (DMEM, low glucose) contained 1% non-essential amino acid solution, 1% antibiotic-antimycotic solution and 10% fetal bovine serum.

0.25% Trypsin solution

- 0.125 g Trypsin (Gibco Life Technologies)
- 0.186 g EDTA (100 mM)
- 50 ml plain medium

0.1% Trypsin inhibitor solution

- 2 mg Trypsin inhibitor (Gibco Life Technologies)
- 2 ml plain medium

0.1% DNA deoxyribonuclease I (DNase I)

- 2 mg DNase I (Roche Diagnostics, IN, USA)
- 2 ml plain medium

4% Bovine serum albumin (Cohn fraction V, Wako Pure Chemical Industries, Osaka, Japan)

- 0.24 g albumin
- 6 ml plain medium

Spinner flask

Flame-polished Pasteur pipettes

Microplate (96-well, Iwaki, Asahi glass Co., Ltd., Tokyo, Japan)

CO₂ incubator

b. Protocol

Primary culture of anterior pituitary cells was prepared from pituitary glands of adult female Wistar Imamichi rats. Anterior pituitary glands were cut into 1 mm³ pieces. Cells were dispersed with 0.25% trypsin (Gibco Life Technologies) and 10 mM EDTA in DMEM supplemented with 2% HEPES solution (pH 7.4) for 40 min at 37°C with slow stirring by using a spinner flask. Tissue pieces were rinsed and mechanically disrupted by passage through flame-polished large bored Pasteur pipettes. The cells were washed and then re-suspended to 10⁶ cells/ml in DMEM supplemented with 1% antibiotic-antimycotic solution (Gibco Life Technologies), 1% non-essential amino acid solution (Gibco Life Technologies) and 10% fetal bovine serum (Gibco Life Technologies). Pituitary cells were seeded into a microplate (96-well, Iwaki) as 100,000 cells per a well. They were maintained in an atmosphere of 95% air, 5% CO₂ and 100% humidity at 37°C. Experiments were performed after 24 or 48 hrs of cell dissociation.

3. Gonadotrope derived cell line, LβT2 cell culture

LβT2 cell was a kind gift from Dr. Pamela Mellon, University of California San Diego [47].

a. Reagents, solutions and equipment

Culture medium

- Dulbecco's Modified Eagle Medium (DMEM; high glucose powder, L-glutamine, sodium pyruvate, pyridoxine hydrochloride, Gibco Life Technologies)

Antibiotic-antimycotic solution (Gibco Life Technologies)

Fetal bovine serum (Equa Fetal, Atlas Biologicals, Fort Collins, CO, USA)

Vacuum-driven filter (Stericup and Steritop, EMD Millipore Corporation)

75 cm² flask (Corning Incorporated, Oneonta, NY, USA)

DMEM preparation

DMEM solution was prepared as described above.

Culture medium (high glucose)

The culture medium (DMEM, high glucose) for L β T2 contained 1% antibiotic-antimycotic solution and 10% fetal bovine serum.

b. Protocol

A gonadotrope derived cell line, L β T2, was cultured in DMEM supplemented with 10% fetal bovine serum and 1 % antibiotic-antimycotic solution. The cells were grown in 75 cm² flask and maintained in an atmosphere of 95% air, 5% CO₂ and 100% humidity at 37°C. The cells were sub-cultured before 80% confluent. The experiment was performed after 24 hrs of sub-culture.

4. Hormone assay

a. Reagents, solutions and equipment

Anti-rabbit gamma globulin

Anti-rabbit gamma globulin goat serum was purified by ammonium sulfate precipitation.

Rabbit anti-rat LH (NIDDK-anti-rat LH-RIA-11; NIDDK-NIH, Bethesda, MD, USA)

Rat luteinizing hormone (NIDDK-rat LH-RP-3; NIDDK-NIH)

EU-labeling kit (Delfia, PerkinElmer Inc., MA, USA)

Enhancement solution (Delfia, PerkinElmer, Inc., Turku, Finland)

Coating buffer (pH 7.4)

- 5 g NaN_3
- 9 g NaCl
- 8.709 g K_2HPO_4
- 1,000 ml Milli Q water

Assay buffer (pH 7.4)

- 6.067 g Tris (2-amino-2- hydroxymethyl-1,3-propanediol)
- 8.5 g NaCl
- 0.5 g Bovine gamma globulin serum
- 7.8 mg DTPA (Diethylene triamide)
- 0.5 g NaN_3 (Sodium diazide)
- 5 g Bovine serum albumin (Sigma-Aldrich, MO, USA)
- 15 mg Phenol red
- 0.1 ml Tween 40
- 1,000 ml Milli Q water

Washing buffer

- 0.605 g Tris
- 0.5g NaN_3
- 1 ml Tween 20
- 8.766 g NaCl
- 1,000 ml Milli Q water

Time-resolved immuno-fluorometric assay (Pharmacia Biotech, Tokyo Japan)

Immunowash (Bio-Rad, Tokyo, Japan)

96 well immunoplate (Nunc, Tokyo, Japan)

b. Protocol

LH levels in the medium samples were measured by time-resolved immunofluorometric assay using NIDDK rat LH assay kit with Delfia system (Pharmacia Biotech). LH-I-9 was labeled with europium using a Delfia EU-labelling kit (Delfia, PerkinElmer Inc.). Anti-rabbit gamma globulin goat serum was prepared and gamma globulin was purified by ammonium sulfate precipitation. A 96 well immunoplate (Nunc) was coated with anti-rabbit gamma globulin and then 1:4,000 dilution of anti-rat LH serum was overlaid. After incubation, optimally diluted EU-labeled hormone was added and further incubated. All procedures were done at 4°C overnight.

5. Administration of rat recombinant ANXA5 to L β T2 cells

a. Reagents, solutions and equipment

Rat recombinant ANXA5

Recombinant ANXA5 was produced from inoculation of rat ANXA5 in insect cells using a baculovirus vector [71].

Culture medium (high glucose)

Microplate (96 well; Iwaki)

b. Protocol

L β T2 cells were seeded at 100,000 cells/well of 96 well microplate. Cells were cultured and maintained as previously described. One day after plating, the medium was

changed to 0, 0.04, 0.4 or 4 µg/ml rat recombinant ANXA5 containing medium and incubated for 24 hrs. LH levels in the medium were measured by time-resolved fluorometric immunoassay using Delfia system (Pharmacia Biotech) as already described.

6. Administration of rat recombinant ANXA5 with GnRH agonist (GnRHa)

a. Reagents, solutions and equipment

Rat recombinant ANXA5

GnRH agonist, (Concereal, Fertirelin acetate, Des-Gly10 [Pro9]-GnRH ethylamide Takeda Pharmaceuticals Co., Osaka, Japan)

Culture medium (low glucose)

Microplate (96 well, Iwaki)

b. Protocol

Primary culture of anterior pituitary cells was seeded at 100,000 cells/well of microplate (96-well, Iwaki). Two days after cell dissociation, the medium was changed to 0, 0.01, 0.1, 1 and 10 nM GnRHa with or without 1µg/ml rat recombinant ANXA5 for 1 hr. LH levels in the medium were measured.

7. Knockdown of ANXA5 by siRNA

a. Reagents, solutions and equipment

Silencer negative control siRNA (Ambion, MA, USA)

- 20 µM control siRNA stock solution in nuclease free water

Silencer select pre-designed siRNA (S62357) of ANXA5 (Ambion)

- 20 μ M ANXA5 siRNA stock solution in nuclease free water

Lipofectamine 2000 (Invitrogen, CA, USA)

GnRH agonist (Concereal, Fertirelin acetate)

Culture medium (low glucose, without antibiotic-antimycotic solution)

Microplate (96 well; Iwaki)

b. Protocol

Pituitary cells were seeded at 100,000 cells/well of microplate (96 well). One day after cell dissociation, the medium was changed to the medium without antibiotic-antimycotic solution. Transfection of pituitary cells with 100 nM control siRNA (Ambion Silencer Select Negative Control siRNA) or ANXA5 siRNA (Ambion Silencer Select Pre-designed siRNA, S62357) using Lipofectamine 2000 according to the protocol described by manufacturer. Briefly, five micro-litter of 20 μ M control siRNA or ANXA5 siRNA was added to 250 μ l medium. Pre-incubation of 5 μ l Lipofectamine 2000 was also prepared in 250 μ l medium for 5 min at room temperature. siRNA solution and pre-incubated Lipofectamine 2,000 was mixed gently and incubated for 20 min. Fifty microliter of the mixture was applied to pituitary cells contained 50 μ l DMEM (final concentration 100 nM siRNA) for 48 hrs. Then the medium was changed. Transfected pituitary cells were challenged with or without 10 nM GnRHa for 1 hr. LH levels in the medium samples were measured.

8. Expression vector of ANXA5

a. Reagents, solutions and equipment

Control vector

ANXA5 vector, pcDNA3.1

Lipofectamine 2000 (Invitrogen)

GnRH agonist (Concereal, Fertirelin acetate)

Culture medium (low glucose)

Microplate (96 well; Iwaki)

b. Protocol

Pituitary cells were seeded at 100,000 cells/well of microplate (96-well). ANXA5 vector (4 µg/ml) was prepared in our laboratory. One day after cell dissociation, the medium was changed to the medium without antibiotic-antimycotic solution. Pituitary cells were transfected with 0.5 µg of control or ANXA5 vector using 0.5 µl Lipofectamine 2000 per well (pcDNA: Lipofectamine 2000; 1:1). After 48 hrs of transfection, pituitary cells were challenged with or without 10 nM GnRH α for 1 hr. LH levels in the medium samples were measured.

9. Cell count

a. Reagents, solutions and equipment

GnRH agonist (Concereal, Fertirelin acetate)

0.25% Trypsin solution

Culture medium (high glucose)

Microplate (96 well, Iwaki)

Counting slides, Dual chamber for cell counter (Bio-Rad, CA, USA)

TC20 automated cell counter (Bio-Rad)

b. Protocol

LβT2 cells were seeded into a microplate (96 well) at 33,000 cells/well. On day 1, the medium was changed. LβT2 cells were incubated with or without 10 nM GnRHa for 24, 48 or 96 hrs. The medium was removed. Adherent cells were detached by 0.25% Trypsin solution. Number of cells was counted by TC20 automated cell counter.

The relation between cell growth and dosage of GnRHa was also examined. LβT2 cells were incubated with 0, 0.01, 0.1, 1, 10 or 100 nM GnRHa for 96 hrs. Number of cells was counted. LH levels in the medium were measured.

10.DNA ladder

a. Reagents, solutions and equipment

GnRH agonist (Concereal, Fertirelin acetate)

Culture medium (high glucose)

Culture dish (Cellstar 100x20 mm; Greiner bio-one, Frickenhausen, Germany)

Apoptotic DNA ladder kit (Roche, Mannheim, Germany)

- Binding/Lysis buffer (6 M guanidine-HCl, 10 mM urea, 10 mM Tris-HCl, 20% Triton X-100 (v/v), pH 4.4)
- Washing buffer (20 mM NaCl and 2 mM Tris-HCl, pH 7.5) and ethanol
- Elution buffer (10 mM Tris, pH 8.5)
- High pure spin filter tubes (Polypropylene tubes and filter tubes)
- Positive control (Lyophilized apoptotic U937 cells)

1.5% Agarose gel

Loading buffer (Takara Bio Inc., Otsu, Japan)

TBE buffer (pH 8.3)

- 10.9 g Tris base
- 5.5 g Boric acid
- 0.465 g EDTA
- 1,000 ml distilled water

5% ethidium bromide solution

Distilled water

Centrifuge 5430R (Eppendorf AG, Hamburg, Germany)

UV-transilluminator

b. Protocol

LβT2 cells were seeded onto 100x20 mm dishes. After 7 days of culture, LβT2 cells were incubated with or without 10 or 100 nM GnRHa for 3, 6 or 24 hrs. Cells were isolated and purified using apoptotic DNA ladder kit according to the protocol supplied by the manufacturer. Briefly, cells were lysed by adding 300 µl lysis buffer. The sample solution or positive control was mixed immediately and incubated for 10 min at 15-25°C. Then they were added 100 µl isopropanol and shaken on a vortex. The sample in the upper reservoir was centrifuged at 7,012 xg for 1 min. The sample in the reservoir was washed 2 times using washing buffer and centrifuged as previously described. Finally, centrifugation for 10 sec at 18,516 xg was performed to remove residual washing buffer. After insertion of the filter tube in clean tube, DNA was eluted from the filter tube using 200 µl of pre-warmed (70°C) elution buffer and centrifuged at 7,012 xg for 1 min. The purified DNA was electrophoresed on 1.5% agarose gel. The gel was stained with 5% ethidium bromide solution for 10 min and de-stained by shaking in distilled water for 5 min. The DNA ladder pattern was detected by UV-transilluminator.

11.Hemi-pituitary organ culture

a. Reagents, solutions and equipment

GnRH agonist (Concereal, Fertirelin acetate)

50 mM KCl

Culture medium (low glucose)

Shaker (Uni thermo shaker NTS-1300 EYELA, Tokyo, Japan)

b. Protocol

Pituitary glands were obtained from female rat on diestrus. Pituitary glands were cut at isthmus to make equivalent halves. Hemi-pituitary was immediately soaked in DMEM (low glucose) supplemented with 10% fetal bovine serum, 1% non-essential amino acid solution and 1% antibiotic-antimycotic solution with or without 100 nM GnRH α or 50 mM KCl for 10 min. The pituitary tissues were maintained in 99.5% O₂ at 37°C.

12.Immunohistochemistry for ANXA5

Paraffin block preparation

a. Reagents and solutions

0.1 M Phosphate buffered saline (PBS, pH 7.4)

- 140 mM NaCl (8.181 g)
- 8 mM Na₂HPO₄·12H₂O (29 g)
- 2.7 mM KCl (2 g)
- 1.4 mM KH₂PO₄ (1.9 g)

- 1,000 ml Distilled water

4% Paraformaldehyde (PFA) in PBS

- Paraformaldehyde (Merk Schuchardt OHG, Hohenbrunn, Germany)
- 0.1 M Phosphate buffer saline

Paraffin (Merk Schuchardt OHG)

b. Protocol

Hemi-pituitary tissues were fixed with 4% paraformaldehyde at 4°C overnight. After washing tissues with PBS, dehydration was performed per standard procedure. Paraffin blocks were used for making tissue sections with 4 µm thickness and dried in an incubator at 37°C overnight.

Immunohistochemistry

a. Reagent, solution and equipment

Antibody binding buffer (ABB, pH 7.4)

- 150 mM NaCl
- 5 mM EDTA
- 0.25% Gelatin
- 0.05% Nonidet P40
- 50 mM Tris
- Milli Q water

Blocking buffer

- 3% normal goat serum in ABB

Primary antibody

- Anti-ANXA5 rabbit serum (MS-2, home made)

Second antibody

- Alexa flour 488 goat anti-rabbit IgG (H+L) (Life Technologies, OR, USA)

Vectashield mounting medium with DAPI (Vector Laboratories, Inc., CA, USA)

Confocal laser microscope (Zeiss 710, Jena, Germany)

b. Protocol

Deparaffinization was done by xylene and ethanol series. Tissue sections were blocked with 3% normal goat serum in ABB for 1 hr at room temperature. Primary antibody incubation with anti-ANXA5 in ABB (1:10,000) was performed overnight at 4°C in a humidified chamber. Tissue sections were washed 3 times in ABB. Alexa flour 488 goat anti-rabbit IgG in ABB (1:1,000) was applied and specimens were maintained for 2 hrs at room temperature. Nucleus was stained with DAPI (Vectashield mounting medium, Vector Laboratories, Inc.). Fluorescence was observed under confocal laser microscope (Zeiss 710).

13.SDS-PAGE and Western blotting for ANXA5

Protein precipitation of EDTA washout of cultured L β T2 cell

a. Reagents, solutions and equipment

GnRH agonist (Concereal, Fertirelin acetate)

50 mM KCl

5 mM EDTA

Acetone (-20°C)

Culture medium (high glucose)

Culture dish (Cellstar; 60x15 mm, Greiner bio-one)

Multipurpose refrigerated centrifuge LX-120 (Tomy; PT. Prolabmas, Jakarta, Indonesia)

Centrifuge 5430R (Eppendorf AG, Hamburg, Germany)

b. Protocol

One day after the seeding of L β T2 cells (5×10^6 cells/dish), the medium was changed. Cells were challenged with 100 nM GnRHa or 50 mM KCl for 24 hrs. Then the medium was removed. Cells were washed with 1 ml pre-warmed 5 mM EDTA. The washout was collected and then centrifuged at 1,220 xg for 5 min at 4°C. The supernatant was transferred to a new tube. Protein was precipitated using 4 volumes of cold acetone and kept at -20°C for 2 days. Precipitated protein was pelleted by centrifugation at 1,220 xg for 10 min at 4°C and then re-suspended with 50 μ l sample buffer. After boiling the suspension at 95°C for 5 min, centrifugation was done at 18,516 xg 4°C for 5 min. The supernatant was collected and subjected to SDS-PAGE and Western-Blotting.

SDS-PAGE and Western-Blotting of protein precipitation

a. Reagents, solutions and equipment

1.5 M Tris-HCl (pH 8.8)

- 1.5 M Tris-base
- Adjust pH to 8.8 using HCl
- Distilled water

0.5 M Tris-HCl (pH 6.8)

- 0.5 M Tris-base

- Adjust pH to 6.8 using HCl
- Distilled water

0.25 M Tris-HCl (pH 6.8)

- 0.25 M Tris-base
- Adjust pH 6.8 using HCl
- Distilled water

30% w/v Acrylamide/ 0.8% w/v Bis solution

- Acrylamide (Bio-Rad)
- Bis (N,N'-methylene bis-acrylamide, Bio-Rad)
- Distilled water

10% SDS

- SDS (Bio-Rad)
- Distilled water

10% Ammonium per sulfate

- Ammonium per sulfate (Bio-Rad)
- Distilled water

TEMED (N,N,N',N'-tetra-methyl ethylenediamine, Bio-Rad)

Sample buffer

- 0.25 M Tris-HCl (pH 6.8)
- Glycerol
- 10% SDS
- 2-Mercaptoethanol (Bio-Rad)
- 0.05% Bromophenol blue

12% Separating gel

- Distilled water (3.245 ml)

- 1.5 M Tris-HCl (2.6 ml)
- 10% SDS (100 μ l)
- 30%/0.8% Acrylamide/Bis (4 ml)
- 10% Ammonium per sulfate (50 μ l)
- TEMED (5 μ l)

4% Stacking gel

- Distilled water (3 ml)
- 0.5 M Tris-HCl (1.25 ml)
- 10% SDS (50 μ l)
- 30%/0.8% Acrylamide/Bis (670 μ l)
- 10% Ammonium per sulfate (25 μ l)
- TEMED (5 μ l)

Electrophoresis buffer (pH 8.3)

- 3.03 g Tris-base
- 14.4 g Glycine
- 1 g SDS
- 1,000 ml Distilled water

Blotting buffer

- 192 mM Glycine
- 25 mM Tris
- 15% Methanol
- Distilled water

0.01% PBS-T

- PBS (pH7.4)
- 0.01% Tween 20

Blocking buffer

- 5% skim milk
- 0.01% PBS-T

Primary antibody

- Anti-ANXA5 rabbit serum (MS-2, home made)

Second antibody

- Anti-rabbit IgG-conjugated with horse radish peroxidase (ICN Biomedicals, Aurora, OH, USA)

Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, Tokyo, Japan)

- Solution A Amersham ECL Prime Luminol enhancer solution
- Solution B Amersham ECL Prime Peroxide solution

Electrophoresis chamber (Bio-Rad)

Electrophoresis power supply (Bio-Rad)

Blotting chamber (Bio-Rad)

PVDF membrane (Amersham Hybond P 0.45 PVDF, Amersham GE Healthcare)

NanoDrop 2000 (Thermo Scientific, DE, USA)

Luminescent image analyzer (Image Quant LAS 4000 series, GE Healthcare)

b. Protocol

The precipitated protein was adjusted to about 1 $\mu\text{g}/\mu\text{l}$ measured by photometry at 280 nm (NanoDrop 2000). Proteins were separated by SDS-PAGE using 12% poly-acrylamide gel. Electrophoretic transfer of protein to PVDF membrane was performed. Membrane was blocked with 5% skim milk in 0.01% PBS-T for 1 hr at room temperature. Immunodetection for ANXA5 was accomplished with anti-ANXA5 in blocking buffer (1:10,000) overnight at 4°C. The membrane was washed in 0.01% PBS-T for 10 min 3 times. Incubation with anti-rabbit

IgG-conjugated horse radish peroxidase in blocking buffer (1:50,000) for 2 hrs at room temperature was followed. Chemiluminescence was performed by pre-incubation of ECL prime reagent (A and B solution) for 5 min before adding to the membrane. The membrane was incubated with ECL prime mixture reagent for 5 min. Chemiluminescence was detected with luminescent image analyzer (ImageQuant LAS 4000 series).

14. Statistical analysis

Each value was presented as mean \pm SEM. Statistical analysis was performed using Tukey test after ANOVA for multiple comparison. P values less than 0.05 were considered to be significant.

Results

1. Stimulatory effect of rat recombinant ANXA5 on LH release of L β T2 cells

The effect of ANXA5 on LH release was examined. Administration of rat recombinant ANXA5 (0.04, 0.4 or 4 μ g/ml) to L β T2 cells resulted in significant increase of LH levels during 24 hrs incubation. Even 40 ng/ml of ANXA5 was effective. The increase of LH levels was in a dose dependent manner to ANXA5 (Fig. 2-1).

2. Stimulatory effect of rat recombinant ANXA5 on GnRH α action on LH release in primary culture of pituitary cells

GnRH α (0, 0.01, 0.1, 1, or 10 nM) was applied to primary culture of pituitary cells with or without 1 μ g/ml rat recombinant ANXA5 for 1 hr. GnRH α stimulated LH release in a dose dependent manner. Rat recombinant ANXA5 augmented the effect of GnRH α on LH release (Fig. 2-2).

3. Inhibitory effect of ANXA5 knockdown by siRNA on GnRH α action on LH release in primary culture of pituitary cells

The effect of ANXA5 knockdown on GnRH α action on LH release was examined with primary culture of pituitary cells. Cells were transfected with siRNA-control or siRNA-ANXA5 for 48 hrs. GnRH α (10 nM) was applied to transfected cells for 1 hr. Knockdown of ANXA5 expression by siRNA resulted in blunting of GnRH α action on LH release (Fig. 2-3).

4. Effect of ANXA5 expression on GnRHa action on LH release in primary culture of pituitary cells

After 48 hrs of ANXA5 vector transfection, GnRHa (10 nM) was administered in the culture medium for 1 hr. ANXA5 vector showed a tendency to increase LH release in the primary pituitary cells (Fig. 2-4).

5. GnRHa suppression of L β T2 cell growth

The effect of GnRHa on L β T2 cell growth was confirmed in the present study. GnRHa administration clearly suppressed the growth of L β T2 cells by 96 hrs incubation (Fig. 2-5).

6. Apoptosis induction by GnRHa suppression of L β T2 cell growth

DNA fragmentation of L β T2 cell after 10 or 100 nM GnRHa treatment for 3, 6 or 24 hrs was examined. DNA ladder was detected all samples of 3, 6 and 24 hrs of 10 nM or 100 nM GnRHa treatment (Fig. 2-6).

7. GnRHa suppression of L β T2 cell growth and stimulation of LH release in a longer period of incubation

The relation between GnRHa suppression of L β T2 cell growth and stimulation of LH release was studied at 96 hrs. L β T2 cells were cultured in presence of GnRHa (0, 0.01, 0.1, 1, 10 or 100 nM) for 96 hrs. The suppressive effect of GnRHa on L β T2 cell growth was observed in a dose dependent manner (Fig. 2-7A) whereas the effect of GnRHa on LH release was biphasic, lower concentration of GnRHa (0-0.1 nM) stimulated LH release in a dose dependent manner while higher dose (1-100 nM) inhibited LH release (Fig. 2-7B).

8. Effect of GnRHa and high potassium on localization of ANXA5 in pituitary glands of rats

GnRHa and non-specific depolarizing stimulation with high potassium on ANXA5 localization in pituitary cells was examined. Hemi-pituitary gland was cultured with or without 100 nM GnRHa or 50 mM KCl for 10 min. The distribution of ANXA5 was observed by immunohistochemistry. Depolarizing stimulation with high potassium treatment induced obvious plasma membrane-association of ANXA5. GnRHa showed similar effect on ANXA5 translocation to the periphery of the cell but lesser extent (Fig. 2-8).

9. Translocation of ANXA5 to outer-surface of plasma membrane

Externalization of ANXA5 was further clarified. Cultured L β T2 was treated with 100 nM GnRHa or 50 mM KCl for 24 hrs. After removing the medium, cultured cell was washed with 5 mM EDTA. The EDTA washout was precipitated and subjected to SDS-PAGE and Western-Blotting for ANXA5. ANXA5 was detected obviously in EDTA washout of L β T2 cells after GnRHa treatment. At 24 hrs, high potassium treatment also found ANXA5 in the washout but lower than GnRHa treatment (Fig. 2-9).

Discussion

Expression of ANXA5 mRNA is increased under the control of GnRH in the gonadotrope [30, 34, 36]. ANXA5 in turn stimulates LH release [30]. It was confirmed in the present study that recombinant ANXA5 augments LH release in L β T2 cells and the action of GnRH on LH secretion. Furthermore, gene knockdown of ANXA5 by siRNA suppressed the response to GnRHa stimulation of LH release. Expression vector of ANXA5 transfected to pituitary cells showed a tendency to increase LH release. These data confirmed that ANXA5 synthesized in the gonadotrope is in favor of LH release although its mechanism is not known.

ANXA5 exists in many cell types and has been proposed as an intracellular protein since it does not contain a signal sequence in its gene sequence [21]. However, ANXA5 has been shown in extracellular fluid such as plasma [66], amniotic fluid [18] and peritoneal lavage [60]. Extracellular ANXA5 was suggested to have a crucial physiological role. For example, ANXA5 on cell surface of syncytiotrophoblast was postulated to prevent inappropriate coagulation in the placenta during the early stage of pregnancy [82]. It was suggested that lower availability of maternal ANXA5 by autoantibodies against ANXA5 in anti-phospholipid syndrome or autoimmune disease causes placental thrombosis and fetal loss [82]. It is suggested that haplotype M2 in the annexin A5 (ANXA5) gene of human in which the expression level of ANXA5 is low and related to fetal loss [78]. Later it was proved that maternal deficiency of ANXA5 gene causes placental platelet thrombosis and fetal loss [80]. While ANXA5 was shown to be synthesized in the gonadotrope, ANXA5 added to the culture medium of gonadotrope augments LH release meaning that extracellular ANXA5 would affect on the gonadotrope. It is not known that ANXA5 in the pituitary gonadotropes exerts its effect inside or outside cells. Site of function and its mechanism are not clarified so far for any function of ANXA5.

GnRHa suppressed the growth of L β T2 cells during 96 hrs incubation. As DNA ladder was detected after 6 hrs of GnRHa treatment, the decrease of cells was suggested to be at least partly by apoptosis. Induction of apoptosis by GnRHa has been well demonstrated in many cell types [4, 52, 84]. Various tumors including breast, ovarian, endometrial and prostate cancer express GnRH receptor [23-25] and GnRH analogs have demonstrated to inhibit the growth of these cells [23-25, 84]. So, GnRHa is suggested to drive a signal transduction mechanism to induce apoptosis also in the gonadotropes, probably through Gi as reported for other tumor cells [27]. The suppressive effect of GnRH on cell growth seems to relate to ANXA5 since ANXA5 is increased by GnRH stimulation and ANXA5 has been reported to suppress cell growth [58].

While the suppressive effect of GnRHa on L β T2 cell growth was shown in a dose dependent manner, the effect of GnRHa on LH release was biphasic. Higher dosage of GnRHa was rather suppressive on LH release when incubation lasts longer period of time. The biphasic action of GnRH suggests different intracellular signals are responsible for these two cellular responses to GnRH or different thresholds exist for each response. Furthermore, since synthesis of ANXA5 is continued under GnRH stimulation, chronic stimulation of GnRHa is hypothesized to be pro-apoptotic and mediated by ANXA5. So, it is necessary to clarify a relationship between a manner of GnRH action, namely frequency, concentration, duration, and cellular response. ANXA5 synthesis seems to primarily facilitate gonadotropin secretion and it would be against cell growth when ANXA5 continues to affect on cells.

ANXA5 changes its localization within a cell by GnRH and it is thought that the movement of ANXA5 in a cell is related to its function. ANXA5 associates with plasma membranes and nuclear envelope of most pituitary cells [35] and it accumulates to the nucleus and plasma membranes under a condition in which GnRH secretion is augmented, namely ovariectomy [37]. Such changes in ANXA5 on gonadotropes were observed also in this study. High concentration of potassium and GnRHa induced plasma membrane association of ANXA5.

Furthermore, ANXA5 was externalized by such stimulations. Interestingly, GnRHa facilitated externalization of ANXA5 more than that seen with high potassium stimulation. Plasma membrane association of ANXA5 was more in high potassium condition. It is suggested that externalization of ANXA5 is controlled by a GnRH specific cellular mechanism. GnRHa is suggested to enhance ANXA5 expression not only in gonadotropes but also in other pituitary cell types [87]. High potassium induces ANXA5 display on pituitary cell surface and increases the stimulation of prolactin and growth hormone release from pituitary cells [35]. GnRHa and high potassium stimulation are thought to provide a different effect on pituitary cells. Increase of ANXA5 translocation to plasma membrane by GnRHa at gonadotropes was suggested to be involved in gonadotropin secretion.

High concentration of continuous GnRHa administration augments ANXA5 mRNA expression for longer period of 24 hrs [36]. Involvement of GnRH stimulation of ANXA5 expression in the cell growth suppression through protein kinase C activation is suggested in human uterine leiomyoma cells [85] and human endometrial cells [70]. In those reports, cell growth was suppressed by PKC activator and PKC inhibitor restrained GnRHa action. GnRH was shown to stimulate ANXA5 synthesis through protein kinase C and MAPK at least during first 2 hrs [36]. These data and present result suggest that high and continuous stimulation with GnRH and ANXA5 would affect cell growth phenomena.

This chapter demonstrated that the stimulating effect of LH release and anti-proliferative effect on cell growth by GnRH were suggested to associate with externalization of ANXA5. Further study is needed to clarify how ANXA5 is translocated to outside of plasma membrane and whether it is involved in stimulation of LH release by GnRH.

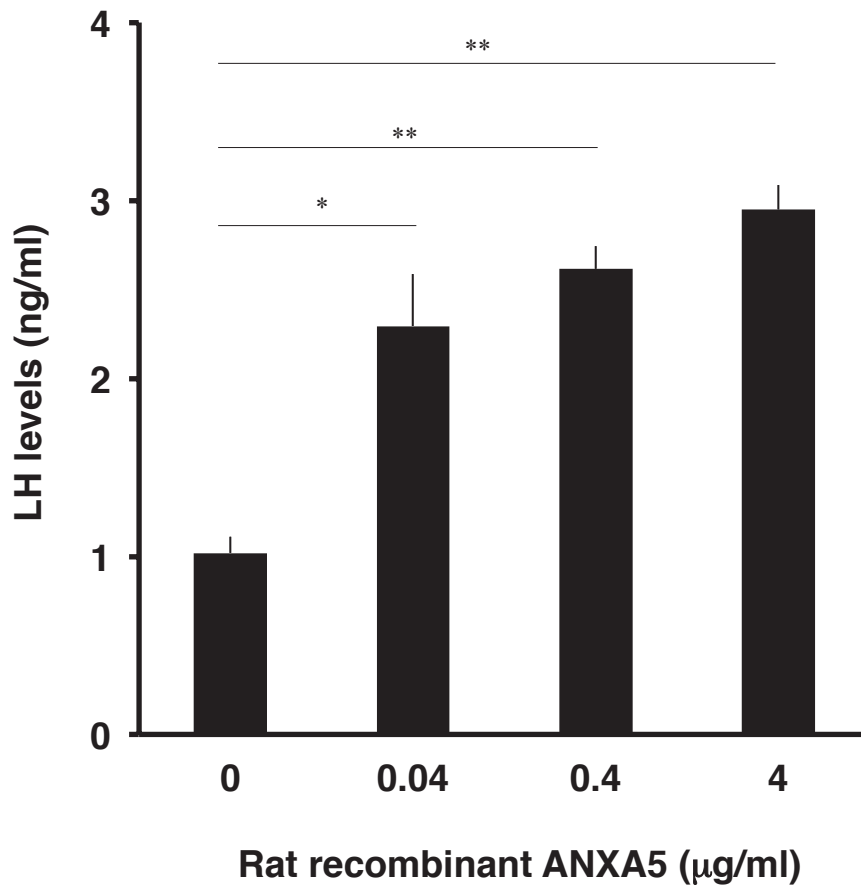


Fig. 2-1 Stimulatory effect of rat recombinant ANXA5 on LH release in LβT2 cells

Rat recombinant ANXA5 was given to LβT2 cells for 24 hrs. Medium content of LH was measured and values are revealed as mean±SEM. Statistical significance, * p<0.01 and ** p<0.0001.

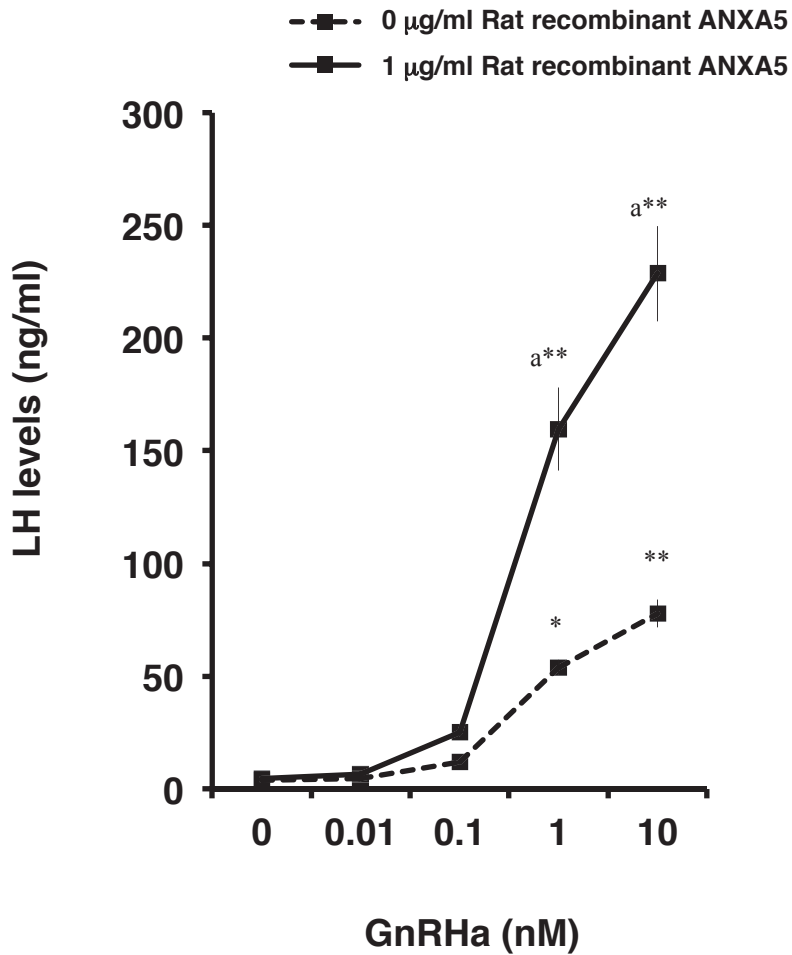


Fig. 2-2 Enhancement of GnRHa action on LH release by rat recombinant ANXA5

Two days after the cell dissociation of pituitary cells, the medium was changed to 0, 0.01, 0.1, 1 and 10 nM of GnRHa with/without rat recombinant ANXA5 (1 µg/ml) and cells were incubated for 1 hr. LH levels are revealed with mean \pm SEM. Statistical significance of values different from that of 0 M are revealed with asterisks respectively, * $p < 0.05$ and ** $p < 0.0001$. "a" is added when significant within the same GnRHa group, $a < 0.0001$.

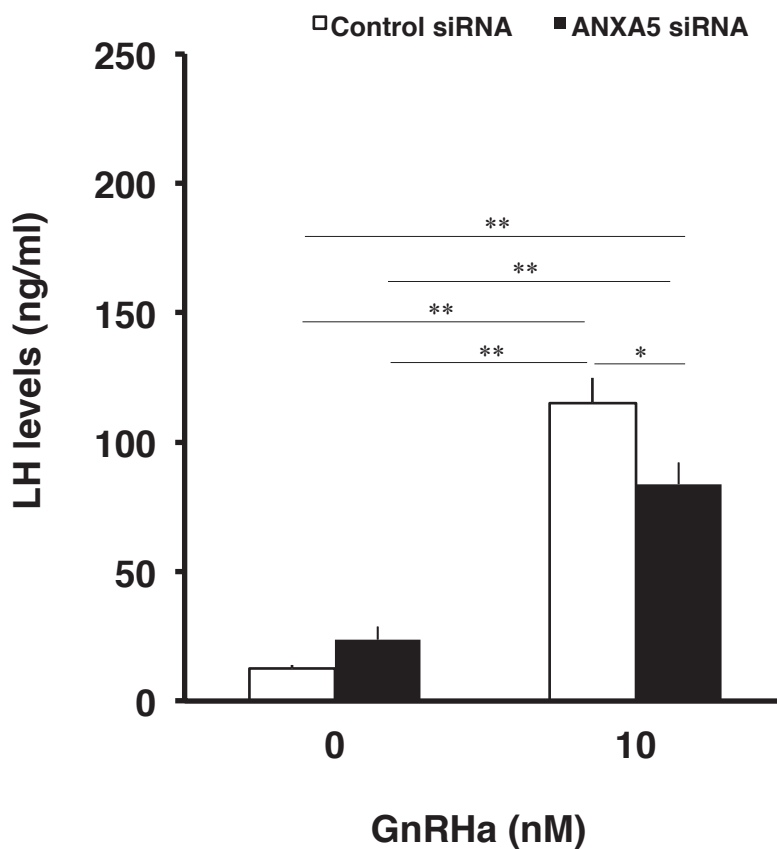


Fig. 2-3 Inhibitory effect of gene knockdown by siRNA for ANXA5 on LH release in primary pituitary cells

Pituitary cells were transfected with 100 nM control siRNA or ANXA5 siRNA using Lipofectamine 2000 and incubated for 48 hrs. Transfected cells were challenged with 10 nM GnRHa for 1 hr. Values are revealed with mean±SEM. Statistical significance, * p<0.05 and ** p<0.0001.

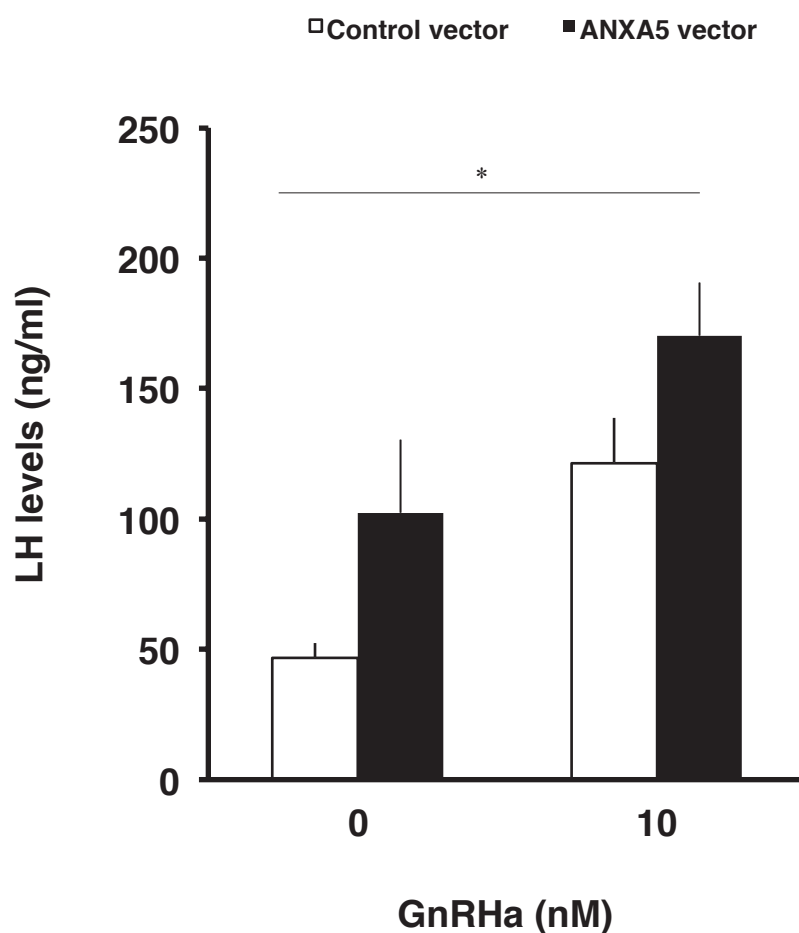


Fig. 2-4 Effect of ANXA5 expression vector on LH release in primary pituitary cells

Pituitary cells were transfected with 0.5 μ g control vector or ANXA5 vector using Lipofectamine 2000 for 48 hrs. Transfected cells were treated with or without 10 nM GnRHa for 1 hr. LH levels in the medium was measured. Statistical significance, * $p < 0.01$.

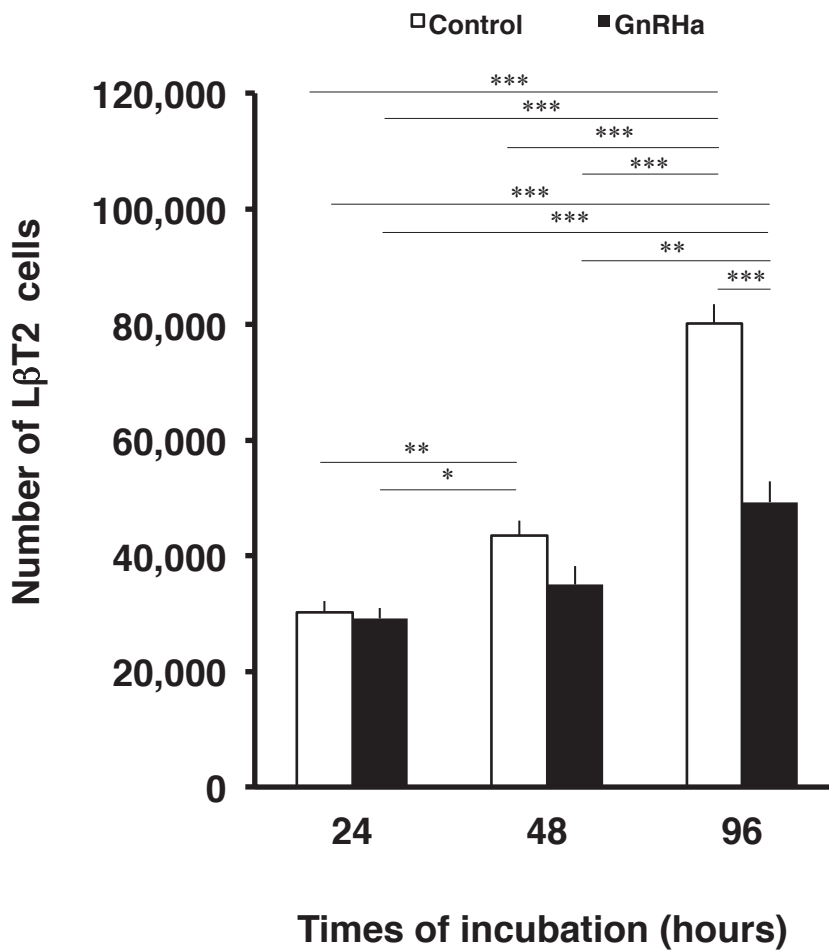


Fig. 2-5 Suppression of LβT2 cell growth by GnRHa

LβT2 cells were seeded into a microplate (96 well). After 1 day, cells were treated with or without 10 nM GnRHa and incubated for 24, 48 or 96 hrs. Number of LβT2 cells (mean±SEM) per well was counted.

Statistical significance, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.0001$.

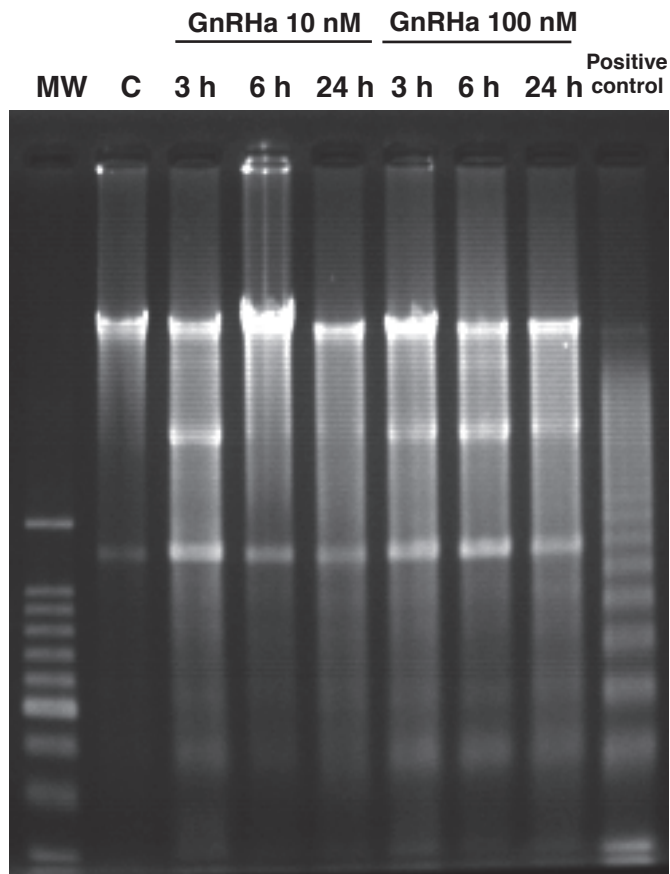


Fig. 2-6 Apoptotic DNA ladder of GnRHa treated L β T2 cells

DNA was isolated and purified from L β T2 cells incubated with 10 or 100 nM GnRHa for 3, 6 or 24 hrs. Control sample (C) was isolated at 0 hr. Purified DNA was electrophoresed and DNA ladder was detected by UV transilluminator.

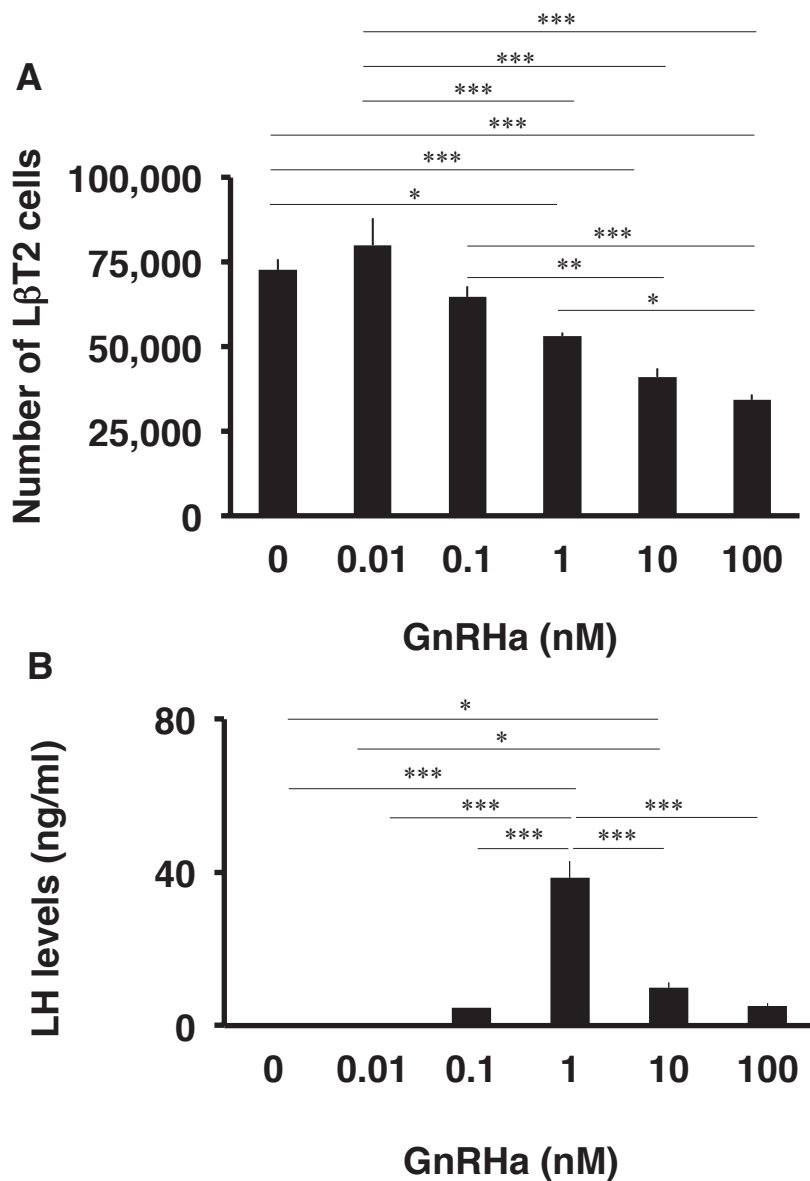


Fig. 2-7 Effect of GnRHa on LβT2 cell growth and LH release

LβT2 cells were seeded into a microplate (96 well). After 1 day, cells were treated with 0, 0.01, 0.1, 1, 10 or 100 nM of GnRHa for 96 hrs. Number of cells was counted. LH level in the medium was measured. (A) Number of LβT2 cell per well (mean±SEM) after incubation with GnRHa for 96 hrs. (B) LH levels in the medium. Statistical significance, * p<0.05, **p<0.01 and ***p<0.0001.

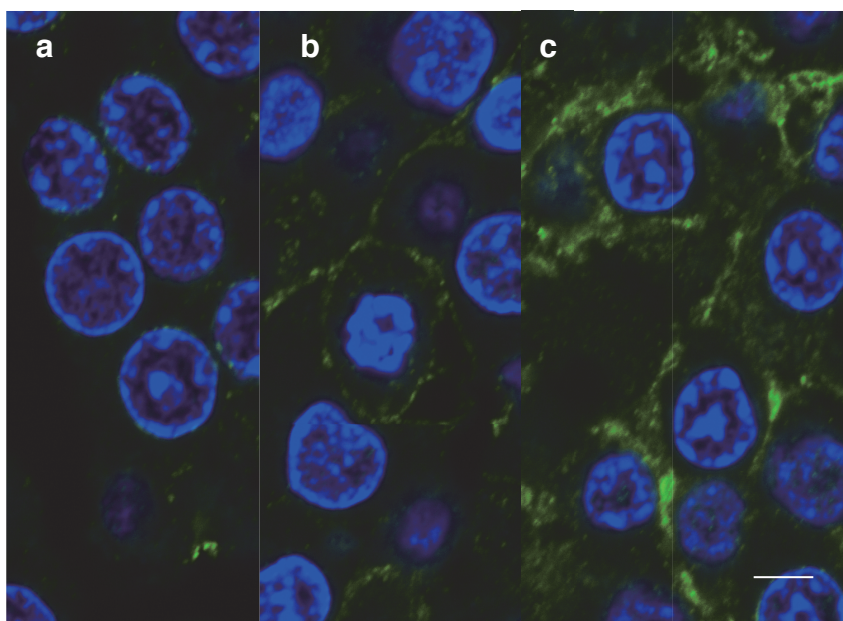


Fig. 2-8 Effects of GnRHa and high potassium on ANXA5 localization in pituitary gland of rats

Hemi-pituitary gland was incubated for 10 min. (a) Control incubation. (b) 100 nM GnRHa was administered. (c) Medium was changed to 50 mM KCl. Green and blue signals indicate ANXA5 and DAPI respectively. Scale bar is 5 μ m.

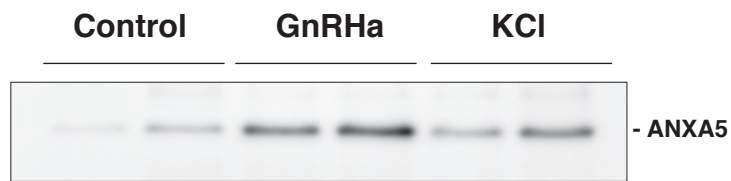


Fig. 2-9 GnRHa induces ANXA5 association with plasma membranes

L β T2 cells were treated with 100 nM GnRHa or 50 mM KCl for 24 hrs. The medium was discarded. Cells were washed with 5 mM EDTA. The EDTA washout was precipitated with cold acetone at -20°C for 2 days. The precipitated protein was subjected to SDS-PAGE and Western blotting for ANXA5.

Chapter 3 GnRH stimulation of ANXA5 containing extracellular vesicle (EV) formation of gonadotropes

Introduction

It was observed in chapter 2 that the externalization of ANXA5 was augmented by GnRH stimulation. ANXA5 does not contain a signal sequence in its sequence and secretory proteins lacking a signal sequence is not exported through plasma membranes by classical endoplasmic reticulum (ER)/Golgi secretory pathway [83]. However, there are many exceptions in which cytoplasmic proteins directly traverse across plasma membrane or extracellular vesicles (EVs, microvesicle and exosome) play a role of transporter [54]. EVs are known as a mechanism by which protein would be released to the extracellular space. Ectosome is generated by shedding of budding (or bleb) from the plasma membrane and with a relatively larger diameter (100-350 nm) while exosome is extracellular vesicles released by exocytosis of intracellular vesicles derived from multi-vesicular bodies and characterized by smaller diameter (50-100 nm) [13]. Intercellular communication by EV is reported for protein, lipid, and RNA [65]. ANXA5 has been reported to be present in exosomes [56, 57, 63, 64] and has proposed to be one of markers for exosomal identification as well as CD9, CD63, CD81, Alix, TGS 101, FLOT1, FLOT2 and ANXA2 [63]. Exosome containing ANXA5 is suggested to be a biomarker of cancer such as colorectal carcinoma [81]. Association of ANXA5 to EV in extracellular fluid is suggested its extracellular functions. So, it is very interesting to clarify how ANXA5 is transported to extracellular space not by classical exocytosis route.

GnRH receptor is typical G-protein coupled receptor (GPCR) characterized by a lack of C terminal intracellular domain. In pituitary gonadotropes, binding of GnRH to its receptor initiates both $G_{\alpha q/11}$ and $G_{\alpha s}$ subunit [44]. $G_{\alpha q/11}$ activate phospholipase C and then

facilitates the hydrolysis of membrane phosphatidylinositol 4,5-bisphosphate (PIP₂) to diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP₃). They result in a rise in intracellular calcium and activation of protein kinase C (PKC) respectively [59]. G α s stimulates cAMP-dependent protein kinase A (PKA) signal transduction [44]. Complex crosstalk between these transductions implicates various actions of GnRH at gonadotropes. It is not known which system is responsible for GnRH stimulation of ANXA5 externalization.

ANXA5 was shown to stimulate LH secretion [30]. GnRH augments biosynthesis of LH β mRNA and LH release at gonadotropes. LH β and α are transported through the Golgi and stored in electron dense granules [15] associated with the storage protein secretogranin II (SgII) [16] which is responsible for regulation of hormone delivery in the regulated secretory pathway of exocytosis [2]. LH associated SgII in electron-dense storage granules are released in response to GnRH signal transductions. The synergistic interaction of cAMP, PKC and calcium induced by GnRH initiates exocytosis of LH [45]. Moreover, cAMP has been demonstrated to induce LH exocytosis with calcium independent manner [45]. It is not known how ANXA5 would affect on GnRH stimulation of LH secretion. As GnRH stimulates the externalization of ANXA5 and administration of recombinant ANXA5 augments LH release, it is suggested that ANXA5 would affect on exocytosis of LH from outside gonadotropes.

This chapter is to clarify the mechanism by which GnRH stimulates ANXA5 externalization of gonadotropes and whether this process would be involved in LH secretion. It was clearly demonstrated that ANXA5 translocation outside the gonadotropes by budding or bleb formation. Blebbing was pinched off from plasma membranes to become shedding microvesicles or ectosome. GnRH induced ANXA5 containing ectosome and it significantly increased LH secretion at gonadotropes. This process mediated through cAMP-dependent PKA signaling. Increase of ANXA5 containing ectosome formation demonstrated physiologically in ovariectomized rats in which GnRH secretion is increased by liberation from negative feedback.

Materials and methods

1. Animals

Adult female Wistar Imamichi rats were maintained in light (5:00-19:00 hr) and temperature ($23\pm 3^{\circ}\text{C}$) controlled room. They were fed with laboratory chow and tap water *ad libitum*. All procedures were done according to the guideline for animal treatment of Kitasato University (#16-089, 16-090).

2. Primary culture of anterior pituitary cells and L β T2 cell culture

Primary culture of anterior pituitary cells and L β T2 cell culture were prepared as described in chapter 2.

3. Poly-L-lysine coated cover slip preparation

a. Reagents and equipment

Poly-L-lysine (Sigma-Aldrich, MO, USA)

Milli Q water

Membrane filter unit (0.45 μM , Advantec, Toyo Roshi Kaisha, Ltd., Tokyo, Japan)

Cover slip (18x18 mm; Matsunami, Kishiwada, Japan)

b. Protocol

Milli Q water was filtrated by membrane filter unit (0.45 μM , Advantec). Poly-L-lysine solution was prepared to make the final concentration of 0.5 mg/ml poly-L-lysine with filtrated Milli Q water. Cover slip was soaked in poly-L-lysine solution for at least 30 min. After drying, they were autoclaved and kept in dry place.

4. Immunocytochemistry of L β T2 cell culture for ANXA5

a. Reagents, solutions and equipment

GnRH agonist (Fertirelin acetate, Takeda Pharmaceuticals)

GnRH antagonist (Cetrorelix, kindly donated by Zentaris GmbH, Frankfurt, Germany)

Dimethyl sulfoxide (DMSO; Sigma)

Protein kinase C inhibitor (GF109203x; Tocris bioscience, Funakoshi, Tokyo, Japan)

- 1 mM stock solution of GF109203x in DMSO was prepared.

MAPK kinase inhibitor (PD98059; Tocris bioscience)

- 25 mM stock solution of PD98059 in DMSO was prepared.

Protein kinase A inhibitor (H89; Funakoshi)

- 20 mM stock solution of H89 in DMSO was prepared.

0.1 M Phosphate buffered saline (PBS, pH 7.4)

- 140 mM NaCl
- 8 mM Na₂HPO₄ 12H₂O
- 2.7 mM KCl
- 1.4 mM KH₂PO₄
- 1,000 ml Distilled water

4% Paraformaldehyde (PFA) in PBS

- Paraformaldehyde (Merk Schuchardt OHG)
- 0.1 M Phosphate buffer saline

Acetone (-20°C)

Antibody binding buffer (ABB, pH 7.4)

- 150 mM NaCl
- 5 mM EDTA
- 0.25% Gelatin
- 0.05% Nonidet P40
- 50 mM Tris
- Milli Q water

Blocking buffer

- 3% normal goat serum in ABB

Primary antibody

- Anti-ANXA5 rabbit serum (MS-2, home made)

Second antibody

- Alexa flour 488 goat anti-rabbit IgG (H+L) (Life Technologies)

Alexa flour 568 Phalloidin (Invitrogen)

Vectashield mounting medium with DAPI (Vector Laboratories, Inc.)

Confocal laser microscope (Zeiss 710)

b. Protocol

Cells were seeded onto poly-L-lysine coated cover slip and were fixed with 4% paraformaldehyde for 15 min at room temperature after experiment. Then they were rinsed with cold acetone (-20°C). Fixed cells were washed with PBS for 5 min 3 times following rinse with ABB once for 5 min. Blocking solution contained 3% normal goat serum in ABB were added on the fixed cells for 1 hr at room temperature. Immunocytochemistry was performed using anti-ANXA5 in ABB (1:10,000) 4°C overnight. Alexa flour 488 goat anti-rabbit IgG in ABB (1:1,000) was used for second antibody for 2 hrs at room temperature. Finally, phalloidin was

applied to stain actin filaments for 20 min at room temperature. Washing with ABB was performed for 5 min 3 times. DAPI was used for DNA staining. Specimen was observed by confocal laser microscope (Zeiss 710).

5. Double staining immunocytochemistry for ANXA5 and LH β

a. Reagents

Reagents and solution were same as those for single antibody staining.

Primary antibody

- Anti-LH β guinea pig serum (NIDDK-NIH)
- Anti-ANXA5 rabbit serum (MS-2, home made)

Second antibody

- Alexa flour 568 goat anti-guinea pig IgG (H+L) (Life Technologies)
- Alexa flour 488 goat anti-rabbit IgG (H+L) (Life Technologies)

b. Protocol

Immunocytochemistry was performed using anti-LH β in ABB (1:10,000) and anti-ANXA5 in ABB (1:10,000). Immunoreaction was detected with Alexa flour 568 goat anti-guinea pig IgG in ABB (1:1,000) and Alexa flour 488 goat anti-rabbit IgG in ABB (1:1,000) with incubation time of 2 hrs at room temperature. Washing with ABB was performed for 5 min 3 times at the end of each process. DAPI was used for DNA staining. Immunocytochemistry of ANXA5 distribution was observed by confocal laser microscope (Zeiss 710).

6. Transmission electron microscopic (TEM) analysis of hemi-pituitary organ culture

a. Reagents, solutions and equipment

Karnovsky solution

- 2% glutaraldehyde
- 2% paraformaldehyde in 0.05M cacodylate buffer (pH 7.4)

1% osmium tetroxide

1.5% potassium ferrocyanide

Epoxy resin

Uranyl acetate

Lead citrate

Ultracut N (Reichert-Nissei, Wein, Austria)

Transmission electron microscope (Hitachi H-7650; Hitachi Ltd., Tokyo, Japan)

b. Protocol

The pituitary tissue samples were fixed by immersion in Karnovsky solution (2% glutaraldehyde, 2% paraformaldehyde in 0.05M cacodylate buffer, pH 7.4). The specimens were excised, trimmed to about 1 mm³ and immersed in the same Karnovsky solution. Tissue samples were post-fixed with 1% osmium tetroxide/1.5% potassium ferrocyanide. The tissue samples were then dehydrated and embedded in epoxy resin. Ultrathin sections were cut using an Ultracut N, stained using uranyl acetate followed by lead citrate and examined using a Hitachi H-7650 transmission electron microscope.

7. Isolation of extracellular vesicles by sequential centrifugation

a. Equipment

Conical tube (50 ml; Cellstar, Greiner bio-one)

Oak ridge tube (Nalgene Products, Thermo Scientific)

Ultra-clear centrifuge tube (16x76 mm, Beckman Counter Inc., CA, USA)

Multipurpose refrigerated centrifuge LX-120 (Tomy; PT. Prolabmas)

High speed refrigerated centrifuge Suprema 21(Tomy Digital Biology Co., Ltd., Tokyo, Japan)

Ultracentrifuge (Beckman Coulter Inc., CA, USA)

b. Protocol

Extracellular vesicles isolation from conditioned medium of L β T2 cells

After performing experiments, conditioned medium was collected to a conical tube (50 ml tube; Cellstar, Greiner bio-one). To collect the remaining particles, each of conditioned-medium was washed with 2 ml of medium. Two fractions were combined to one conical tube and placed on ice. The sample medium was centrifuged at 300xg at 4°C for 10 min (Multipurpose refrigerated centrifuge LX-120; Tomy, PT. Prolabmas). The supernatant was poured to an oak ridge tube (Nalgene Products, Thermo Scientific) and then, centrifuged at 2,000xg (High speed refrigerated centrifuge Suprema 21; Tomy Digital Biological Co., Ltd.) at 4°C for 20 min. Next, the supernatant was transferred to a new autoclaved-oak ridge tube and centrifuged at 20,000xg at 4°C for 45 min. The 20,000 xg supernatant was moved to the Ultra-clear centrifuge tube. They were subjected to ultracentrifugation (Ultracentrifuge, Beckman Coulter Inc.) at 110,000 xg, 4°C for 60 min. The 20,000 xg pellets, ectosomes and the 110,000 xg, exosomes fraction were collected. Pellets were washed with PBS (Fig. 3-1).

8. Negative staining of extracellular vesicles

a. Reagents, solutions and equipment

4% filtrated paraformaldehyde (pH 7.4)

Copper film coated grids (ALLIANCE Biosystems, Osaka, Japan)

10 % EM stainer (Nisshin-EM, Inc., Tokyo, Japan)

ddH₂O

Transmission electron microscope (Hitachi H-7650; Hitachi Ltd., Tokyo, Japan)

b. Protocol

The isolated extracellular vesicles were fixed in 4% filtrated paraformaldehyde (pH 7.4) 4°C overnight. Fixed samples (10 µl) were incubated on the film coated copper grids (ALLIANCE Biosystems) for 5 min. The grids were negatively stained with 10% EM stainer (Nisshin-EM, Inc.) in ddH₂O for 5 min. A Hitachi H-7650 transmission electron microscope was used for imaging.

9. SDS-PAGE and Western blotting for ANXA5

a. Reagents, solutions and equipment

1.5 M Tris-HCl (pH 8.8)

0.5 M Tris-HCl

0.25 M Tris-HCl (pH 6.8)

30% w/v Acrylamide/ 0.8% w/v Bis solution

10% SDS

10% Ammonium per sulfate

TEMED (N,N,N',N'-tetra-methyl ethylenediamine, Bio-Rad)

Sample buffer

12% Separating gel

4% Stacking gel

Electrophoresis buffer (pH 8.3)

Blotting buffer

0.01% PBS-T

Blocking solution

- 5% skim milk
- 0.01% PBS-T

Primary antibody

- Anti-ANXA5 rabbit serum (MS-2, home made)

Second antibody

- Anti-rabbit IgG-conjugated with horse radish peroxidase (ICN Immuno-biological laboratories, MN, USA)

Amersham ECL Prime Western Blotting Detection Reagent (GE Health care)

- Solution A Amersham ECL Prime Luminol enhancer solution
- Solution B Amersham ECL Prime Peroxide solution

Electrophoresis chamber (Bio-Rad)

Electrophoresis power supply (Bio-Rad)

Blotting chamber (Bio-Rad)

PVDF membrane (Amersham Hybond P 0.45 PVDF, GE Healthcare)

NanoDrop 2000 (Thermo Scientific, DE, USA)

Luminescent image analyzer (Image Quant LAS 4000 series)

b. Protocol

SDS-PAGE and Western blotting was done as described in chapter 2. Briefly, SDS-PAGE of protein was run in 12% poly-acrylamide gel. Electrophoretic transfer of protein to PVDF membrane was performed. Membrane was blocked with 5% skim milk for 1 hr at room temperature. Immunodetection for ANXA5 was accomplished with anti-ANXA5 in ABB (1:10,000) and anti-rabbit IgG-conjugated horse radish peroxidase in ABB (1:50,000). Chemiluminescence was detected by ImageQuant LAS 4000 series. Each band was analyzed and shown as fold increase of ANXA5 intensity.

10.Hormone assay

LH levels in medium were measured by time resolved fluorometric immunoassay using Delfia system as described in chapter 2.

11.Experimental designs

11.1 L β T2 cells

Effects of GnRHa on ANXA5 distribution in L β T2 cells

L β T2 cells (100,000 cells) were seeded onto poly-L-lysine coated cover slips. The medium was replaced next day to those with or without 100 nM GnRHa and cells were incubated for 10 and 30 min.

Negative staining of extracellular vesicles

L β T2 cells were grown in the Petri dish until 80% confluent. Before the experiment, the medium was removed and cells were washed 2 times with pre-warmed medium. Cultured cells were treated with or without 100 nM GnRHa for 30 min (2 dishes each). The conditioned

medium was collected. Cultured cells were washed with pre-warmed medium 2 times. Two fractions of medium were combined and then processed to isolate extracellular vesicles by sequential centrifugation. The 20,000 xg and 110,000 xg precipitates were fixed with 4% paraformaldehyde for negative staining of vesicles.

Detection of ANXA5 in culture medium by Western blotting

After washing L β T2 cells, they were treated with or without 100 nM GnRHa for 10, 30 and 180 min (4 dishes each). Extracellular vesicles were isolated by sequential centrifugation. The washed pellets were re-suspended in 20 μ l sample buffer and subjected to SDS-PAGE and Western blotting for ANXA5.

Examining the effect of 20,000 xg pellet on LH releases

L β T2 cells were incubated with or without 100 nM GnRHa for 3 hrs (4 dishes each). The 20,000 xg pellet was obtained by sequential centrifugation. Pre-incubation without GnRHa was also performed for 3 hrs (8 dishes). Isolation of the 20,000 xg pellet was collected from 4 dishes each group. The isolated 20,000 xg pellet fraction was incubated with or without 100 nM GnRHa for 3 hrs. Pellet was washed with serum free medium and then centrifuged at 20,000xg for 45 min. The washed pellets were re-suspended in 280 μ l of medium. The suspension was examined the effect on LH release of L β T2 cell for 24 hrs incubation. LH levels in the medium samples were measured by time-resolved fluorometric immunoassay.

Study of inhibition of extracellular vesicle formation

Immunocytochemical observation of ANXA5 in L β T2 cells after treatment with Cetrorelix (GnRH antagonist), GF109203x (protein kinase C inhibitor), PD98059 (MAPK kinase inhibitor) or H89 (protein kinase A inhibitor). Inhibitors for GnRHa related signal

transduction were shown (Fig. 3-2). One day after seeding cells, L β T2 cells were pretreated with 100 nM Cetrorelix, 10 nM GF109203x, 28 μ M PD58059 or 30 μ M H89 for 1 hr. Then, the cells were incubated with or without 100 nM GnRHa for 30 min. Cells were fixed and processed for immunocytochemistry with anti-ANXA5 (MS-2).

The same experiments were performed and ANXA5 in extracellular vesicle was examined with Western blotting. L β T2 cells were grown in the Petri dish (100x20 mm) until 80% confluent. Cells were treated with 0 or 100 nM GnRHa for 30 min (2 dishes each). Pre-treatment with 100 nM Cetrorelix (GnRH antagonist), 10 nM GF 109203x (protein kinase C inhibitor), 25 μ M PD 98059 (MAPK kinase inhibitor) or 30 μ M H89 (protein kinase A inhibitor) for 1 hr was performed. Then, the cells were incubated with or without 100 nM GnRHa (2 dishes) for 30 min. The 20,000 xg pellet was re-suspended in 20 μ l sample buffer and subjected to SDS PAGE and Western blotting.

11.2 Primary culture of anterior pituitary cells

Primary culture of anterior pituitary cells (100,000 cells) were seeded onto poly-L lysine coated cover slip. Two days after the cell dissociation, the medium was changed with 0 or 100 nM GnRHa and incubated for 10 and 30 min. Cells were fixed and subjected to the double staining immunocytochemistry with anti-ANXA5 (MS-2) and anti-LH β (NIDDK-NIH).

11.3 Hemi-pituitary organ culture

Hemi-pituitary organ culture was prepared as previously described in chapter 2. The hemi-pituitary glands were incubated with 100 nM GnRHa for 10 or 30 min. Tissues were subjected to the observation with electron microscopy.

11.4 Ovariectomy

Adult female Wistar Iamichi rats were ovariectomized (OVX, n=3). Sham operated rats were prepared. Plasma was collected 1 week later from abdominal aorta using heparinized syringe. All procedures were done under isoflurane anesthesia. Heparinized plasma was consecutively centrifuged as described. After 2,000 xg centrifugation, 4 ml of plasma was then subjected to sequential centrifugation to isolate 20,000 xg and 110,000 xg pellets. Each pellet fraction was washed with cold PBS and followed centrifugation at 20,000xg for 45 min and 110,000 xg for 60 min at 4°C. After washing, the 20,000 xg and 110,000 xg pellet were dissolved in 50 µl sample buffer for SDS-PAGE and Western blotting.

Detection of free ANXA5 in the plasma after ultracentrifugation was also performed. Two hundred microliter of post-ultracentrifuged plasma was precipitated using cold acetone (800 µl) and kept at -20°C for 2 days. The precipitated sample was then centrifuged at 685 xg (2500 rpm) for 10 min at 4°C. The pellet was re-suspended with sample buffer. Total protein was adjusted to 1 µg/µl. Twenty microliter of suspension was subjected to SDS-PAGE and Western Blotting for ANXA5.

12.Statistical analysis

Each value was presented as mean±SEM. Statistical analysis was performed using Student's *t* test for the comparison of two groups and Tukey test after ANOVA for multiple comparison. P values less than 0.05 were considered to be significant.

Results

1. GnRHa induces the formation of blebs containing ANXA5 from gonadotropes

Immunocytochemistry for ANXA5 showed that GnRHa induced obvious membrane blebs on the surface of L β T2 cells (Fig. 3-3A). These blebs were shown to contain ANXA5. This phenomenon was also demonstrated for gonadotropes of primary pituitary cells. Double staining of primary pituitary cells with anti-ANXA5 and -LH β revealed that ANXA5 distributed intra-nucleus, cytoplasm and translocated to periphery of plasma membrane blebs during 10 and 30 min of GnRHa incubation (Fig. 3-3B). The optical sectioning of L β T2 cells treated with GnRHa for 10 min was demonstrated (Fig. 3-4).

2. Changes in ultrastructure after GnRHa treatment of hemi-pituitary organ culture

Changes in ultrastructure were observed in the pituitary tissues after GnRHa treatment. Hemi-pituitary gland was incubated with or without GnRHa for 10 and 30 min. Although each cell was clearly observed in control tissue, the boundary of the cell became obscure by GnRHa treatment. Cells with different sized round secretogranules were assumed to be gonadotropes and at higher magnification of a 30 min GnRHa treatment many bubble like particles appeared at periphery of gonadotrope like cells. Arrows indicate the area of boundary of cells (Fig. 3-5).

3. Characterization of extracellular vesicles of L β T2 after GnRHa treatment

Extracellular vesicles in the medium of L β T2 after GnRHa stimulation were isolated by sequential centrifugation for harvesting the 20,000 xg ectosome fraction and 110,000 xg exosome fraction. Each pellet was examined by negative staining. EV was larger in 20,000 xg than 110,000 xg fraction. Further, the size was much bigger in GnRHa treated sample. The diameter of ectosome was about 200 nm after GnRHa treatment. The particle size less than 100

nm was shown in the 110,000 xg fraction. These 20,000 xg and 110,000 xg particles were increased by GnRHa treatment for 30 min. Particles are indicated by arrows (Fig. 3-6).

4. Changes in ANXA5 content of extracellular vesicles after GnRHa treatment

LβT2 cells were incubated with or without 100 nM GnRHa for 10, 30 and 180 min. Ectosome and exosome fractions were isolated from the medium by sequential centrifugation. ANXA5 in the fraction was detected by Western blotting. ANXA5 was detected profoundly in the 20,000 xg pellet prepared from cultured medium after treatment with GnRHa for 10, 30 and 180 min and it was increased until 180 min. ANXA5 in 110,000 xg pellet was also increased at 180 min of GnRHa treatment. Each band was analyzed and shown as fold increase intensity of ANXA5. (Fig. 3-7).

5. GnRH stimulation of ANXA5 containing extracellular vesicles is beneficial for LH release

The effect of ANXA5 containing extracellular vesicles stimulated by GnRHa on LH release was performed. The 20,000 xg pellet was isolated from medium of LβT2 cell treated with 0 or 100 nM GnRHa for 3 hrs (Control pellet or Pellet isolated from GnRHa treatment). A part of control pellet was incubated with medium or 100 nM GnRHa for 3 hrs (Pellet incubated with medium or Pellet incubated with GnRHa). GnRHa treated 20,000 xg pellet fraction significantly enhanced LH release (Fig. 3-8).

6. Enhancement of LH release by GnRHa treated 20,000 xg pellet fraction in a dose dependent manner

The 20,000 xg pellet was isolated from medium of L β T2 cell treated with 0 or 100 nM GnRHa for 3 hrs (Control pellet or Pellet isolated from GnRHa treatment). A part of control pellet or pellet isolated from GnRHa treatment was prepared by 2-fold serial dilutions (0.0625, 0.125, 0.25, 0.5 and 1). The stimulatory effect of 20,000 xg pellet fraction by GnRHa on LH release showed in a dose dependent manner and it significantly increased from 2 times dilution (Fig. 3-9).

7. ANXA5 profoundly detected in the 20,000 xg pellet fraction of plasma of 1 week ovariectomized rats

Membrane particles were isolated from blood plasma obtained from sham or 1 week ovariectomized rats. ANXA5 contents in the 20,000 xg and 110,000 xg pellets were examined. Contents of ANXA5 were analyzed and shown as fold increase intensity of ANXA5. Ovariectomy significantly augmented ANXA5 in the 20,000 xg pellet fraction. Free ANXA5 in the post-ultracentrifugated plasma precipitation of ovariectomized rats also significantly increased (Fig. 3-10).

8. Immunocytochemistry observation of inhibition of GnRHa stimulated extracellular vesicles containing ANXA5 in L β T2

Effect of GnRH antagonist (Cetrorelix), protein kinase C inhibitor (GF 109203x), MAPKK inhibitor (PD98059) and protein kinase A inhibitor (H89) on the inhibitory effect of GnRHa stimulation of extracellular vesicle containing ANXA5 in L β T2 was clarified. Immunocytochemistry for ANXA5 and staining of actin with phalloidin demonstrated that the extracellular vesicle containing ANXA5 by GnRHa stimulation was inhibited by GnRH

antagonist and protein kinase A inhibitor, but not by protein kinase C inhibitor or MAPKK inhibitor (Fig. 3-11).

9. Western blotting analysis of inhibition of GnRHa stimulated extracellular vesicles containing ANXA5 in L β T2

The effect of GnRH antagonist (Cetrorelix), protein kinase C (GF 109203x), MAPKK inhibitor (PD98059) and protein kinase A inhibitor (H89) of GnRHa stimulation of extracellular vesicle containing ANXA5 in L β T2 was also confirmed by Western blotting. The 20,000 xg pellet fraction was prepared from the medium of treated cells. ANXA5 in the 20,000 xg pellet was lower in the pretreatment of GnRH antagonist and protein kinase A inhibitor in presence of GnRHa (Fig. 3-12).

Discussion

It was shown in chapter 2 that GnRHa stimulated the externalization of ANXA5 in gonadotropes. Although the extracellular ANXA5 has been already demonstrated by other groups despite lacking a signal sequence in its gene [21, 60], hormonal stimulation of externalization seen in this study is a new observation. The process of ANXA5 transportation to extracellular space is thought to be a regulated cell function. As ANXA5 could not be secreted by classical or ER/Golgi dependent pathway [83], a mechanism for the transportation of ANXA5 to outside cells is interesting.

In the present study, abundant blebs appeared on the surface of gonadotropes shortly after GnRH administration. Blebs are suggested to be pinched off and to become ectosomes. Extracellular vesicles (EVs) are known as two different type vesicles. They are ectosome and exosome, those are different for their origin, size in diameter, protein marker and way of release [13]. It has been reported that after 2,000 xg centrifugation of conditioned medium, ectosome enriched fraction is isolated at 20,000 xg precipitate and exosome enrich fraction is further centrifuged at 100,000 xg following purification on linear sucrose gradient [17]. There are reports to show the association of ANXA5 with exosomes but not ectosomes [1, 57, 63].

In the present study, the precipitate of 20,000 xg was shown to consist of relatively large (200 nm) membrane particles containing ANXA5 and they were increased by GnRHa. So, the externalization of ANXA5 would occur at least partly by forming ectosomes.

The bleb formation by GnRHa stimulation has been reported by others and the authors claimed the retraction of blebs [53]. They proposed that blebbing is for cell migration and did not think that blebs are pinched off from cells. The existence of pinched-off membrane particles was confirmed in 20,000xg pellet in this study. The 20,000xg pellet fraction showed the specific characteristics of ectosome, namely large particle size more than about 200 nm in diameter.

While the diameter of 110,000 xg pellet was thought to be that of exosome as about or less than 100 nm. Although the origin of 110,000 xg pellet was not demonstrated in the present study, the 110,000 xg pellet fraction was assumed to be exosome fraction mainly by their size. GnRHa augmented ANXA5 of ectosome and ANXA5 in exosome fraction was increased later. As exosome was postulated to be produced from endosome [13], externalized ANXA5 by ectosome shortly after GnRHa stimulation would be internalized and used for producing exosome later. ANXA5 was shown in exosome fraction only at 180 min sample in this study.

Present study demonstrates that GnRHa enhances the bleb and ectosome formation as a specific cell function. It was interesting which signal driven by GnRHa is responsible for this process. So, various inhibitors were examined whether they would interfere GnRHa action on bleb formation and the externalization of ANXA5. Protein kinase A (PKA) inhibitor was demonstrated to suppress both GnRHa stimulation of bleb formation and the externalization of ANXA5. The major signal transduction of GnRH receptor is through $G\alpha_q/11$ [61]. However, $G\alpha_s$ has been reported to couple with GnRH receptor also [44]. GnRH activates $G\alpha_s$ -adenylate cyclase and PKA [44]. Activated PKA would be necessary to form blebs containing ANXA5 and to pinch off them.

It was reported that the process by which GnRH induces bleb formation is through extracellular signal regulated kinase (ERK1/2) and RhoA-ROCK [53]. It was not seen in the present study that MAPKK inhibitor PD98059 inhibited blebbing and externalization of ANXA5 in this study. It is not clear why PD98059 did not show any effects in this study. Although further study would be necessary for the signal transduction and bleb formation, cAMP-PKA would be necessary step for ANXA5 translocation and blebs induced by GnRH.

ANXA5 containing ectosome was demonstrated to stimulate LH secretion. Although, it was assumed that other proteins were included in the particles, ANXA5 was clearly proven the stimulatory effect on LH secretion and ANXA5 containing ectosome was increased by

GnRHa treatment. Membrane particles carrying specific protein has been postulated to be integrated to membranes of the target cells, thereby releasing of its content in a context of paracrine signal [38]. Integration of β -cells with microparticles containing its specific endothelial receptor and ANXA1 released by endothelial cells treated with an anticoagulant serine protease (aPC) promotes signal transduction of islet cytoprotective effect [38]. Although it is reasonable to postulate that ANXA5 containing ectosome would fuse and is integrated to exert positive effects on LH release, ANXA5 itself was shown to stimulate LH release. In the present study, delayed association of ANXA5 with exosome was found after GnRHa administration and this result suggests free molecule of ANXA5 would be internalized by cells.

Release of free ANXA5 from ectosomes in extracellular space would happen and it would be a functioning form for stimulation of LH secretion. ANXA1 has been demonstrated to function both by direct interaction with phospholipid plasma membrane and by its specific receptor, formyl peptide receptor (FPR), FPR2/ALX which is a G protein couple receptor [3, 46]. Inhibition of adrenocorticotrophic hormone (ACTH) exocytosis by ANXA1 has been reported to be triggered through this receptor [46]. Unlikely, a receptor for ANXA5 has not been reported and it has not yet been proven that the action of ANXA5 would mediate through its specific receptor. Annexins have proposed to be involved in membrane associated exocytosis [55]. So, it is very interesting whether a common mechanism for annexins to affect exocytosis exists.

GnRH stimulated the formation of ANXA5 containing ectosome and it facilitated LH secretion shown by experiments with L β T2 cells. ANXA5 containing ectosomes were also increased in blood plasma after one week of ovariectomy. In the ovariectomized rats, negative feedback of ovarian hormones disappears and GnRH secretion is increased. So, augmented GnRH secretion facilitated ectosome formation at pituitary gonadotropes. Free ANXA5 was also increased in the post-ultracentrifuged plasma of ovariectomized rats. It was suggested that

free ANXA5 would be released from ectosomes. These changes in extracellular ANXA5 would stimulate LH secretion *in vivo*.

The present study demonstrated that ANXA5 of gonadotropes is externalized primarily by ectosome formation under GnRH-cAMP signal. Ectosome containing ANXA5 derived from the gonadotrope was demonstrated to stimulate LH release and this ectosome formation physiologically takes place *in vivo*. Ectosome formation was demonstrated to play a role in an autocrine and paracrine fashion for a novel intercellular communication by ANXA5. The summary of the study was illustrated (Fig. 3-13).

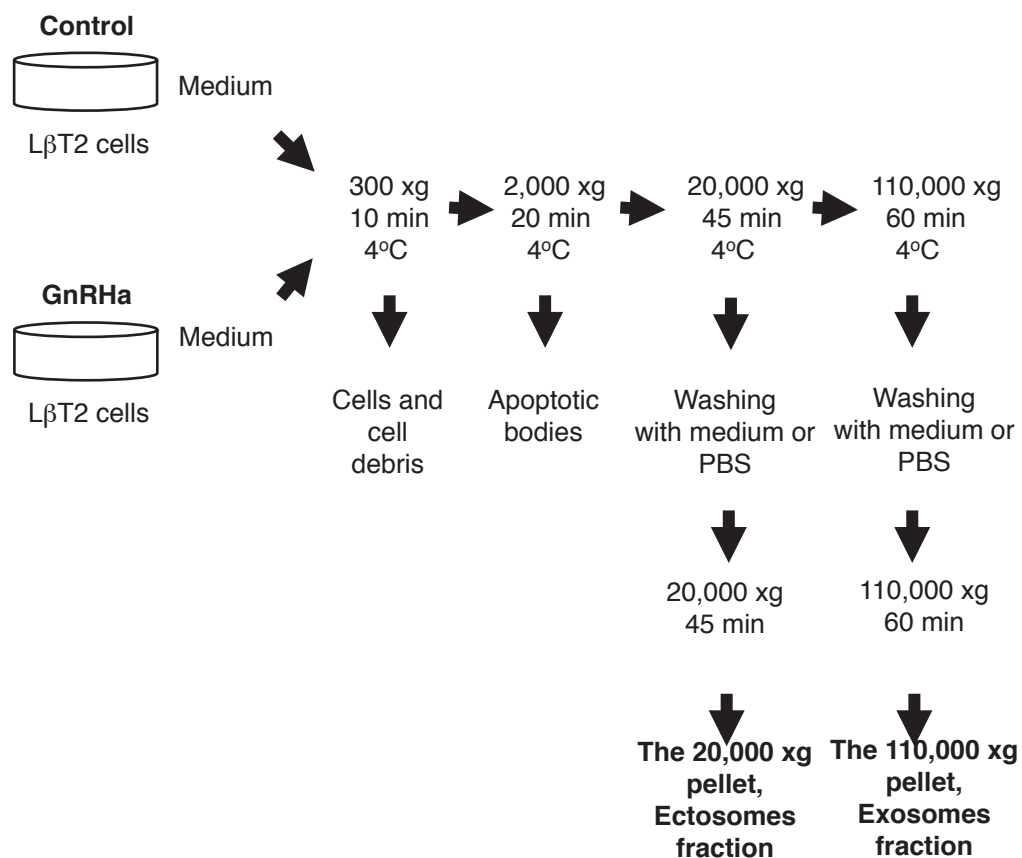


Fig. 3-1 Isolation of particulate fraction by sequential centrifugation

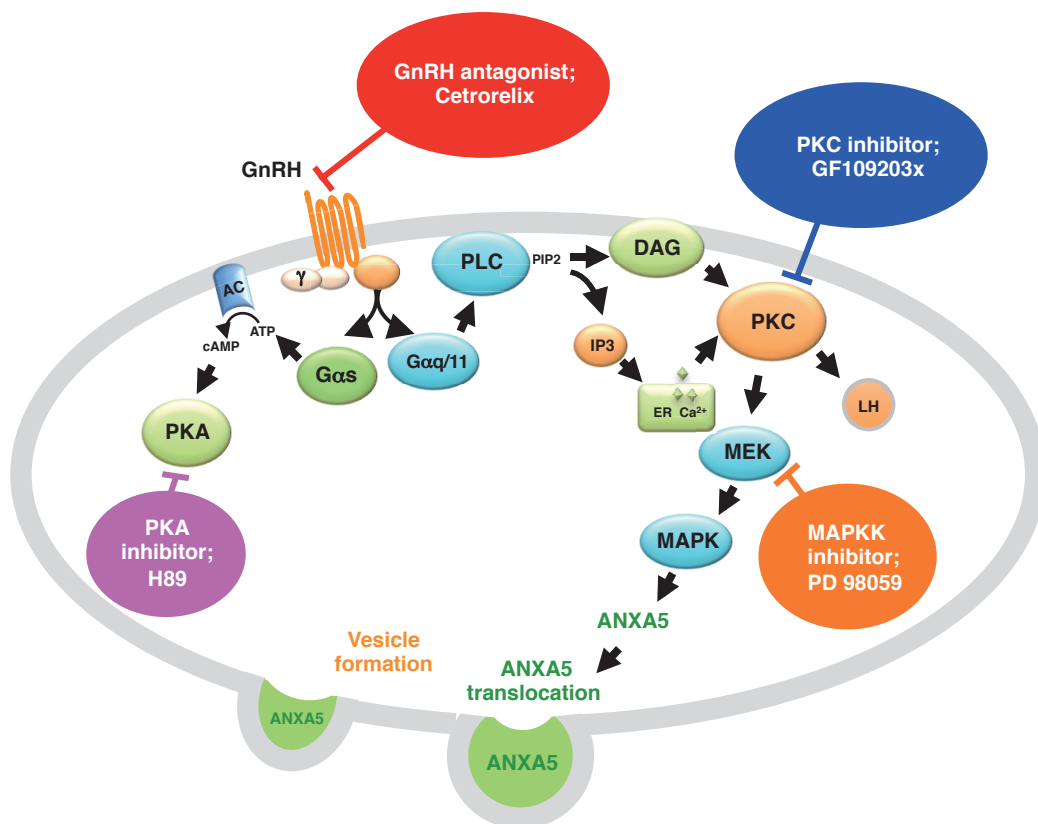
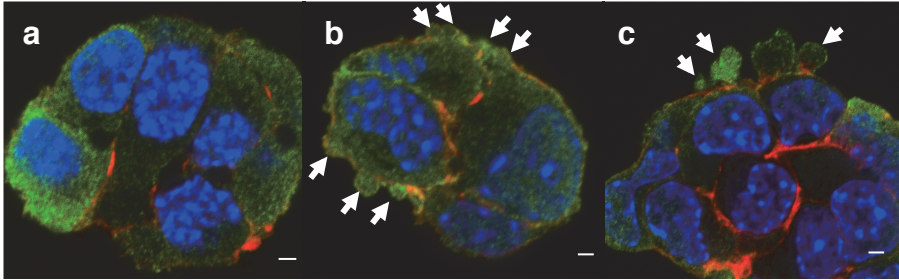


Fig. 3-2 Inhibitors for GnRH stimulated signal transduction

Pretreatment with GnRH antagonist (Cetorelix), protein kinase C inhibitor (GF 109203x), MAP kinase kinases (MAPKK) inhibitor (PD98059) or protein kinase A inhibitor (H89) were examined. AC; adenylyl cyclase, ATP; adenosine triphosphate, cAMP; cyclic adenosine monophosphate, PKA; protein kinase A, PLC; phospholipase C, PIP₂; phosphatidylinositol 4,5-bisphosphate, DAG; diacylglycerol, IP₃; inositol 1,4,5-triphosphate, ER; endoplasmic reticulum, PKC; protein kinase C, MEK; mitogen-activated protein kinase/extracellular signaling-regulated kinase (MAPK/ERK kinase), MAPK; mitogen activated protein kinase, LH; luteinizing hormone.

A



B

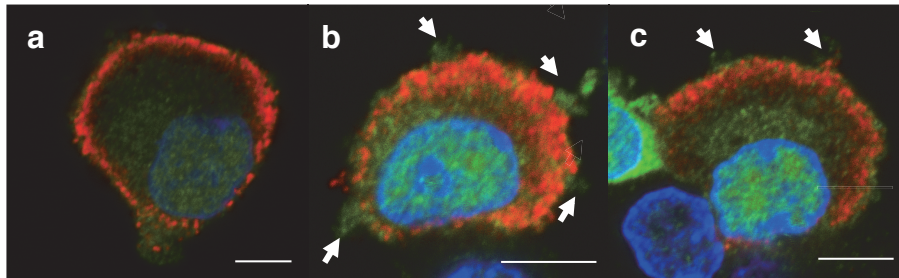


Fig. 3-3 Immunocytochemistry for ANXA5; (A) LβT2 and (B) primary culture of pituitary cell

Cells were incubated with or without 100 nM GnRHa. (a) control incubation, (b) and (c) incubation with GnRHa for 10 and 30 min respectively. Immunocytochemistry for ANXA5 of LβT2 cells were performed. Green, blue and red signals indicate ANXA5, nucleus and actin respectively. Pituitary cells were prepared for immunocytochemistry with anti-ANXA5 and -LHβ. Green, blue and red signals indicate ANXA5, nucleus and LHβ respectively. Scale bar represents 5 μm.

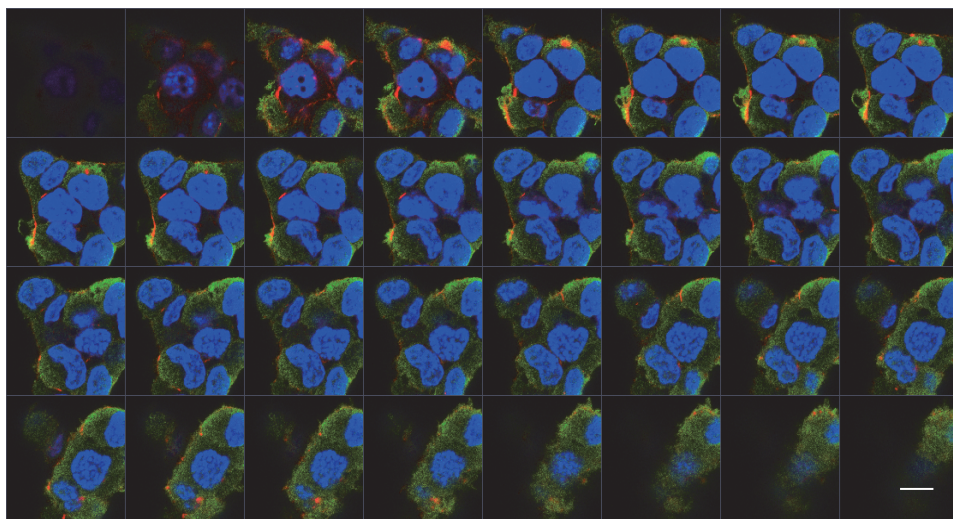
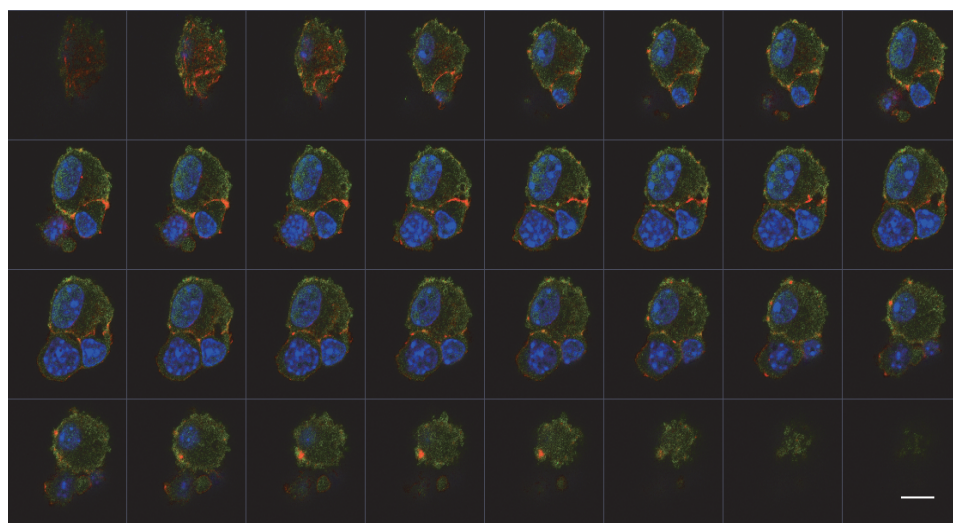
A**B**

Fig. 3-4 Optical sectioning LβT2 cells

Cells were incubated with (B) or without (A) 100 nM GnRHα for 10 min. Images were made with confocal laser microscope LSM710. Scale bar represents 10 μm .

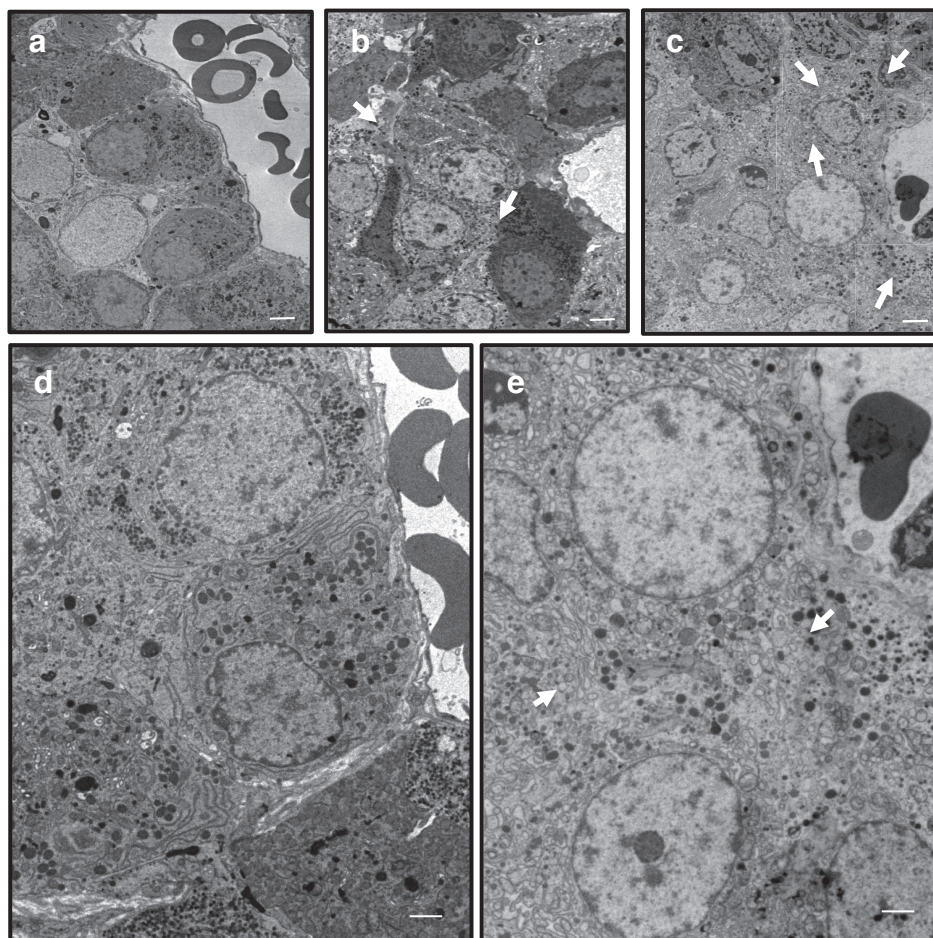


Fig. 3-5 Electron microscope observation of hemi-pituitary organ culture after GnRHa treatment

Hemi-pituitary glands were incubated with or without 100 nM GnRHa. (a) control incubation, (b) and (c) are 10 and 30 min incubation with GnRHa, (d) and (e) are higher magnification of control incubation and 30 min incubation with GnRHa respectively. Pituitary tissue was subjected to transmission electron microscope observation. Scale bar represents 2 μ m.

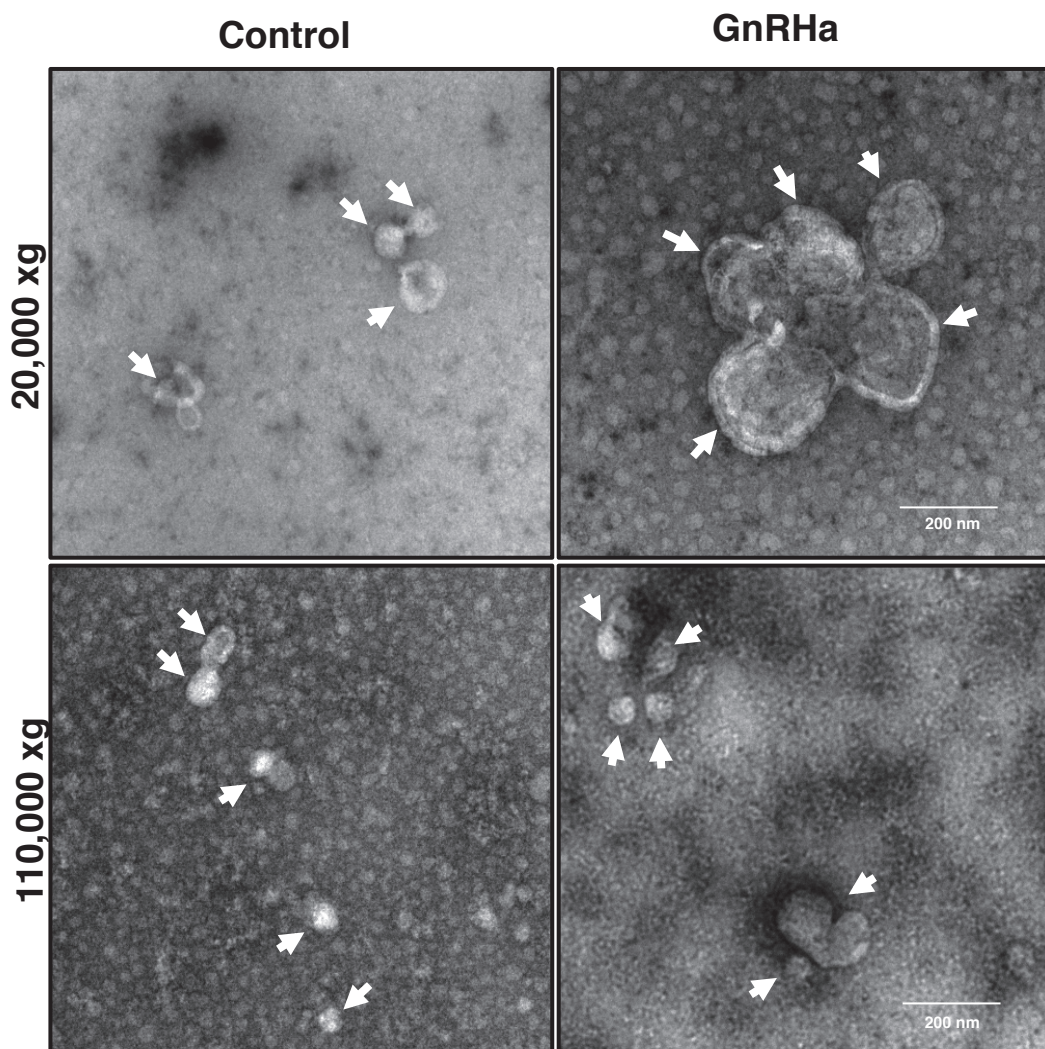


Fig. 3-6 Negative staining of 20,000 xg and 110,000 xg pellet fraction

LβT2 cells were treated with or without 100 nM GnRHa for 30 min. The pellet fractions, 20,000 xg and 110,000 xg were isolated from the medium by sequential centrifugation and then fixed with 4% paraformaldehyde at 4°C overnight. Fixed sample was performed for negative staining observation. Scale bar is 200 nm. The large particles with diameter more than about 200 nm are found in the 20,000 xg pellet fraction. The particles less than 100 nm are observed in the 110,000 xg pellet fraction. Increase of the particles is shown by GnRHa treatment. Arrows indicate the particles.

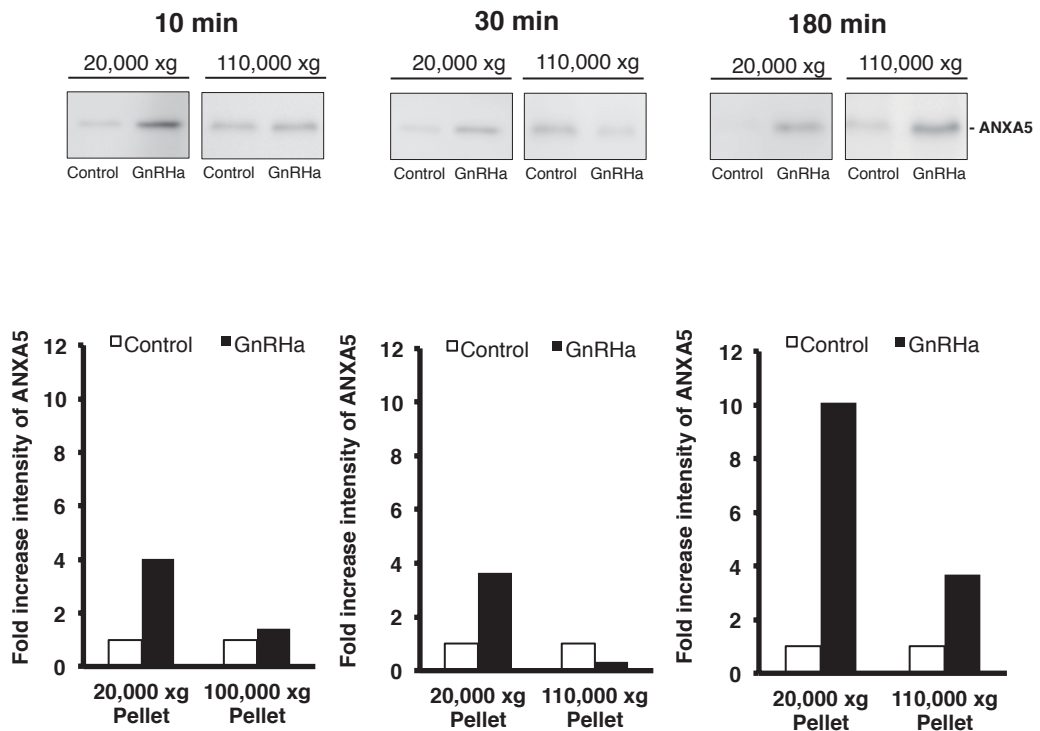


Fig. 3-7 Western blotting of extracellular vesicle protein for ANXA5

LβT2 cells were incubated with 0 or 100 nM GnRHa for 10, 30 and 180 min. Extracellular vesicle, the 20,000 xg and 110,000 xg precipitates were isolated from the medium by sequential centrifugation. The pellet fractions were re-suspended in sample buffer and subjected to SDS-PAGE and Western blotting for ANXA5. Each band was analyzed and shown as fold increase intensity of ANXA5.

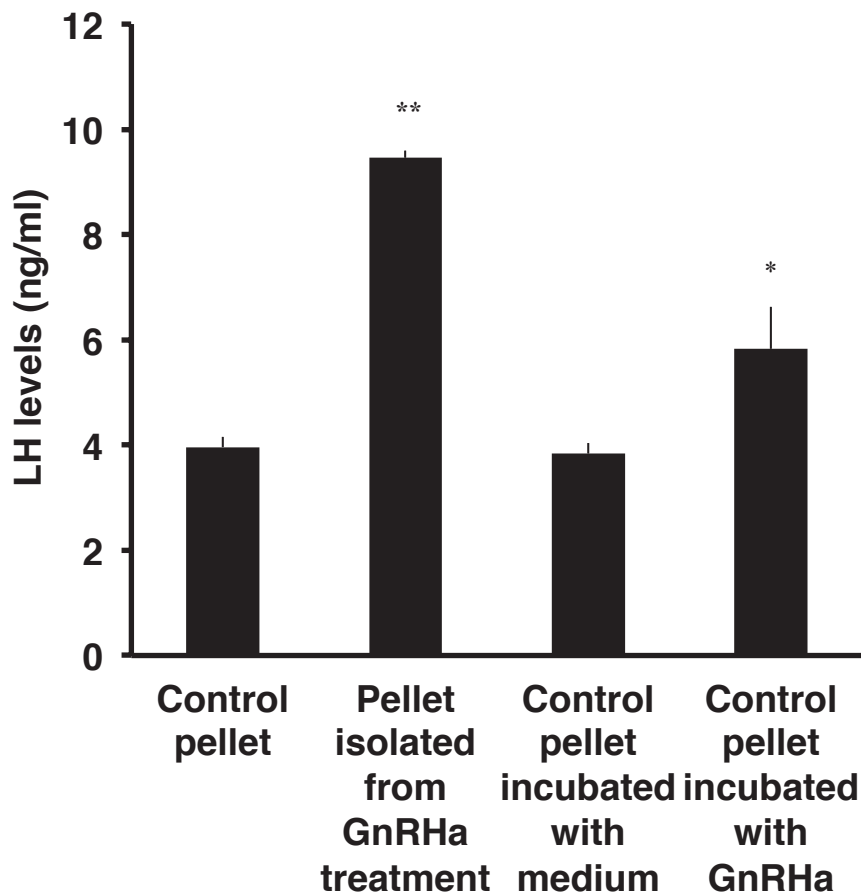
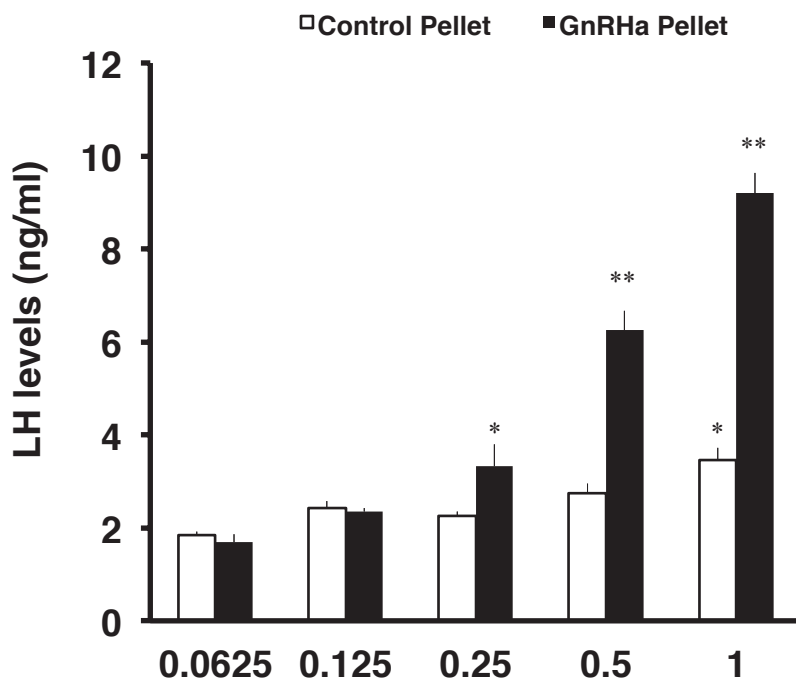


Fig. 3-8 The effect of ANXA5 containing extracellular vesicles on LH release

The effect of ANXA5 containing extracellular vesicles stimulated by GnRHa on LH release was examined. The 20,000 xg pellet was isolated from medium of L β T2 cell treated with 0 or 100 nM GnRHa for 3 hrs (Control pellet or Pellet isolated from GnRHa treatment). A part of control pellet was incubated with medium or 100 nM GnRHa for 3 hrs (Pellet incubated with medium or Pellet incubated with GnRHa). Statistical significance, * $p < 0.05$ when compared to control values, ** $p < 0.0001$ when compared to all other groups.



The 20,000 xg pellet ratio

Fig. 3-9 Augmentation of GnRHa stimulated ANXA5 containing extracellular vesicle on LH release in a dose dependent manner

The 20,000 xg pellet was isolated from medium of L β T2 cells treated with 0 or 100 nM GnRHa for 3 hrs (Control pellet or Pellet isolated from GnRHa treatment). Control pellet and pellet isolated from GnRHa treatment was diluted (0.0625, 0.125, 0.25, 0.5 and 1) and the effect on LH release was confirmed. Statistical significance, * $p < 0.05$ when compared to 0.0625 group, ** $p < 0.0001$ when compared to all other groups.

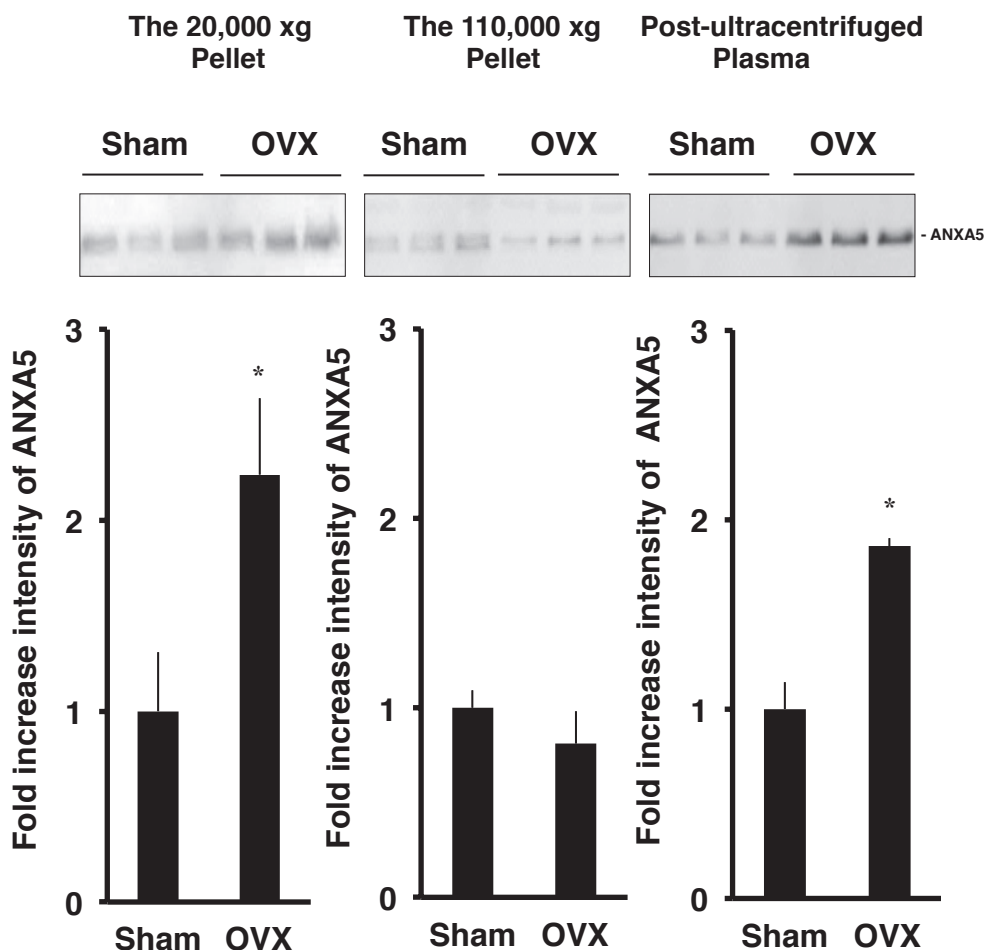


Fig. 3-10 Detection of ANXA5 in the extracellular vesicle and free ANXA5 in the post-ultracentrifuged supernatant after 1 week ovariectomized rat

Membrane particles were isolated from blood plasma obtained from sham or 1 week ovariectomized rats (n=3). ANXA5 contents in the 20,000 xg and 110,000 xg pellets were examined. Contents of ANXA5 were analyzed and shown as fold increase of ANXA5 intensity. Statistical significance, * p<0.05.

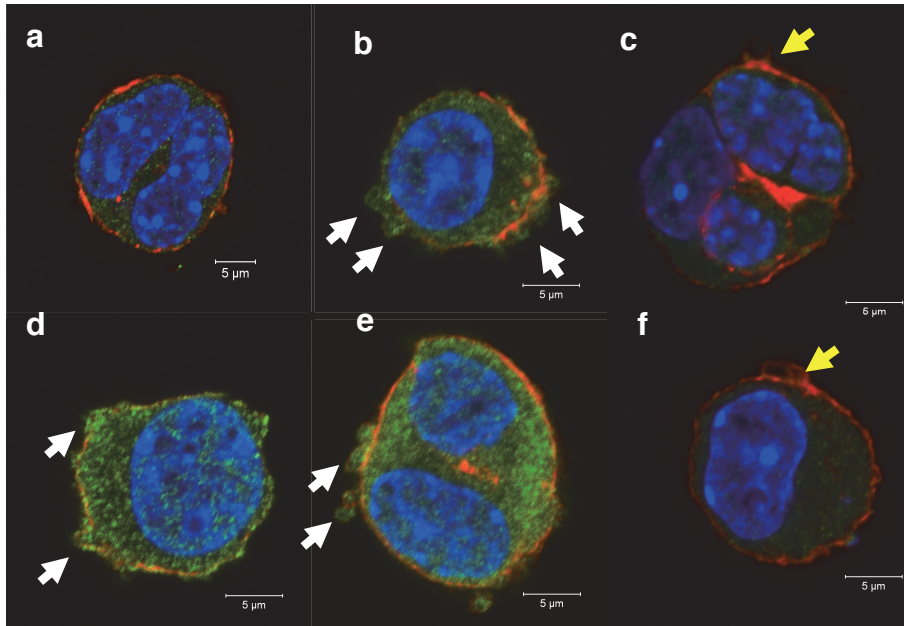


Fig. 3-11 The effect of inhibitors on bleb formation

Immunocytochemistry for ANXA5; (a) control, (b) GnRHa treatment, (c), (d), (e) and (f) are pretreatment of GnRH antagonist (Cetrorelix), protein kinase C inhibitor (GF 109203x), MAPKK inhibitor (PD98059) or protein kinase A inhibitor (H89) respectively in the presence of GnRHa for 30 min. Scale bar is 5 μ m. White arrows indicate blebs containing ANXA5. Yellow arrows indicate inhibitory effect of the formation with an absence of ANXA5 in the blebs.

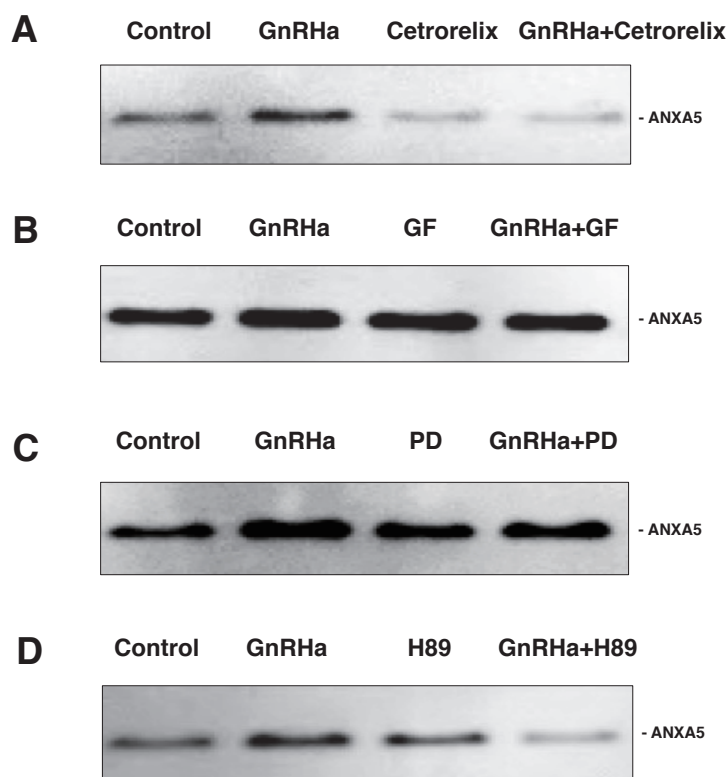


Fig. 3-12 Inhibition GnRHa stimulation of bleb containing ANXA5 in L β T2

Western blotting for ANXA5; Pretreatment with (A) GnRH antagonist (Cetorelix), (B) protein kinase C inhibitor (GF 109203x), (C) MAPKK inhibitor (PD98059) or (D) protein kinase A inhibitor (H89) respectively with or without GnRHa for 30 min was performed. The 20,000 xg pellet fraction was isolated from the medium. Detection of ANXA5 in the 20,000 xg pellet fraction was performed.

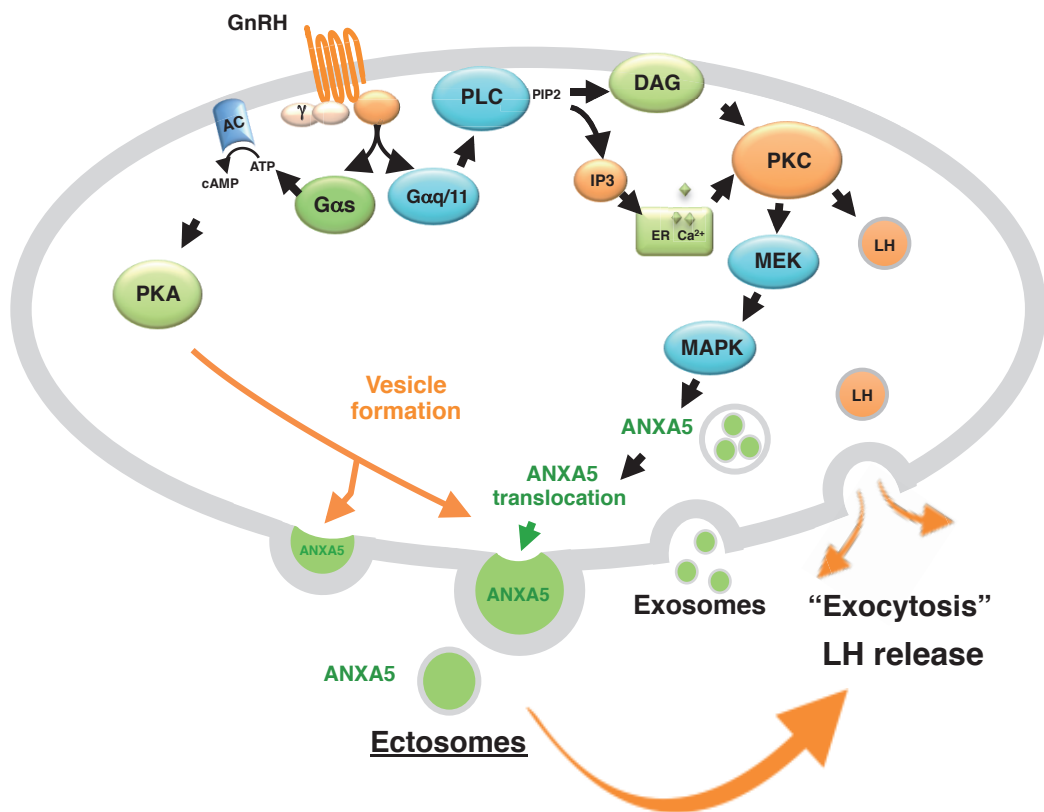


Fig. 3-13 GnRH-cAMP facilitates ANXA5 containing ectosome formation

GnRH activates Gαs through its specific GPCR, adenylyl cyclase and PKA. The activation of PKA initiates the formation of ectosome containing ANXA5. Ectosome carrying ANXA5 is demonstrated to function as paracrine and/or autocrine mediator of gonadotropes and to facilitates LH release.

Chapter 4 GnRH stimulation of annexin A1 (ANXA1) expression

Introduction

ANXA5 is shown to be involved in LH secretion in chapters 2 and 3. GnRH stimulates transportation of ANXA5 to outer space of cells by ectosome formation. This process is essential autocrine and/or paracrine of ANXA5 necessary for LH secretion. As ANXA5 belongs to annexin family proteins, it is interesting to know whether other annexins are also involved in GnRH function. So, chapter 4 is to clarify whether the expression of other annexins is regulated by GnRH.

GnRH is a central regulator of hypothalamo-hypophyseal-gonadal axis. However, GnRH mRNA and GnRH receptor have been also demonstrated in peripheral tissues such as placenta [11] and mammary gland [40] indicating biological function of GnRH other than hypothalamo-hypophyseal-gonadal axis. GnRH is also implicated in non-reproductive process such as immune system [28]. GnRHa has been demonstrated to alter about 200 genes by both up- and down- regulation in L β T2 cell and the different profile is shown on the distinct interval of treatment [29]. ANXA5 gene expression in L β T2 cell is regulated by GnRHa and it is upregulated continuously for 24 hrs [36]. ANXA5 is involved in various functions and some of those functions have been demonstrated to be promoted by GnRH [30, 85].

All annexins have been examined their expression under the control of GnRHa and extreme increase of ANXA1 mRNA expression has been shown [22]. ANXA1 was originally referred to macrocortin, renocortin, lipomodulin or lipocortin-1 [42]. ANXA1 displays an anti-inflammatory property with its inhibitory effect on phospholipase A₂ (PLA₂) activity [19]. It has been demonstrated that ANXA1 released from monocytes and leukocytes associates with extracellular anti-inflammatory action induced by glucocorticoid [14]. ANXA1 mediates glucocorticoid action in the anterior pituitary gland and hypothalamus by which the synthesis

and association with plasma membrane of ANXA1 are induced [7, 72]. ANXA1 presents abundantly in folliculostellate cells (FS cells) [77, 79]. It is suggested that ANXA1 is not expressed in the corticotropes [77]. It is proposed that ANXA1 associated inhibitory effect of glucocorticoid on ACTH secretion at corticotropes is dependent upon ANXA1 produced by FS cells since ANXA1 has found abundantly in FS cell line (TtT/GF cell) but not detected in corticotrope cell line (AtT20 clone D1). AtT20 cells co-cultured with TtT/GF cell have been shown to respond to glucocorticoid inhibition of ACTH secretion but it was not occurred when AtT20 was cultured alone [77]. Inhibitory effect of glucocorticoid on ACTH secretion by ANXA1 is involved in exocytosis prevention by actin polymerized enhancement through formyl peptide receptor and Rho kinase signaling [46].

This chapter demonstrated that intact gonadotropes showed a low expression of ANXA1 and the expression of ANXA1 is significantly stimulated by GnRH α . The ectosome associated extracellular transportation of ANXA1 induced by GnRH α was not obvious as ANXA5. ANXA1 was proposed to share the function of GnRH.

Materials and methods

1. Animals

Adult female Wistar Imamichi rats were bred in our laboratory and prepared as described in chapter 2. All procedures were done according to the guideline for animal treatment of Kitasato University and experimental plan was approved by the committee (#16-089, 16-090).

2. Primary culture of anterior pituitary cells and L β T2 cell culture

Primary culture of anterior pituitary cells and L β T2 cell culture were prepared as described in chapter 2.

3. Immunocytochemistry of L β T2 cell culture for ANXA1

a. Reagents, solutions and equipment

Poly-L-lysine (Sigma)

Cover slip (18x18 mm, Matsunami)

Culture dish (Cellstar; 35x10 mm, Greiner bio-one)

GnRH agonist (Fertirelin acetate, Takeda Pharmaceuticals)

0.1 M Phosphate buffered saline (PBS, pH 7.4)

4% Paraformaldehyde (PFA) in PBS

Acetone (-20°C)

Antibody binding buffer (ABB, pH 7.4)

Blocking buffer

- 3% normal goat serum in ABB

Primary antibody

- Anti-ANXA1 rabbit serum (Thermo Scientific)

Second antibody

- Alexa flour 488 goat anti-rabbit IgG (H+L) (Life Technologies)

Alexa flour 568 Phalloidin (Invitrogen)

Vectashield mounting medium with DAPI (Vector Laboratories, Inc.)

Confocal laser microscope (Zeiss 710)

b. Protocol

LβT2 cells (about 100,000 cells) were seeded onto poly-L-lysine coated cover slip. Cells were treated with or without 100 nM GnRHa for 10 and 30 min. Treated cells were fixed with 4% paraformaldehyde for immunohistochemistry using anti-ANXA1 in ABB (1:1,000). All procedures are described in chapter 3.

4. SDS-PAGE and Western blotting

a. Reagents, solutions and equipment

1.5 M Tris-HCl (pH 8.8)

0.5 M Tris-HCl

0.25 M Tris-HCl (pH 6.8)

30% w/v Acrylamide/ 0.8% w/v Bis solution

10% SDS

10% Ammonium per sulfate

TEMED (N,N,N',N'-tetra-methyl ethylenediamine, Bio-Rad)

Sample buffer

12% Separating gel

4% Stacking gel

Electrophoresis buffer (pH 8.3)

Blotting buffer

0.01% PBS-T

Blocking buffer

- 5% skim milk
- 0.01% PBS-T

Primary antibody

- Anti-ANXA1 rabbit serum (Thermo Scientific)
- Mouse β -actin monoclonal IgG (Santa Cruz Biotechnology)
- Anti-ANXA5 rabbit serum (MS-2, home made)

Second antibody

- Anti-rabbit IgG-conjugated with horse radish peroxidase (ICN Biomedicals)
- Anti-mouse IgG-conjugated with horse radish peroxidase (ICN Biomedicals)

Amersham ECL Prime Western Blotting Detection Reagent (GE Health care)

- Solution A Amersham ECL Prime Luminol enhancer solution
- Solution B Amersham ECL Prime Peroxide solution

Electrophoresis chamber (Bio-Rad)

Electrophoresis power supply (Bio-Rad)

Blotting chamber (Bio-Rad)

PVDF membrane (Amersham Hybond P 0.45 PVDF, Amersham GE Healthcare)

NanoDrop 2000 (Thermo Scientific)

Luminescent image analyzer (Image Quant LAS 4000 series)

b. Protocol

LβT2 cells treated with GnRHα or whole pituitary glands of 2 weeks ovariectomized (ovx) rats were prepared. SDS-PAGE and Western blotting was done as described in chapter 2. Immunodetection of ANXA1 was accomplished with anti-ANXA1 in blocking buffer (1:10,000) and anti-rabbit IgG-conjugated with horse radish peroxidase in blocking buffer (1:50,000). Membrane was stripped off the first antibody and re-probed with mouse β-actin monoclonal IgG in blocking buffer (1:1,000) overnight at 4°C. Anti-mouse IgG-conjugated with horse radish peroxidase in blocking buffer (1:20,000) was utilized for second antibody. Chemiluminescence was detected by ImageQuant LAS 4000. Each band was normalized with the intensity of β-actin and shown as fold increase intensity of ANXA5.

The 20,000 xg pellet was obtained from conditioned medium. SDS-PAGE and Western blotting of re-suspended pellet was performed with anti-ANXA1 in blocking buffer (1:10,000). Membrane was stripped off the first antibody and re-probed with anti-ANXA5 (MS-2) in blocking buffer (1:10,000) as described in chapter 2.

5. Double staining immunocytochemistry for ANXA1 and LHβ

a. Reagents, solutions and equipment

0.1 M Phosphate buffered saline (PBS, pH 7.4)

4% Paraformaldehyde (PFA) in PBS

Acetone (-20°C)

Antibody binding buffer (ABB, pH 7.4)

Blocking buffer

- 3% normal goat serum in ABB

Primary antibody

- Anti-LH β guinea pig serum (NIDDK-NIH)
- Anti-ANXA1 rabbit serum (Thermo Scientific)

Second antibody

- Alexa flour 568 goat anti-guinea pig IgG (H+L) (Life Technologies)
- Alexa flour 488 goat anti-rabbit IgG (H+L) (Life Technologies)

Vectashield mounting medium with DAPI (Vector Laboratories, Inc.)

Confocal laser microscope (Zeiss 710)

b. Protocol

Pituitary cells were seeded on poly-L-lysine coated micro cover slip. One day after seeding, cells were treated with or without 1 nM GnRHa for 48 hrs. The medium was removed. Cells were fixed with 4% paraformaldehyde for 15 min at room temperature. Fixed cells were subjected to double staining immunocytochemistry with anti-ANXA1 in ABB (1:1,000) and anti-LH β (1:10,000) as mentioned in chapter 3. Immunocytochemical distribution of ANXA1 was observed by confocal laser microscope (Zeiss 710).

6. Immunohistochemistry of pituitary after 2 weeks of ovariectomy for ANXA1

Paraffin block preparation

a. Reagents, solutions and equipment

0.1 M Phosphate buffered saline (PBS, pH 7.4)

4% Paraformaldehyde (PFA) in PBS

Paraffin (Merk Schuchardt OHG)

b. Protocol

Hemi-pituitary tissues were fixed with 4% paraformaldehyde at 4°C overnight. After washing tissues with PBS, dehydration was performed per standard procedure. Paraffin blocks were used for making tissue sections with 4 µm thickness and dried in an incubator at 37°C overnight.

Immunohistochemistry for ANXA1

a. Reagents, solutions and equipment

Antibody binding buffer (ABB, pH 7.4)

1% Hydrogen peroxide in methanol

Primary antibody

- Anti-ANXA1 rabbit serum (Thermo Scientific)

ImmPRESS HRP Reagent kit peroxidase anti-rabbit IgG (Vector Laboratories, Inc.)

- 2.5% normal horse serum
- Anti-rabbit IgG

Mayer's Hematoxylin stain (Muto Pure Chemical Co., Ltd., Tokyo, Japan)

DAB substrate (Roche Diagnostics, IN, USA)

- DAB metal concentrate
- Peroxide buffer

Distilled water

Microscope (Olympus BX50; Olympus Optical Co., Ltd., Tokyo, Japan)

b. Protocol

Deparaffinization was done by xylene and ethanol series. Endogenous peroxidase activity was eliminated by pretreatment of tissue sections with 1% hydrogen peroxide in methanol for 20 minutes. Tissue sections were then blocked with 2.5% normal horse serum for 1 hr at room temperature. Primary antibody incubation with rabbit anti-ANXA1 in ABB (1:1,000) was performed overnight at 4°C in a humidified chamber. Tissue sections were washed for 3 times in ABB. Peroxidase conjugated anti-rabbit IgG was applied and specimens were maintained for 2 hrs at room temperature. The sections were washed with ABB 3 times before staining with DAB substrate: Buffer (1:9). Stained sections were soaked in water flow for 5 min. Tissue sections were stained with Mayer's Hematoxylin for 5 min. After washing, the sections were dehydrated with ethanol series and mounting. ANXA1 immunoreactivity was observed with microscope (Olympus BX50).

7. Sample preparation

7.1L β T2

Immunocytochemistry

L β T2 cells (about 100,000 cells) were seeded onto poly-L-lysine coated cover slip. One day of seeding, the medium was changed to 100 nM GnRH α for 10 or 30 min. Control was also prepared. Cells were fixed and subjected to immunocytochemistry for ANXA1.

Protein electrophoresis and Western blotting

L β T2 cells (200,000 cells) were cultured in 35 mm dishes. Two days of culture, the medium was replaced to 100 nM GnRH α for 3 hrs. A part of intact cells was used for control (n=4). Cells were washed with PBS and then lysed with sample buffer. The lysed cells were

then boiled at 95°C for 5 min. Centrifugation of sample solution at 18,516 xg (13,000 rpm) 4°C for 5 min was done. Protein concentration was adjusted to 1 µg/µl. Twenty microliter of this was subjected to SDS-PAGE and Western blotting for ANXA1.

LβT2 cells were plated onto culture dishes (100x20 mm; 4 dishes) for 7 days. Cells were treated with 0 or 100 nM GnRHa for 30 min or 24 hrs. The 20,000 xg pellet was isolated from conditioned medium by sequential centrifugation according to the description in chapter 2. The pellet fraction was re-suspended in sample buffer and subjected to SDS-PAGE and Western blotting for ANXA1 and A5.

7.2 Primary culture of pituitary cells

Double staining for ANXA1 and LHβ

Primary culture of pituitary cells was established as already described. Pituitary cells (100,000 cells) were seeded onto poly-L-lysine coated cover slip. Two days after cell dissociation, cells were incubated with 1 nM GnRHa for 48 hrs. Control condition was obtained from intact primary culture of pituitary cells. Cells were fixed and processed for double staining with anti-ANXA1 (Thermo Scientific) and anti-LHβ (NIDDK-NIH).

7.3 Ovariectomy of rats

Adult female Wistar Imamichi rats were utilized for ovariectomy. The experiment was performed under anesthesia with isoflurane. After 2 weeks of the operation, anterior pituitary glands were harvested. Pituitary tissues were fixed and subjected to immunohistochemistry for ANXA1. A part of whole pituitary glands (n=4) prepared for protein electrophoresis and Western blotting was also performed.

8. Statistical analysis

Each value was presented as mean \pm SEM. Statistical analysis was performed using Student's *t* test for the comparison of two groups. P values less than 0.05 were considered to be significant.

Results

1. ANXA1 in the blebs after GnRHa treatment

L β T2 cells were treated with 100 nM GnRHa for 10 and 30 min. Immunocytochemistry for ANXA1 was performed. ANXA1, actin and nucleus were shown as green, red and blue respectively. At 10 and 30 min of GnRHa treatment, ANXA1 was represented in the blebs as indicated by arrows (Fig. 4-1).

2. Content of ANXA1 of the 20,000 xg particulate fraction

L β T2 cells were incubated with 0 or 100 nM GnRHa for 30 min and 24 hrs. The 20,000 xg precipitate was isolated from the medium by sequential centrifugation. The pellet was re-suspended in sample buffer and subjected to SDS-PAGE and Western blotting for ANXA1 and re-probed with anti-ANXA5. ANXA1 in the 20,000 xg was undetectable at 30 min of incubation (Fig. 4-2 A). Until 24 hrs, ANXA1 was slightly detected in 20,000 xg pellet of GnRHa treatment. ANXA5 was abundantly existed in 20,000 xg pellet and was augmented by GnRHa as in chapter 3 (Fig. 4-2 B).

3. GnRHa augments ANXA1 protein expression in L β T2 cells

GnRHa (100 nM) was administrated to the culture of L β T2 cells. ANXA1 protein expression of L β T2 cells was examined by SDS-PAGE and Western blotting. β -actin was used as internal control. ANXA1/ β -actin was analyzed and shown as mean \pm SEM ($p < 0.05$). Three hrs of GnRHa treatment of L β T2 cells showed the increase of ANXA1 protein expression (Fig. 4-3).

4. Increase of ANXA1 protein expression in gonadotrope by GnRHa

Primary culture of pituitary cells of rats was prepared. Pituitary cells were treated with 1 nM GnRHa for 48 hrs. Control was prepared from intact cells. Double staining of pituitary cells with anti-ANXA1 and -LH β was shown as green and red signal respectively. Nucleus was stained as indicated by blue signal. ANXA1 in the intact gonadotrope was very low. GnRHa increased the ANXA1 protein expression in the nucleus and cytoplasm of gonadotrope (Fig. 4-4).

5. Change in ANXA1 distribution in the pituitary tissue of ovariectomized rats

The distribution of ANXA1 was observed in pituitary tissues of 2 weeks ovariectomized rats. While the gonadotropes were not identified in the anterior pituitary tissues of sham operated rats, large cells or so called castration cells were obviously found in the pituitary tissues of ovariectomized rats. ANXA1 seemed to localize in the nucleus and at the periphery of large cell of pituitary tissue of ovariectomized rat (Fig. 4-5). Smaller and irregular shaped cells were intensely stained with ANXA1.

6. Increase of ANXA1 protein expression in pituitary gland of ovariectomized rats

ANXA1 protein expression in whole pituitary glands of 2 weeks ovariectomized rats was observed by SDS-PAGE and Western blotting (Fig. 4-6). ANXA1/ β -actin was analyzed and shown as mean \pm SEM ($p < 0.05$). ANXA1 expression of whole pituitary gland increased after ovariectomy.

Discussion

It was already reported that the expression of ANXA1 mRNA is stimulated by GnRH in gonadotropes [22]. GnRH α dramatically augmented the expression of ANXA1 in the pituitary gonadotrope cell line, L β T2, and it was shown in this study. Expression of other annexins was not affected by GnRH α [22]. Thus, of the 12 mammalian annexins, ANXA5 and A1 are the only GnRH-responsive annexins. ANXA5 plays a significant role in the GnRH functions of gonadotropin secretion [30] and apoptosis [85], the present data suggest an important, but as yet undefined role for ANXA1 in GnRH function.

The suppressive activity of ANXA1 on adrenocorticotrophic hormone (ACTH) release by the pituitary gland in response to cortisol has been well-studied [6, 46, 73]. Negative feedback of cortisol on ACTH release is mediated by ANXA1. ANXA1 was also shown to be involved in thyrotropin-releasing hormone (TSH) and prolactin secretion by the same research group [74-76]. Folliculostellate cells contain ANXA1, and paracrine regulation of ANXA1 by folliculostellate cells in anterior pituitary tissues has been observed [79]. In the present study, small and irregular shaped cells were stained with anti-ANXA1 in the pituitary tissues of ovariectomized rats. It is suggested that these cells would be folliculostellate cells and they would be affected by ovariectomy also.

ANXA1 was found to function primarily as a mediator of the anti-inflammatory action of cortisol [20]. Cortisol suppresses phospholipase A₂ by means of ANXA1 [12]. Despite these studies, no study has yet examined ANXA1 in gonadotrope cells. In an ANXA1 knockout mouse (ANXA1^{-/-}), aberrant inflammation and resistance to glucocorticoids were reported [26, 69]. The relationship between GnRH and ANXA1 that was indicated in the present study suggests that ANXA1^{-/-} mice would exhibit reproductive failure; however, to date there has been no report regarding gonadotropin secretion or any reproductive complications in ANXA1^{-/-} mice.

GnRH stimulates the synthesis of ANXA5, and ANXA5 is involved in gonadotropin secretion. Although it was previously found a significant stimulatory effect of ANXA5 on gonadotropin secretion, an intact estrous cycle and ovulation were found in ANXA5^{-/-} mice. Further studies are therefore necessary to clarify the existence of a compensatory mechanism for the depletion of ANXA1 and/or A5. Analysis of the phenotype of ANXA1 and A5 double-knockout mice would also be interesting in this respect.

The expression of ANXA1 and A5 in pituitary tissue was similar but not identical [22]. ANXA1 was present in the primary pituitary cells but the intensity was very low in the gonadotropes. It was dramatically increased after GnRHa stimulation. Immunoreactivity was seen in the nucleus and cytoplasm of gonadotropes *in vitro*. Furthermore, blebs formed by GnRH were also shown to contain ANXA1. However, ANXA1 content in the ectosome fraction of 30 min GnRHa stimulation of cultured LβT2 cells was very low when compared with ANXA5. Sustained GnRHa treatment for 24 hrs showed slight increase of ANXA1 in the ectosome fraction. ANXA5 and A1 would share the function by GnRH, but they would be distinctive.

GnRH is a phylogenetically very old peptide hormone. GnRH has been reported even for protochordates that have neither a hypothalamus nor a pituitary gland [43, 50, 62]. Two GnRH molecules have been reported in mammals, GnRH I and II [10]. GnRH I is synthesized in the medial preoptic area of the hypothalamus and is transported to the anterior pituitary gland through the hypophyseal portal system. GnRH I is also synthesized in peripheral tissues. GnRH I is expressed outside of the hypothalamus, for example in the ovary, testis, and mammary gland [33, 68, 86]. GnRH II is found mostly in the mid-brain in mammals. We previously found that GnRH augmented ANXA5 expression by treating with a GnRHa and have proposed that ANXA5 is a useful biomarker of GnRH action. The present results suggest that ANXA1 would also be a sensitive biomarker of GnRH action.

In the present study, we examined the effect of GnRH on the localization of ANXA1 in gonadotropes and found that GnRHa induced localization of ANXA1 to the cell periphery and blebs. It has been reported that ANXA1 is translocated to the outer surface of cells by cortisol in various cell types [5]. It is interesting to know the difference between the effect of cortisol and GnRH in the gonadotropes.

Expression of ANXA1 mRNA has shown to be increased 3 hrs after GnRH incubation [22]. This chapter confirmed that augmentation of ANXA1 protein expression of L β T2 cells by GnRH. The externalization of ANXA1 by bleb formation at gonadotropes was also shown. ANXA1 exhibits in microparticles has been reported and is implicated in autocrine and paracrine signal [38]. Synthetic nanoparticle applied from extracellular vesicle formation of ANXA1 is shown to promote its function on wound healing of intestinal mucosa [39]. These data reveal that extracellular vesicle formation of ANXA1 is implicated in its intracellular function.

This chapter clearly demonstrated that ANXA1 is expressed in gonadotrope cells and that its expression is under the control of GnRH. ANXA1 was also demonstrated to be a content of blebs formed by GnRH. It was hypothesized that bleb formation would be a mechanism of externalization of ANXA1 like as ANXA5. These data suggest that ANXA1 and A5 share GnRH-related functions in gonadotropes and probably also in peripheral tissues.

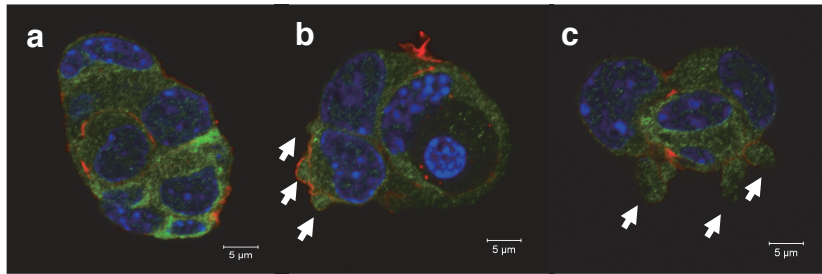
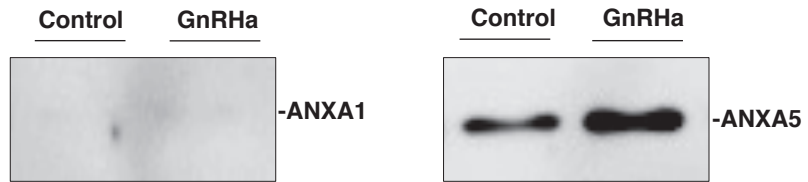


Fig. 4-1 Immunocytochemistry of ANXA1 in LβT2

LβT2 cells were treated with or without 100 nM GnRHa for 10 and 30 min; (a) control, (b) and (c) are 100 nM GnRHa treatment for 10 min and 30 min respectively. Green, red and blue signals represent ANXA1, actin and DNA. Scale bar is 5 μm.

A. 30 min



B. 24 hrs

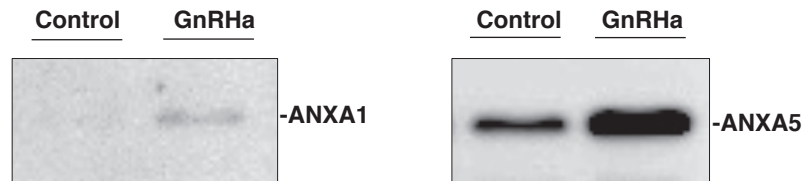


Fig. 4-2 Western blotting of ectosome fraction for ANXA1 and A5

LβT2 cells were incubated with 0 or 100 nM GnRHa for 30 min (A) or 24 hrs (B). Extracellular vesicle, the 20,000 xg precipitate, was isolated from the medium by sequential centrifugation. The pellet fractions were re-suspended in sample buffer and subjected to SDS-PAGE and Western blotting for ANXA1 and re-probed with ANXA5.

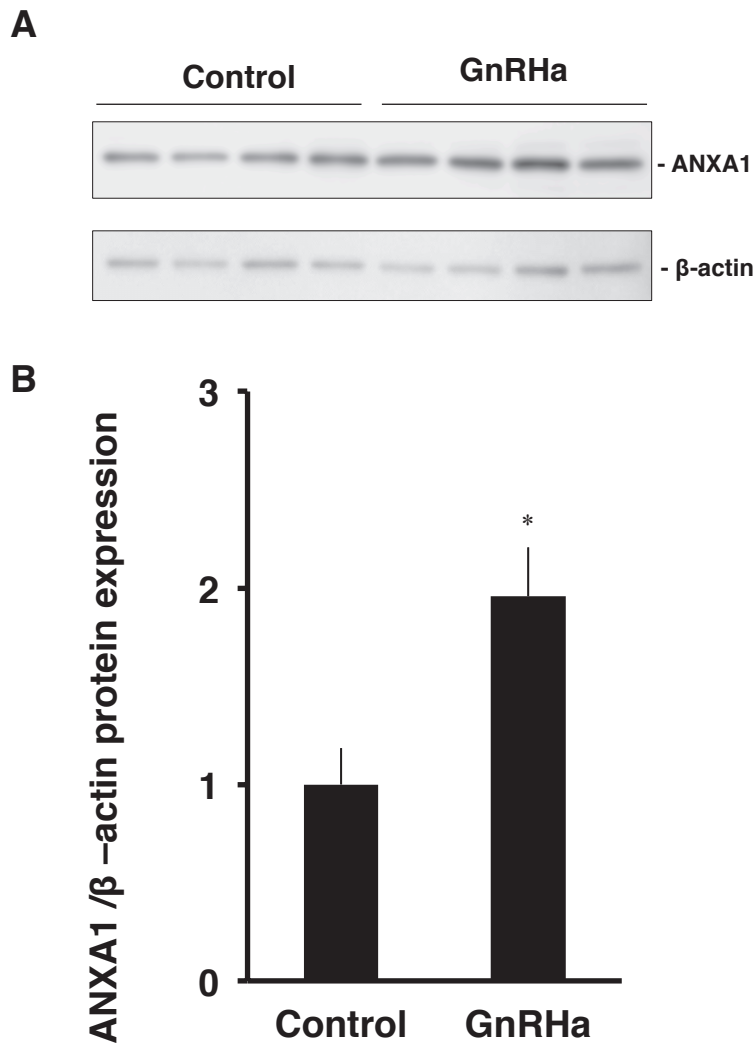


Fig. 4-3 ANXA1 in 3 hrs GnRHa treated LβT2 cells

LβT2 cells were treated with or without 100 nM GnRHa for 3 hrs (n=4). LβT2 cells were harvested and dissolved with sample buffer. Lysed cells were subjected to SDS-PAGE and Western blotting for ANXA1 (A). β-actin was utilized as internal control. ANXA1/β-actin was shown as mean ±SEM (B). Statistical significance, * p<0.05.

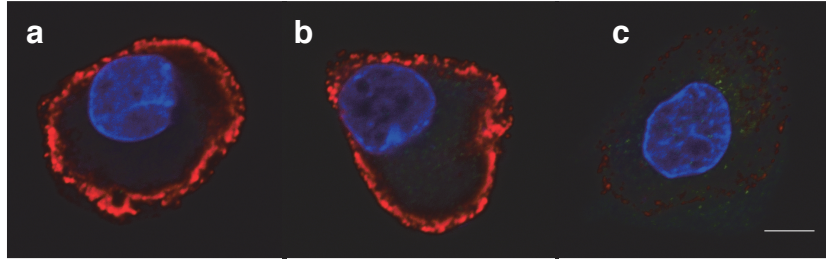


Fig. 4-4 Double staining for anti-ANXA1 and -LH β of primary culture of pituitary cells of rats

Primary culture of pituitary cells was treated with or without 1 nM GnRHa for 48 hrs; (a) negative control, (b) control and (c) GnRHa. Green, Red and blue signals represent ANXA1, LH β and DNA respectively. Scale bar is 5 μ m.

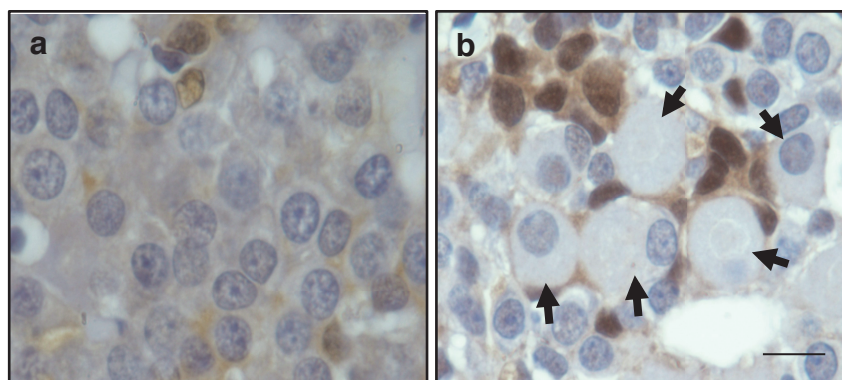


Fig. 4-5 Immunohistochemistry for ANXA1 in the pituitary tissues of 2 week ovariectomized rat

Pituitary tissues of sham operated (a) and 2 weeks ovariectomized (ovx) (b) were subjected to immunohistochemistry for ANXA1. Scale bar is 10 μ m. Large castration cells (arrows) are found in the pituitary of 2 weeks ovx rat.

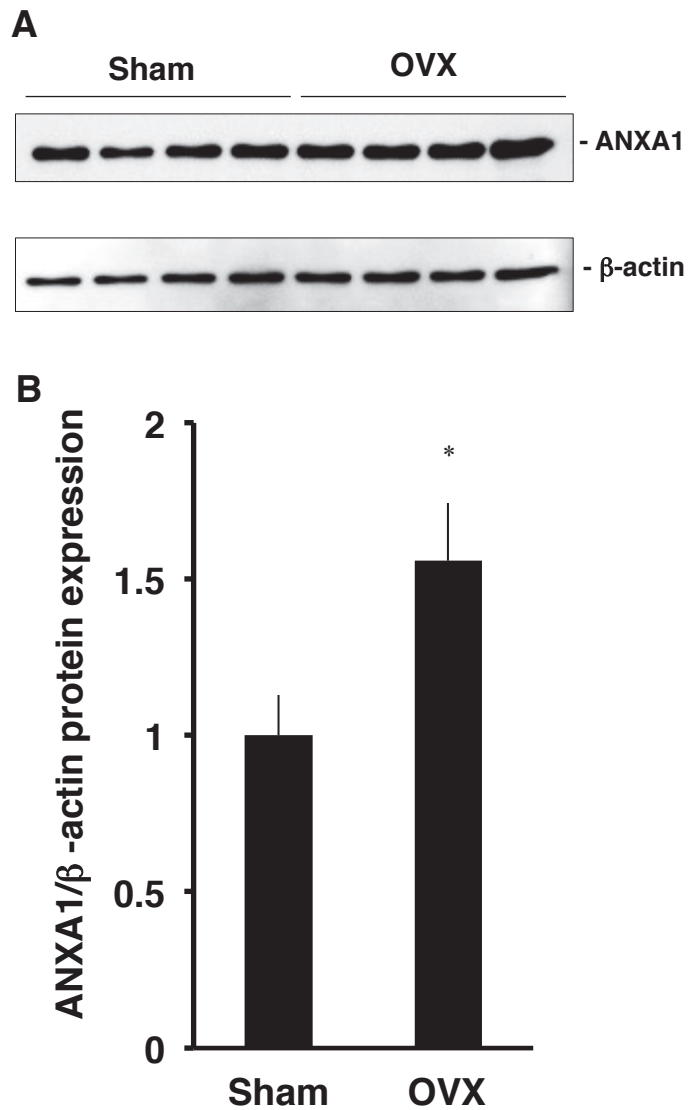


Fig. 4-6 ANXA1 protein expression in the pituitary tissues of 2 week ovariectomized rats

Whole pituitary gland of sham operated or 2 weeks ovariectomized rats (n=4) were homogenized and sonicated in sample buffer. Protein was subjected to SDS-PAGE and Western blotting (A). β -actin was utilized as internal control. The expression of ANXA1/ β -actin was shown as mean \pm SEM (B). Statistical significance, * p<0.05.

Chapter 5 Summary

Inter-cellular communication is prerequisite for a synchronized response and function performed by multicellular organisms. Vertebrates have developed various mechanisms for cell communication, e.g. endocrine, juxtacrine, autocrine, paracrine, gap junction and so on. Recently, a novel communication method via extracellular vesicles that is probably evolutionarily old is attracting attention. Annexin A5 (ANXA5) is a member of annexin family of proteins that is characterized by a calcium-dependent phospholipid binding. Gonadotropin releasing hormone (GnRH) stimulates the synthesis of ANXA5 in the pituitary gonadotrope. ANXA5 has been shown to be involved in GnRH stimulation of gonadotropin secretion. However, how ANXA5 augments LH release at gonadotrope is still obscure. Although ANXA5 does not contain a signal sequence in its gene sequence, ANXA5 was demonstrated both in and out of cells. In the present study, a mechanism for the augmentation of gonadotropin secretion by ANXA5, an effect of GnRH on ANXA5 localization in the gonadotropes and the relationship between another annexin member ANXA1 and GnRH were studied. Inter-cellular communication via extracellular vesicles formed by GnRH will be discussed.

Function of Annexin A5 in the pituitary gonadotropes: Involvement of ANXA5 in LH release was already shown and it is confirmed in this study. Recombinant rat ANXA5 augmented LH release in L β T2 gonadotrope cell culture. Recombinant ANXA5 augmented GnRH agonist (GnRHa) stimulation of LH release in the primary culture of anterior pituitary cells of rats. Knockdown of ANXA5 by siRNA in the primary culture of pituitary cells resulted in the blunting of GnRH action on LH release. Furthermore, increase of intracellular ANXA5 by expression vector of ANXA5 tended to increase the GnRH action on LH release. These data confirm that ANXA5 synthesized in the gonadotrope is in favor of LH release. It has been demonstrated that proliferation suppressive action of GnRH on hormone dependent cancer in

many studies. Suppression of L β T2 growth by GnRHa was confirmed also in the present study. GnRHa administration suppressed the growth of L β T2 by 96 hrs of incubation. DNA ladder was observed after incubation with GnRHa suggesting an induction of apoptosis by GnRH. The suppressive effect of GnRHa on L β T2 growth was in a dose response manner, but the effect of GnRHa on LH release was biphasic. Lower concentration of GnRHa stimulated LH release in a dose dependent manner, while higher dosage and longer period rather inhibited LH secretion. This diversity suggests different intracellular signals responsible for these two cellular responses to GnRH. To see the effect of GnRH on the distribution of ANXA5, immunohistochemistry for ANXA5 of cultured pituitary tissue was performed. Depolarizing stimulation with high potassium treatment induced obvious plasma membrane-association of ANXA5 in hemi-pituitary organ culture. GnRHa showed similar effect on ANXA5 translocation to the periphery of the cell but lesser extent. ANXA5 was detected in EDTA-washout of L β T2 cells after GnRHa and high potassium treatment, suggesting augmentation of externalization of ANXA5 to outer space of cells by GnRHa. It was demonstrated that the stimulating effect of LH release and anti-proliferative effect on cell growth by GnRH were suggested to associate with externalization of ANXA5.

GnRH stimulation of ANXA5-containing extracellular vesicle (EV) formation of gonadotropes: Translocation of ANXA5 in the gonadotropes after GnRH stimulation was examined more precisely. Immunocytochemistry of L β T2 cells for ANXA5 was performed after GnRHa administration. GnRHa induced blebs containing ANXA5 even after only 10 and 30 min incubation of L β T2 cells. Double staining of primary pituitary cells with anti-ANXA5 and -LH β showed blebs containing ANXA5 in the gonadotropes also after 10 and 30 min. Hemi-pituitary gland was cultured with GnRHa and subjected to the observation with transmission electron-microscope (TEM). The boundary of GnRHa stimulated gonadotrope-like cell became obscure with many bubble like particles after 30 min incubation. The conditioned medium of

cultured L β T2 was sequentially centrifuged at 20,000 xg and 110,000 xg to obtain membrane particle fractions, namely ectosome and exosome respectively. Negative staining of extracellular vesicles (EVs) showed the increase of large particles with a diameter more than about 200 nm in 20,000xg pellet. The particle size less than 100 nm was found in the 110,000 xg fraction. These 20,000 xg and 110,000xg particles were increased by the GnRHa treatment. ANXA5 was detected dominantly in 20,000 xg pellet after treatment with GnRHa for 10, 30 and 180 min. It increased until 180 min. ANXA5 in 110,000 xg pellet was also shown at 180 min. GnRHa treated 20,000 xg particulate fraction significantly stimulated LH release in a dose dependent manner. Membrane fraction prepared from plasma of one-week ovariectomized rats, in which GnRH secretion was expected to be augmented, showed significant increase of ANXA5 in the 20,000 xg pellet. Furthermore, augmentation of free ANXA5 was detected from post-ultracentrifuged plasma. It was suggested that free ANXA5 would be released from those membrane fractions. GnRH stimulates the formation of ANXA5 containing ectosome and it facilitates LH secretion. GnRH antagonist, Cetrorelix, was confirmed to inhibit EV formation by GnRH. Protein kinase C inhibitor, GF 109203x, MAPKK inhibitor, PD98059 and protein kinase A inhibitor, H89 were applied to GnRHa stimulation of bleb formation in L β T2 cells. Immunocytochemistry for ANXA5 demonstrated that the ANXA5 containing bleb formation by GnRH stimulation was inhibited by H89, but not by GF109203 and PD 98059. Western-blotting showed the decrease of ANXA5 in the 20,000 xg pellet obtained from the conditioned medium of GnRHa treated cells after pretreatment with H89. It is suggested that G α s signaling is necessary for GnRH stimulation of ANXA5 containing ectosome. The present study demonstrates that ANXA5 of gonadotropes is externalized primarily by ectosome formation under GnRH-cAMP signal. ANXA5 containing ectosome of gonadotrope was demonstrated to stimulate LH release. This ectosome formation is a physiological process and suggest a novel intercellular communication by ANXA5.

GnRH stimulation of annexin A1 (ANXA1) expression: As it has been reported that ANXA1 mRNA expression is augmented also by GnRH in L β T2 cells, changes in ANXA1 protein and its distribution in the gonadotropes were examined. Western-blotting showed that ANXA1 protein expression in L β T2 was increased by GnRHa stimulation for 3 hrs. Blebs formed by GnRH stimulation was demonstrated also containing ANXA1. Double-staining immunocytochemistry observation of primary culture of pituitary cells with anti-ANXA1 and -LH β showed the expression of ANXA1 in intact gonadotrope was very low. Furthermore, extracellular ANXA1 was very low even after GnRHa stimulation. After GnRHa treatment for 48 h, ANXA1 in the nucleus and cytoplasm of gonadotrope increased. Immunohistochemistry for ANXA1 in pituitary of 2-weeks ovariectomized rats demonstrated that ANXA1 seemed to be expressed in the nucleus and at periphery of large castrate cells. Western-blotting of whole pituitary gland of 2 weeks ovariectomized rats revealed that annexin A1 protein expression was increased. These data suggest that ANXA1 gene was a novel target of GnRH and ANXA1 is not transported to extracellular space as ANXA5. So, it is suggested that ANXA1 and A5 have distinct physiological role under the effect of GnRH in gonadotropes.

Conclusions: Present study clearly demonstrates that ANXA5 augments LH secretion and reveals that ANXA5 of gonadotropes is externalized primarily by means of ectosome formation under GnRH-cAMP signal and the release of exosome is followed. Ectosome containing ANXA5 was demonstrated to be bioactive for LH release and this ectosome formation is a physiological process. ANXA1 was also demonstrated to be a novel target of GnRH stimulation. This study first demonstrate the existence of hormonal regulation of ectosome formation. Transfer bioactive molecule under the control of hormone is suggested to be a novel style of inter-cellular communication (Fig. 5).

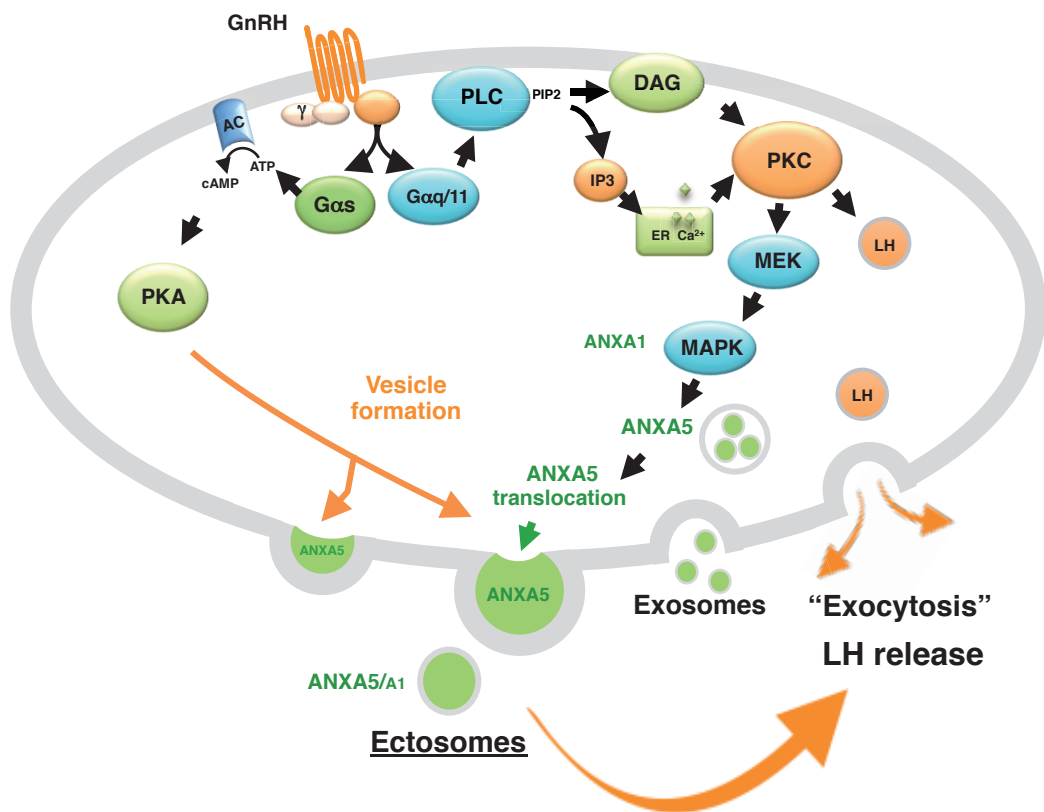


Fig. 5 Summary of the study

GnRH stimulates the exocytosis of LH at gonadotropes by activating $G\alpha_q/11$ and PKC. MAPK was shown to augment the expression of ANXA5 in gonadotropes. ANXA5 stimulates LH secretion. GnRH also activates $G\alpha_s$ and PKA and this is prerequisite for blebbing and forming ectosomes containing ANXA5. The expression of ANXA1 is stimulated by GnRH also but the externalization of ANXA1 is not significant. Ectosomes containing ANXA5 and free ANXA5 facilitate LH release.

Acknowledgements

My background work is concerning small animal clinical medicine. The study on molecular physiology reorganizes my perspective about the modern clinical applications in veterinary medicine. I gained valuable experience whilst studying and living in Japan. As that day fulfilled with inexperience, many people gave me the chance, patience, assist, guidance, motivation and encouragement.

I would like to express my special thanks of gratitude to my supervisor, Prof. Dr. Mitsumori Kawaminami who gave me the golden opportunity to do this research which opens my perspective about molecular research of physiological endocrinology. He manifests the characteristic of the genius with his widespread attitude. I always recognize him as a role model for the great scientist and the teacher. A very special thankfulness for his granting advice and teaching about scientific methodologies as well as suitable social association. My dissertation is created completely with his guidance and kind support.

I would like to express a very special gratitude to Assoc. Prof. Dr. Shiro Kurusu and Assist. Prof. Dr. Ryota Terashima for their great advice, kindness and assist.

I would like to express a very special gratitude to Assist. Prof. Dr. Makoto Sugiyama for technical support and guidance about electron microscopic study and his kindness.

A special thanks to Ms. Miyoko Nakata for her support, kindness, generosity and advice. She is always willing to assist me about the study and livelihood in Japan.

With a special mention to my classmate, Ms. Tungmahasuk Doungrat, and student of laboratory of veterinary physiology, Ms. Ando Midori and all members of laboratory for friendship, and the great support. It was very great sharing laboratory with all of you during last four years.

A special thanks to all staff at Kitasato University for their advice, kindness, helpfulness and support during a Ph.D. study.

With a special mention to Kitasato University, Towada city is a peaceful place and has a beautiful scenery and the great culture.

I would like to express my special thanks of gratitude to prior dean of faculty of Veterinary Medicine, Khon Kaen University, Assoc. Prof. Dr. Suneerat Aiumlamai, for motivation and giving me a great opportunity to communicate with Prof. Dr. Mitsumori Kawaminami.

I would like to express my special thanks of gratitude to current dean Assoc. Prof. Dr Chuchat Kamollerd and my colleagues of faculty of Veterinary Medicine, Khon Kaen University for their kindness and guidance.

I would like to express my sincere gratitude to Khon Kaen University and faculty of Veterinary Medicine for providing me with a scholarship support throughout my Ph.D. study.

With a special mention to Ms. Ancharin Ounthaisong and Ms. Saowanee Namthachan for helping about annual documentary preparation for the KKU scholarship and kindness.

I would like to express my sincere gratitude to Dr. Titaree Laoharatchatathanin for introducing a research work, advice and kindness.

I am also very thankful to the experimental animals for their sacrifices for undertake this research task. The study would not have been possible without animals. I wish this research would be applied for clinical diagnosis and therapy for both human and animals.

Finally, I would like to express a deep sense of gratitude to my family and my friends, especially to my mother, who has always held me, my father, my sister and my friends for their excellent encouragement, constant love and blessings. Above all, truthful thanks to my pets for their honesty and constant love, it is wonderful encouragement and motivation.

References

1. Aoki, N., Jin-no, S., Nakagawa, Y., Asai, N., Arakawa, E., Tamura, N., Tamura, T., and Matsuda, T., 2007, Identification and characterization of microvesicles secreted by 3T3-L1 adipocytes: redox- and hormone-dependent induction of milk fat globule-epidermal growth factor 8-associated microvesicles. *Endocrinology*, **148**:3850-3862.
2. Bartolomucci, A., Possenti, R., Mahata, S.K., Fischer-Colbrie, R., Loh, Y.P., and Salton, S.R., 2011, The extended granin family: structure, function, and biomedical implications. *Endocr Rev*, **32**:755-797.
3. Bena, S., Brancalone, V., Wang, J.M., Perretti, M., and Flower, R.J., 2012, Annexin A1 interaction with the FPR2/ALX receptor identification of distinct domains and downstream associated signaling. *The Journal of Biological Chemistry*, **287**:24690-24697.
4. Billig, H., Furuta, I., and Hsued, A.J., 1994, Gonadotropin-releasing hormone directly induces apoptotic cell death in the rat ovary: biochemical and in situ detection of deoxyribonucleic acid fragmentation in granulosa cells. *Endocrinology*, **134**:245-252.
5. Buckingham, J.C., 1996, Fifteenth Gaddum Memorial Lecture December 1994. Stress and the neuroendocrine-immune axis: the pivotal role of glucocorticoids and lipocortin 1. *Br J Pharmacol*, **118**:1-19.
6. Buckingham, J.C. and Flower, R.J., 1997, Lipocortin 1: a second messenger of glucocorticoid action in the hypothalamo-pituitary-adrenocortical axis. *Mol Med Today*, **3**:296-302.
7. Buckingham, J.C., John, C.D., Solito, E., Tierney, T., Flower, R.J., Christian, H., and Morris, J., 2006, Annexin 1, glucocorticoids, and the neuroendocrine-immune interface. *Ann N Y Acad Sci*, **1088**:396-409.

8. Caohuy, H., Srivastava, M., and Pollard, H.B., 1996, Membrane fusion protein synexin (annexin VII) as a Ca^{2+} /GTP sensor in exocytotic secretion. *Proc Natl Acad Sci USA*, **93**:10797-10802.
9. Chen, A., Kaganovsky, E., Rahimipour, S., Ben-Aroya, N., Okon, E., and Koch, Y., 2002, Two forms of Gonadotropin-releasing Hormone (GnRH) are expressed in human breast tissue and overexpressed in breast cancer: a putative mechanism for the antiproliferative effect of GnRH by down-regulation of acidic ribosomal phosphoproteins P1 and P2. *Cancer Research*, **62**:1036-1044.
10. Chen, C.C. and Fernald, R.D., 2008, Review paper GnRH and GnRH receptors: distribution, function and evolution. *Journal of Fish Biology*, **73**:1099-1120.
11. Cheng, K.W., Nathwani, P.S., and Leung, P.C., 2000, Regulation of human gonadotropin-releasing hormone receptor gene expression in placental cells. *Endocrinology*, **141**:2340-2349.
12. Cirino, G., Peers, S.H., Flower, R.J., Browning, J.L., and Pepinsky, R.B., 1989, Human recombinant lipocortin 1 has acute local anti-inflammatory properties in the rat paw edema test. *Proc Natl Acad Sci USA*, **86**:3428-3432.
13. Cocucci, E. and Meldolesi, J., 2015, Ectosomes and exosomes: shedding the confusion between extracellular vesicles. *Trends in Cell Biology*, **25**:364-372.
14. Comera, C. and Russo-Marie, F., 1995, Glucocorticoid-induced annexin 1 secretion by monocytes and peritoneal leukocytes. *Br J Pharmacol*, **115**:1043-1047.
15. Crawford, J.L., Currie, R.J., and McNeilly, A.S., 2000, Replenishment of LH stores of gonadotrophs in relation to gene expression, synthesis and secretion of LH after the preovulatory phase of the sheep oestrous cycle. *J Endocrinol*, **167**:453-463.
16. Crawford, J.L., McNeilly, J.R., Nicol, L., and McNeilly, A.S., 2002, Promotion of intragranular co-aggregation with LH by enhancement of secretogranin II storage

- resulted in increased intracellular granule storage in gonadotrophs of GnRH-deprived male mice. *Reproduction*, **124**:267-277.
17. Dujardin, S., Begard, S., Caillierez, R., Lachaud, C., Delattre, L., Carrier, S., Loyens, A., Galas, M.-C., Bousset, L., Melki, R., Auregan, G., Hantrate, P., Brouillet, E., Buee, L., and Colin, M., 2014, Ectosomes: a new mechanism for non-exosomal secretion of tau protein. *PLOS ONE*, **9**:1-10.
 18. Eerden, P.V., Wu, X.-X., Chazotte, C., and Rand, J.H., 2006, Annexin A5 levels in midtrimester amniotic fluid: association with intrauterine growth restriction. *American Journal of Obstetrics and Gynecology*, **194**:1371-1376.
 19. Errasfa, M. and Russo-Marie, F., 1989, A purified lipocortin shares the anti-inflammatory effect of glucocorticosteroids in vivo in mice. *Br J Pharmacol*, **97**:1051-1058.
 20. Flower, R.J., 1986, The mediators of steroid action. *Nature*, **320**:20.
 21. Funakoshi, T., Hendrickson, L.E., McMullen, B.A., and Fujikawa, K., 1987, Primary structure of human placental anticoagulant protein. *Biochemistry*, **26**:8087-8092.
 22. Fungbun, N., Tungmahasuk, D., Terashima, R., Kurusu, S., and Kawaminami, M., 2018, Annexin A1 is a novel target gene of gonadotropin-releasing hormone in L β T2 gonadotrope cells. *J Vet Med Sci*, **80**:116-124.
 23. Gnanapragasam, V.J., Darby, S., Khan, M.M., Lock, W.G., Robson, C.N., and Leung, H.Y., 2005, Evidence that prostate gonadotropin-releasing hormone receptors mediate and anti-tumourigenic response to analogue therapy in hormone refractory prostate. *Journal of Pathology*, **206**:205-213.
 24. Grundker, C., Andreas, G.R., Robert, M.P., and Gunter, E., 2002, Expression of gonadotropin-releasing hormone II (GnRH-II) receptors in human endometrial and

- ovarian cancer cells and effects of GnRH-II on tumor cell proliferation. *The Journal of Clinical Endocrinology and Metabolism*, **87**:1427-1430.
25. Gunthert, A.R., Carsten, G., Agnes, O., Julia, L., Nicola, E., and Gunter, E., 2005, Analogs of GnRH-I and GnRH-II inhibit epidermal growth factor-induced signal transduction and resensitize resistant human breast cancer cells to 40H-tamoxifen. *European Journal of Endocrinology*, **153**:613-625.
 26. Hannon, R., Croxtall, J.D., Getting, S.J., Roviezzo, F., Yona, S., Paul-Clark, M.J., Gavins, F.N., Perretti, M., Morris, J.F., Buckingham, J.C., and Flower, R.J., 2003, Aberrant inflammation and resistance to glucocorticoids in annexin 1-/- mouse. *FASEB J*, **17**:253-255.
 27. Imai, A., Takagi, H., Horibe, S., and Fuseya, T., 1996, Coupling of gonadotropin-releasing hormone receptor to Gi protein in human reproductive tract tumors. *Journal of Clinical Endocrinology and Metabolism*, **81**:3249-3253.
 28. Jacobson, J.D., Ansari, M.A., Mansfield, M.E., McArthur, C.P., and Clement, L.T., 1999, Gonadotropin-releasing hormone increases CD4 T-lymphocyte numbers in an animal model of immunodeficiency. *J Allergy Clin Immunol*, **104**:653-658.
 29. Kakar, S.S., Winters, S.J., Zacharias, W., Miller, D.M., and Flynn, S., 2003, Identification of distinct gene expression profiles associated with treatment of LβT2 cells with gonadotropin-releasing hormone agonist using microarray analysis. *Gene*, **308**:67-77.
 30. Kawaminami, M., Etoh, S., Miyaoka, H., Sakai, M., Nishida, M., Kurusu, S., and Hashimoto, I., 2002, Annexin 5 messenger ribonucleic acid expression in pituitary gonadotropes is induced by gonadotropin-releasing hormone (GnRH) and modulates GnRH stimulation of gonadotropin release. *Neuroendocrinology*, **75**:2-11.

31. Kawaminami, M., Kawamoto, T., Tanabe, T., Yamaguchi, K., Mutoh, K., Kurusu, S., and Hashimoto, I., 1998, Immunocytochemical localization of annexin 5, a calcium-dependent phospholipid-binding protein, in rat endocrine organs. *Cell Tissue Res*, **292**:85-89.
32. Kawaminami, M., Okazaki, K., Uchida, S., Marumoto, N., Takehara, K., Kurusu, S., Hashimoto, I., and Walker, A.M., 1996, Intrapituitary distribution and effects of annexin 5 on prolactin release. *Endocrine*, **5**:9-14.
33. Kawaminami, M., Shibata, Y., Yaji, A., Kurusu, S., and Hashimoto, I., 2003, Prolactin inhibits annexin 5 expression and apoptosis in the corpus luteum of pseudopregnant rats: involvement of local gonadotropin-releasing hormone. *Endocrinology*, **144**:3625-3631.
34. Kawaminami, M., Tsuchiyama, Y., Saito, S., Katayama, M., Kurusu, S., and Hashimoto, I., 2002, Gonadotropin-releasing hormone stimulates annexin 5 messenger ribonucleic acid expression in the anterior pituitary cells. *Biochemical and Biophysical Research Communications*, **291**:915-920.
35. Kawaminami, M., Uchida, S., Marumoto, N., Naito, K., Okazaki, K., Sato, Y., Kurusu, S., Hashimoto, I., Mutoh, K., and Walker, A.M., 1994, Simulation of secretion by membrane depolarization increases extracellular plasma membrane association of annexin 5 in the anterior pituitary gland. *Endocrine Journal*, **2**:357-362.
36. Kawaminami, M., Uematsu, N., Funahashi, K., Kokubun, R., and Kurusu, S., 2008, Gonadotropin releasing hormone (GnRH) enhances annexin A5 expression through mitogen activated protein kinase (MAPK) in LβT2 pituitary gonadotrope cells. *Endocrine Journal*, **55**:1005-1014.
37. Kawaminami, M., Yamaguchi, K., Miyagawa, S., Numazawa, S., Ioka, H., Kurusu, S., and Hashimoto, I., 1998, Ovariectomy enhances the expression and nuclear

- translocation of annexin 5 in rat anterior pituitary gonadotrophs. *Molecular and Cellular Endocrinology*, **141**:73-78.
38. Kreutter, G., Kassem, M., Habhab, A.E., Baltzinger, P., Abbas, M., Julie, B.-H., Amoura, L., Peluso, J., Yver, B., Fatiha, Z., Ubeaud-Sequier, G., Kessler, L., and Toti, F., 2017, Endothelial microparticles released by activated protein C protect beta cells through EPCR/PAR1 and annexin A1/FPR2 pathway in islet. *J. Cell. Mol. Med.*, **21**:2759-2772.
 39. Leoni, G., Neumann, P.A., Kamaly, N., Quiros, M., Nishio, H., Jones, H.R., Sumagin, R., Hilgarth, R.S., Alam, A., Fredman, G., Argyris, I., Rijcken, E., Kusters, D., Reutelingsperger, C., Perretti, M., Parkos, C.A., Farokhzad, O.C., Neish, A.S., and Nusrat, A., 2015, Annexin A1-containing extracellular vesicles and polymeric nanoparticles promote epithelial wound repair. *J Clin Invest*, **125**:1215-1227.
 40. Levi, L.N., Ben-Aroya, N., Tel-Or, S., Palmon, A., Burstein, Y., and Koch, Y., 1996, Expression of the gene for the receptor of gonadotropin-releasing hormone in the rat mammary gland. *FEBS Lett*, **379**:186-190.
 41. Liemann, S. and Lewit-Bentley, A., 1995, Annexins: a novel family of calcium- and membrane-binding proteins in search of a function. *Structure*, **3**.
 42. Lim, L.H. and Pervaiz, S., 2007, Annexin 1: the new face of an old molecule. *FASEB J*, **21**:968-975.
 43. Lin, X.W., Otto, C.J., and Peter, R.E., 1998, Evolution of neuroendocrine peptide systems: gonadotropin-releasing hormone and somatostatin. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol*, **119**:375-388.
 44. Liu, F., Usui, I., Evans, L.G., Austin, D.A., L., M.P., Olefsky, J.M., and Webster, J.G., 2002, Involvement of both Gq/11 and Gs proteins in gonadotropin-releasing hormone-mediated signaling in L β T2 cells. *J Biol Chem.*, **277**:32099-32108.

45. Macrae, M.B., Davidson, J.S., Millar, R.P., and van der Merwe, P.A., 1990, Cyclic AMP stimulates luteinizing-hormone (lutropin) exocytosis in permeabilized sheep anterior-pituitary cells. Synergism with protein kinase C and calcium. *Biochem J*, **271**:635-639.
46. McArthur, S., Yazid, S., Christian, H., Sirha, R., Flower, R., Buckingham, J., and Solito, E., 2009, Annexin A1 regulates hormone exocytosis through a mechanism involving actin reorganization. *The FASEB Journal*, **23**:4000-4010.
47. Mellon, P.L., Windle, J.J., and Weiner, R.I., 1991. Immortalization of neuroendocrine cells by targeted oncogenesis. *Recent Prog Horm Res*, **47**: 69-96.
48. Millar, R.P., 2005, GnRHs and GnRH receptors. *Anim Reprod Sci*, **88**:5-28.
49. Miyamoto, K., Hasegawa, Y., Nomura, M., Igarashi, M., Kangawa, K., and Matsuo, H., 1984, Identification of the second gonadotropin-releasing hormone in chicken hypothalamus: evidence that gonadotropin secretion is probably controlled by two distinct gonadotropin-releasing hormones in avian species. *Proc Natl Acad Sci USA*, **81**:3874-3878.
50. Morgan, R.O. and Fernandez, M.P., 1997, Annexin gene structures and molecular evolutionary genetics. *Cell Mol Life Sci*, **53**:508-515.
51. Moss, S.E. and Morgan, R.O., 2004, The annexins. *Genome Biol*, **5**:219.
52. Motomura, S., 1998, Induction of apoptosis in ovarian carcinoma cell line by gonadotropin-releasing hormone agonist. *Kurume Medical Journal*, **45**:27-32.
53. Navi, L.R.-B., Tsukerman, A., Feldman, A., Melamed, P., Tomic, M., Stojikovic, S.S., Boehm, U., Seger, R., and Naor, Z., 2017, GnRH induces ERK-dependent bleb formation in gonadotrope cells, involving recruitment of members of a GnRH receptor-associated signalosome to the blebs. *Frontiers in Endocrinology*, **8**:1-16.

54. Nickel, W., 2010, Pathways of unconventional protein secretion. *Curr Opin Biotechnol*, **21**:621-626.
55. Oling, F., Bergsma-Schutter, W., and Brisson, A., 2001, Trimers, dimers of trimers, and trimers of trimers are common building block of annexin A5 two-dimensional crystals. *Journal of Structural Biology*, **133**:55-63.
56. Palazzolo, G., Albanese, N.N., G, D.I.C., Gygax, D., Vittorelli, M.L., and Pucci-Minafra, I., 2012, Proteomic analysis of exosome-like vesicles derived from breast cancer cells. *Anticancer Res*, **32**:847-860.
57. Park, J.E., Tan, H.S., Datta, A., Lai, R.C., Zhang, H., Meng, W., Lim, S.K., and Sze, S.K., 2010, Hypoxic tumor cell modulates its microenvironment to enhance angiogenic and metastatic potential by secretion of proteins and exosomes. *Mol Cell Proteomics*, **9**:1085-1099.
58. Park, N. and Chun, Y.J., 2014, Auranofin promotes mitochondrial apoptosis by inducing annexin A5 expression and translocation in human prostate cancer cells. *J Toxicol Environ Health A*, **77**:1467-1476.
59. Pawson, A.J. and McNeilly, A.S., 2005, The pituitary effects of GnRH. *Anim Reprod Sci*, **88**:75-94.
60. Pepinsky, R.B., Tizard, R., Mattaliano, R.J., Sinclair, L.K., Miller, G.T., Browning, J.L., Chow, E.P., Burne, C., Huang, K.-S., Pratt, D., Wachter, L., Hession, C., Frey, A.Z., and Wallner, B.P., 1988, Five distinct calcium and phospholipid binding proteins share homology with lipocortin I. *The Journal of Biological Chemistry*, **263**:10799-10811.
61. Perrett, R.M. and McArdle, C.A., 2013, Molecular mechanisms of gonadotropin-releasing hormone signaling: integrating cyclic nucleotides into the network. *Front Endocrinol (Lausanne)*, **4**:180.

62. Powell, J.F., Reska-Skinner, S.M., Prakash, M.O., Fischer, W.H., Park, M., Rivier, J.E., Craig, A.G., Mackie, G.O., and Sherwood, N.M., 1996, Two new forms of gonadotropin-releasing hormone in a protochordate and the evolutionary implications. *Proc Natl Acad Sci USA*, **93**:10461-10464.
63. Principe, S., Jones, E.E., Kim, Y., Sinha, A., Nyalwidhe, J.O., Brooks, J., Semmes, O.J., Troyer, D.A., Lance, R.S., Kislinger, T., and Drake, R.R., 2013, In-depth proteomic analyses of exosomes isolated from expressed prostatic secretions in urine. *Proteomics*, **13**:1667-1671.
64. Raj, D.A., Fiume, I., Capasso, G., and Pocsfalvi, G., 2012, A multiplex quantitative proteomics strategy for protein biomarker studies in urinary exosomes. *Kidney Int*, **81**:1263-1272.
65. Raposo, G. and Stoorvogel, W., 2013, Extracellular vesicles: exosomes, microvesicles, and friends. *J Cell Biol*, **200**:373-383.
66. Ravassa, S., Gonzalez, A., Lopez, B., Beaumont, J., Querejeta, R., Larman, M., and Diez, J., 2007, Upregulation of myocardial annexin A5 hypertensive heart disease: association with systolic dysfunction. *European Heart Journal*, **28**:2785-2791.
67. Rescher, U. and Gerke, V., 2004, Annexins-unique membrane binding proteins with diverse functions. *J Cell Sci*, **117**:2631-2639.
68. Rieanrakwong, D., Laoharatchathanin, T., Terashima, R., Yonezawa, T., Kurusu, S., Hasegawa, Y., and Kawaminami, M., 2016, Prolactin Suppression of Gonadotropin-Releasing Hormone Initiation of Mammary Gland Involution in Female Rats. *Endocrinology*, **157**:2750-2758.
69. Roviezzo, F., Getting, S.J., Paul-Clark, M.J., Yona, S., Gavins, F.N., Perretti, M., Hannon, R., Croxtall, J.D., Buckingham, J.C., and Flower, R.J., 2002, The annexin-1

- knockout mouse: what it tells us about the inflammatory response. *J Physiol Pharmacol*, **53**:541-553.
70. Shibata, S., Hirokazu, S., Hirotaka, O., Akihiro, K., Osamu, T., and Toshinobu, T., 1997, Involvement of annexin v in antiproliferative effects of gonadotropin-releasing hormone agonists on human endometrial cancer cell line. *Gynecologic oncology*, **66**:217-221.
 71. Takehara, K., Uchida, S., Marumoto, N., Asawa, T., Osugi, S., Kurusu, S., Hashimoto, I., and Kawaminami, M., 1994, Secretion of recombinant rat annexin 5 by insect cells in a baculovirus expression system. *Biochemical and Biophysical Research Communications*, **200**:1421-1427.
 72. Taylor, A.D., Christian, H.C., Morris, J.F., Flower, R.J., and Buckingham, J.C., 1997, An antisense oligodeoxynucleotide to lipocortin 1 reverses the inhibitory actions of dexamethasone on the release of adrenocorticotropin from rat pituitary tissue in vitro. *Endocrinology*, **138**:2909-2918.
 73. Taylor, A.D., Cowell, A.M., Flower, J., and Buckingham, J.C., 1993, Lipocortin 1 mediates an early inhibitory action of glucocorticoids on the secretion of ACTH by the rat anterior pituitary gland in vitro. *Neuroendocrinology*, **58**:430-439.
 74. Taylor, A.D., Cowell, A.M., Flower, R.J., and Buckingham, J.C., 1995, Dexamethasone suppresses the release of prolactin from the rat anterior pituitary gland by lipocortin 1 dependent and independent mechanisms. *Neuroendocrinology*, **62**:530-542.
 75. Taylor, A.D., Flower, R.J., and Buckingham, J.C., 1995, Dexamethasone inhibits the release of TSH from the rat anterior pituitary gland in vitro by mechanisms dependent on de novo protein synthesis and lipocortin 1. *J Endocrinol*, **147**:533-544.
 76. Taylor, A.D., Philip, J.G., John, C.D., Cover, P.O., Morris, J.F., Flower, R.J., and Buckingham, J.C., 2000, Annexin 1 (lipocortin 1) mediates the glucocorticoid

- inhibition of cyclic adenosine 3',5'-monophosphate-stimulated prolactin secretion. *Endocrinology*, **141**:2209-2219.
77. Tierney, T., Christian, H.C., Morris, J.F., Solito, E., and Buckingham, J.C., 2003, Evidence from studies on co-cultures of TtT/GF and AtT20 cells that Annexin 1 acts as a paracrine or juxtacrine mediator of the early inhibitory effects of glucocorticoids on ACTH release. *J Neuroendocrinol*, **15**:1134-1143.
 78. Tiscia, G., Colaizzo, D., Chinni, E., Pisanelli, D., Sciannone, N., Favuzzi, G., Margaglione, M., and Grandone, E., 2009, Haplotype M2 in the annexin A5 (ANXA5) gene and the occurrence of obstetric complications. *Thrombosis and Haemostasis*, **102**:309-313.
 79. Traverso, V., Christian, H.C., Morris, J.F., and Buckingham, J.C., 1999, Lipocortin 1 (annexin 1): a candidate paracrine agent localized in pituitary folliculo-stellate cells. *Endocrinology*, **140**:4311-4319.
 80. Ueki, H., Mizushima, T., Laoharatchathanin, T., Terashima, R., Nishimura, Y., Rieanrakwong, D., Yonezawa, T., Kurusu, S., Hasegawa, Y., Brachvogel, B., Poschl, E., and Kawaminami, M., 2012, Loss of maternal annexin A5 increases the likelihood of placental platelet thrombosis and foetal loss. *Scientific Reports*, **2**:1-5.
 81. Valcz, G., Galamb, O., Krenacs, T., Spisak, S., Kalmar, A., Patai, A.V., Wichmann, B., Dede, K., Tulassay, Z., and Molnar, B., 2016, Exosomes in colorectal carcinoma formation: ALIX under the magnifying glass. *Mod Pathol*, **29**:928-938.
 82. Wang, X., Campos, B., Kaetzel, M.A., and Dedman, J.R., 1999, Annexin V is critical in the maintenance of murine placental integrity. *American Journal of Obstetrics and Gynecology*, **180**:1008-1016.
 83. Wang, X., Campos, B., Kaetzel, M.A., and Dedman, J.R., 2001, Secretion of annexin V from cultured cells requires a signal peptide. *Placenta*, **22**:837-845.

84. Weng, H., Fenghua, L., Shuiwang, H., Li, L., and Yifeng, W., 2014, GnRH agonist induce endometrial epithelial cell apoptosis via GRP 78 down regulation. *Journal of Translational Medicine*, **12**:1-10.
85. Yamamoto, H., Sato, H., Shibata, S., Murata, M., Fukuda, J., and Tanaka, T., 2001, Involvement of annexin V in the antiproliferative effect of GnRH agonist on cultured human uterine leiomyoma cells. *Molecular Human Reproduction*, **7**:169-173.
86. Yao, B. and Kawaminami, M., 2008, Stimulation of annexin A5 expression by gonadotropin releasing hormone (GnRH) in the Leydig cells of rats. *J Reprod Dev*, **54**:259-264.
87. Yonezawa, T., Aiko, W., Kurusu, S., and Kawaminami, M., 2015, Gonadotropin-releasing hormone is prerequisite for the constitutive expression of pituitary annexin A5. *Endocrine Journal*, **62**:1127-1132.