学位論文要旨

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

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

北里大学大学院獣医学系研究科

獣医学専攻博士課程

ナンファ・ファンブン

Numfa Fungbun

指導教授　汾陽　光盛

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Introduction: 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 LβT2 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 confirms 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 LβT2 growth by GnRHa was confirmed also in the present study. GnRHa administration suppressed the growth of LβT2 by 96 hrs of incubation. DNA ladder was observed after 6 hrs incubation with GnRHa suggesting an induction of apoptosis by GnRH. The suppressive effect of GnRHa on LβT2 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 LβT2 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 LβT2 cells for ANXA5 was performed after GnRHa administration. GnRHa induced blebs containing ANXA5 even after only 10 and 30 min incubation of LβT2 cells (Fig. 1). Double staining of primary pituitary cells with anti-ANXA5 and -LHβ showed blebs containing ANXA5 in the gonadotropes also after 10 and 30 min (Fig. 1). 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 LβT2 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 (Fig. 2). The particle size less than 100 nm was found in the 110,000 xg fraction (Fig. 2). These 20,000 xg and 110,000xg particles were increased by the GnRHa treatment. ANXA5 was detected dominantly in 20,000 xg pellet after

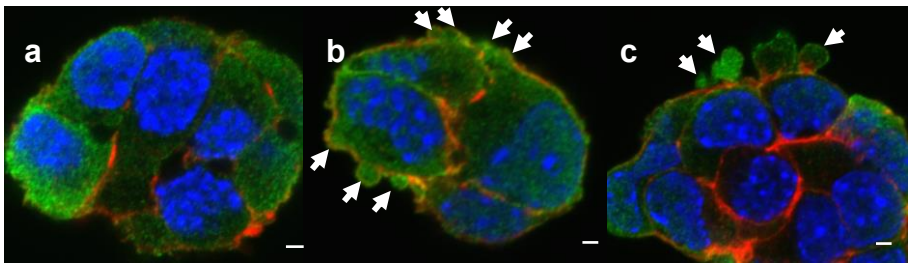
treatment with GnRHa for 10, 30 and 180 min. It increased until 180 min. ANXA5 in 110,000 xg pellet was also shown at 180 min. GnRHa treated 20,000 xg particulate fraction significantly stimulated LH release in a dose dependent manner. Membrane fraction prepared from plasma of one-week ovariectomized 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 LβT2 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 (Fig. 3). 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 $G_{\alpha s}$ 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 LβT2 cells, changes in ANXA1 protein and its distribution in the gonadotropes were examined. Western-blotting showed that ANXA1 protein expression in LβT2 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 -LHβ 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 hrs, ANXA1 in cytoplasm of gonadotrope increased. Immunohistochemistry for ANXA1 in pituitary of 2-weeks ovariectomized rats demonstrated that ANXA1 seemed to be expressed at periphery of large castrate cells. Western-blotting of whole pituitary gland of 2 weeks ovariectomized rats revealed that ANXA1 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 (Fig. 4). 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 mechanism of inter-cellular communication.

A



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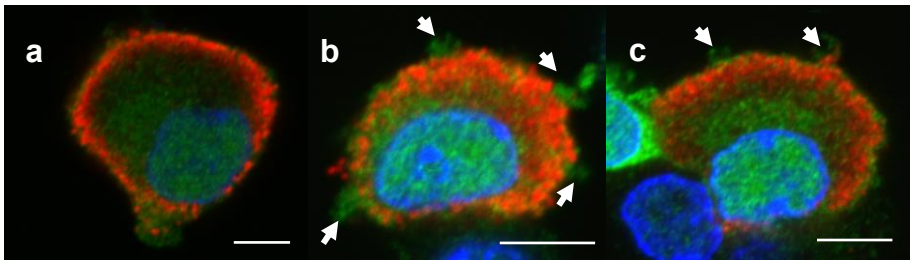

Fig 1 Immunocytochemistry for ANXA5; (A) LβT2 and (B) primary culture of pituitary cell

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.

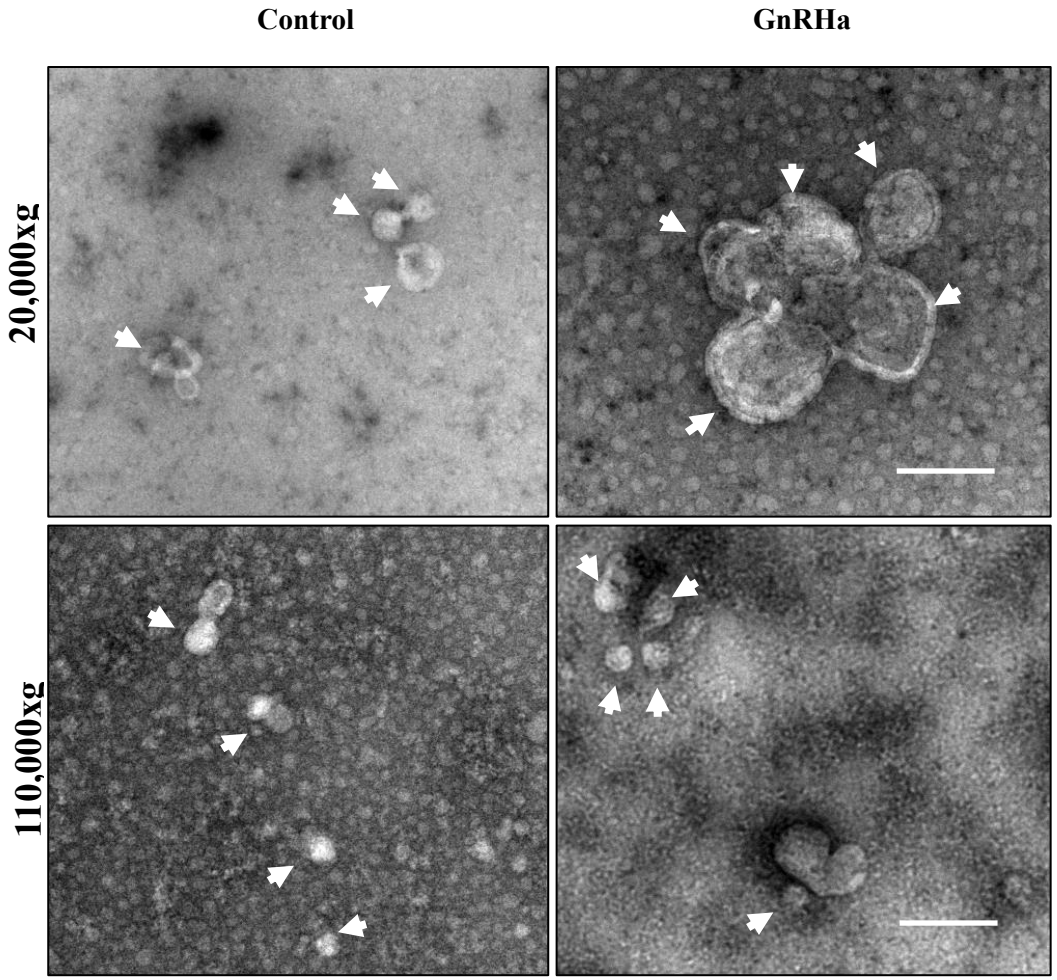

Fig. 2 Negative staining of 20,000 xg and 110,000 xg pellet fraction

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^{o}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.

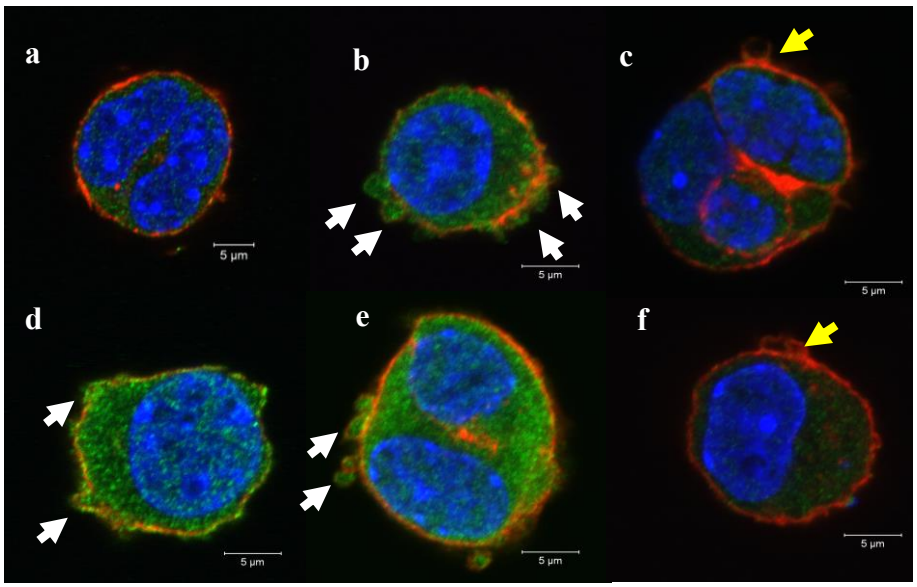

Fig. 3 The effect of inhibitors on bleb formation

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 μm. White arrows indicate blebs containing ANXA5. Yellow arrows indicate inhibitory effect of the formation with an absence of ANXA5 in the blebs.

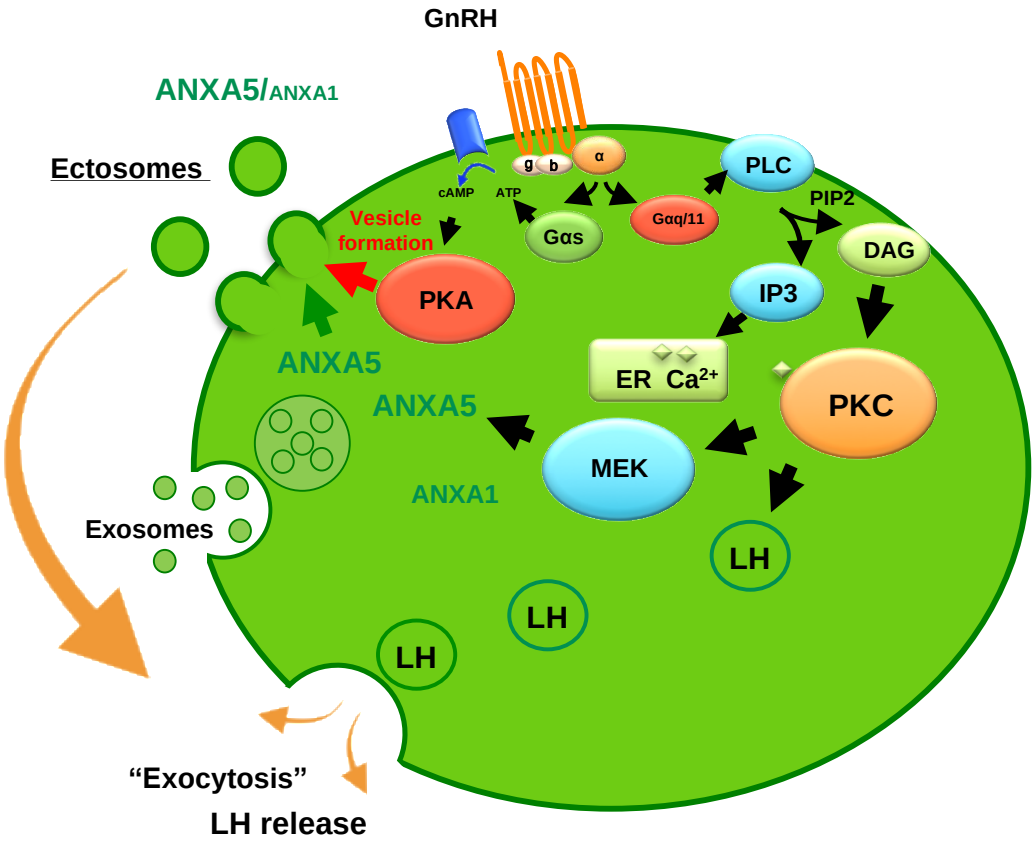

Fig. 4 Summary for ectosome formation

GnRH binds its specific GPCR and activates Gαs. ATP is converted to cAMP by adenylate cyclase and cAMP activates PKA. This results in the formation of ectosome containing ANXA5. Ectosomes and free ANXA5 facilitate LH release.