論文審査の要旨および担当者

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学位論文題目	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
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論文審査の要旨

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 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 gonadotrope 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 which was 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 gene by siRNA in the primary culture of pituitary cells resulted in the blunting of GnRH action on LH release. Furthermore, overexpression of intracellular ANXA5 by ANXA5 expression vector 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 is suppressed by GnRH in hormone-dependent cancer. Suppression of LβT2 growth by GnRHa (96 hrs) was confirmed in the present study. 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 incubation 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, an immunohistochemistry for ANXA5 was performed in cultured pituitary tissue. Depolarizing stimulation with a high potassium treatment induced obvious plasma membrane-association of ANXA5 in hemi-pituitary organ culture. GnRHa showed a 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 be associated with externalization of

ANXA5.

GnRH stimulation of ANXA5-containing extracellular vesicle (EV) formation in 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. Double staining of primary pituitary cells with anti-ANXA5 showed blebs containing ANXA5 in the gonadotropes after 10 and 30 min stimulation. Hemi-pituitary gland was cultured with GnRHa and subjected to an observation with transmission electron-microscope. 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 20,000 xg 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. 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 which 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. 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 in gonadotropes is externalized primarily by ectosome formation under GnRH-cAMP signal. ANXA5 containing ectosome in gonadotrope was demonstrated to stimulate LH release. This ectosome

formation is a physiological process and suggests 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 were also demonstrated to contain ANXA1. Double-staining immunocytochemistry in primary culture of pituitary cells with anti-ANXA1 and -LHβ showed the expression of ANXA1 was very low in intact gonadotrope. Furthermore, extracellular ANXA1 was very low even after GnRHa stimulation. After GnRHa treatment for 48 hrs, ANXA1 increased in cytoplasm of gonadotrope. 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 increased. These data suggest that ANXA1 gene was a novel target of GnRH and that ANXA1 is not transported to extracellular space like 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 in gonadotropes is externalized primarily by means of ectosome formation under GnRH-cAMP signal. Ectosome containing ANXA5 was demonstrated to be bioactive for LH release. ANXA1 was also demonstrated to be a novel target of GnRH stimulation. This study for the first time demonstrates 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.

【論文審査の結果】

本研究は、主に下垂体細胞を用いて GnRH によるアネキシン A5 (ANXA5)を介した LH 放出の機序を検討した。結果、cAMP/PKA シグナルを介した ANXA5 含有エクトソームの形成が関わるという新規メカニズムを発見した。一方、GnRH はエクトソーム非依存性に ANXA1 の発現を亢進するという興味深い現象も見出した。今後、本研究結果を礎として、新たな視点に立った性線刺激ホルモンの分泌機構が解明されることが期待される。

本論文の著者はタイ王国からの留学生で、在学 4 年間でこの成果をまとめて論文を提出した。審査員一同は、本論文が新規の知見を多く含み獣医学や内分泌学の発展に寄与するものとの認識で一致した。更に、著者が真摯な研究態度と豊かな人間性を持つことから、博士（獣医学）の学位の授与に値すると判断した。