学 位 論 文 の 要 旨

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学 位 論 文 名 Kisspeptin Induces Expression of Gonadotropin-Releasing Hormone Receptor in GnRH-producing GT1-7 cells Overexpressing G Protein-Coupled Receptor 54

発 表 雑 誌 名 General and Comparative Endocrinology
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論 文 内 容 の 要 旨

INTRODUCTION

Reproductive functions in mammalians are regulated by the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), from anterior pituitary gonadotrophs, which are involved in sex steroid hormone synthesis, follicular growth and oocyte maturation. LH and FSH are mainly under the control of the hypothalamic peptide, gonadotropin-releasing hormone (GnRH). GnRH is released from hypothalamus in a pulsatile manner, and GnRH pulse frequency determines the specificity of gonadotropin synthesis and secretion. The more rapid frequencies of GnRH pulses increased the secretion of LH, whereas slower frequencies of GnRH pulses are decreased LH secretion but increased FSH secretion.

Kisspeptin, previously known as metastin, was originally identified as a product of the metastasis suppressor gene, KiSS-1, and is a natural ligand of G protein-coupled receptor 54 (GPR54; also known as KiSS-1R). In 2003, inactivating mutations in GPR54 were found in patients with idiopathic hypogonadotropic hypogonadism, which is characterized by low levels of sex steroids and gonadotropin. In addition, it was found that KiSS-1- and GPR54-knockout mice exhibit infertility. Thus, kisspeptin is currently recognized as a potent stimulator of GnRH release. Furthermore, it has been reported that GnRH neurons possess their own GnRH receptor (GnRHR) and respond to GnRH. However, little is known about the direct effect of kisspeptin and GnRH on single population of GnRH neurons still largely unknown. In this study, we have investigated the action of kisspeptin using mouse GnRH-producing cell models, GT1-7. The effects of GnRH on GT1-7 cells were also examined.

MATERIALS AND METHODS

Cell culture

GT1–7 cells, a mouse GnRH-producing cell lines or mouse pituitary LβT2 cells were plated in

35-mm tissue culture dishes and incubated in high-glucose DMEM containing 10% heat-inactivated FBS and 1% penicillin–streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 in air. After 24 h, the culture medium was changed to high-glucose DMEM containing 1% heat-inactivated FBS and 1% penicillin–streptomycin and incubated without (control) or with test reagents for the indicated periods.

Reporter plasmid construct and luciferase assay

Construction of the human GPR54 expression vector and human GnRHR expression vector were transiently transfected to the cells by electroporation. An empty vector (pCI-neo) served as a mock control. When GnRHR promoter activity or the activities of the promoter containing Sre and Cre were measured, the cells were transfected together with either GnRHR-Luc, pSre-Luc or pCre-Luc vectors. Luciferase activities were measured using luminometer and determined by the ratio of firefly to the Renilla luciferase activities.

Western blotting

The cell extracts were subjected to SDS-PAGE in 10% acrylamide gel and the protein was transferred onto polyvinylidene difloride membranes. The membranes were incubated with phosphorylated-extracellular signal-regulated kinases (ERK) antibody. For total ERK determination, after strip washing, membranes were re-probed with anti-ERK antibody.

RNA preparation, reverse transcription, and real-time quantitative RT-PCR

Total RNA from untreated or treated GT1-7 cells was extracted using the extraction method Trizol-S. To obtain cDNA, 1.0 μg of total RNA was reverse transcribed using an oligo-dT primer, and was prepared using a First Strand cDNA Synthesis Kit in reverse transcription (RT) buffer. Messenger RNA (mRNA) was reverse transcribed into single stranded cDNA. Quantification of GnRHR mRNA was obtained through real-time quantitative PCR using specific primer for GnRHR.

Measurement of cAMP accumulation

Cells overexpressing GPR54 or GnRHR were plated in 96-well plates at a density of 10^5 cells/well and cultured for 48 h. Cells were then stimulated with kisspeptin or GnRH in serum-free DMEM medium for 1 h. Intracellular cAMP levels were measured using the direct cAMP ELISA kit.

Statistical analysis

Values were expressed as means±SEM. Statistical analysis was performed using Student t-test or one-way repeated ANOVA followed by Dunnett test or Newman-Keuls test for multiple comparisons. $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

To clarify whether kisspeptin and GnRH has a direct effect on GT1-7 cells, we examined the Sre- and Cre-luciferase promoters activity in GT1–7 cells. Sre is a DNA domain in the promoter region that binds to ERK-mediated transcription factors, and activation of Sre promoters reflects the activity of ERK mediated signaling pathways. On the other hand, the Cre promoter is a known target of the Cre-binding protein, and the Cre-luciferase reporter system

reflects the activity of the cAMP/PKA pathway. In GT1-7 cells, both kisspeptin and GnRH failed to stimulate either Sre or Cre promoter activity. Because we suspected that the expression of GPR54 and GnRHR in GT1-7 cells might have been reduced by changes in cell character due to multiple passages, cells were transfected with GPR54 or GnRHR expression vectors to determine the effect of kisspeptin and GnRH. After overexpression of GPR54 and GnRHR, both kisspeptin and GnRH increased Sre promoter activity. This observation suggests that kisspeptin and GnRH have an ability to stimulate intracellular signaling pathway when their receptors were abundantly expressed. Although Cre promoter activity was significantly activated by kisspeptin, it was not significantly increased by GnRH. That is, both kisspeptin and GnRH increased Sre promoter activity, however, the Cre promoter was increased by kisspeptin, but not by GnRH. These observations implied that GPR54, but not GnRHR has the ability to stimulate both ERK and cAMP/PKA pathways. Indeed, kisspeptin, but not GnRH increased the accumulation of intracellular cAMP in these cells. Next, we examined the effect of kisspeptin and GnRH on GnRHR promoter activity. In mock-transfected GT1–7 cells, kisspeptin failed to stimulate GnRHR promoter activity, while in the cells with GPR54 overexpression, kisspeptin significantly increased GnRHR promoter activity. On the other hand, the effect of GnRH on GnRHR promoters was limited and not significant, even when GnRHR was overexpressed. To exclude the possibility that exogenous induction of GPR54 artificially increases GnRHR expression, we overexpressed GPR54 in pituitary gonadotrophic LβT2 cells. LβT2 cells with overexpressed GPR54 significantly increased Cre promoter activity via kisspeptin stimulation, but no GnRHR transcriptional activity was induced by kisspeptin. Thus, we speculated that both the cAMP/PKA and ERK pathways are necessary for inducing GnRHR expression in GT1-7 cells. To further confirm this, pFC-MEKK and pFC-PKA vectors, which induce constitutively active MEKK (which ultimately stimulates ERK) and PKA were applied. Overexpression of MEKK and PKA increased GnRHR transcriptional activity as well as mRNA expression, respectively. Furthermore, combined expression of MEKK and PKA further increased GnRHR promoters. Notably, combined expression of MEKK and PKA further increased Cre promoter activity. On the other hand, PKA expression did not modify the effect of MEKK on Sre promoters. The potentiated patterns of GnRHR promoter activity by combined overexpression of MEKK and PKA were quite similar to those of Cre promoters. These observations suggest the importance of the cAMP/PKA pathways in the induction of GnRHR. The observation that GnRH, which did not induce a significant increase in GnRHR promoter activity, further stimulated the GnRHR promoter in the presence of cAMP supports this hypothesis.

CONCLUSION

The present findings suggest that kisspeptin is a potent stimulator of GnRHR expression in GnRH-producing neurons. Also we speculate that both ERK and PKA activation induced by kisspeptin are necessary to stimulate GnRHR expression.

論文審査及び最終試験又は学力の確認の結果の要旨

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学位論文名	Kisspeptin induces expression of gonadotropin-releasing hormone receptor in GnRH-producing GT1-7 cells overexpressing G protein-coupled receptor 54	
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論文審査の結果の要旨

申請者はGonadotropin-releasing hormone (GnRH)ニューロンに対する視床下部キスペプチン及びGnRHの直接作用について、GnRH産生ニューロンのモデル細胞であるGT1-7細胞を用いて検討した。受容体シグナルの反応性を高めるため、GT1-7細胞にキスペプチン受容体であるG-protein coupled receptor 54 (GPR54)、あるいはGnRH受容体自体を過剰発現させた細胞を作製し検討を行った。キスペプチンはGT1-7細胞においてExtracellular signal-regulated kinse (ERK)経路及びcAMP/protein kinase A(PKA)経路の両経路を活性化させることが明らかになった。一方、GnRHはERK経路のみを活性化させ、cAMP/PKA経路の活性化は認められなかった。キスペプチンおよびGnRHはGT1-7細胞においてGnRHの発現を促進しなかったが、キスペプチンのみがGnRH受容体発現を増加させ、GnRHにはその作用は認められなかった。恒常活性化型MEKK及び恒常活性化型PKAをGT1-7細胞へ同時導入し、ERK及びcAMP/PKAの両経路を活性化させるとGnRH受容体発現が増強した。またERK経路の活性化しか認められないGnRH刺激と共に、cAMP刺激を加えることでGnRH受容体発現の増強が認められたことから、申請者らはGnRH受容体発現にはERK及びcAMP/PKA経路の両経路の活性化が重要であると結論付けた。

最終試験又は学力の確認の結果の要旨

申請者はGnRH産生ニューロンに対するキスペプチンの作用およびシグナル伝達系について、GT1-7細胞を用いて検討し、ERK経路およびPKA経路の活性化によりGnRH受容体発現が増加することを明らかにした。これらの結果はキスペプチンによる女性生殖機能の制御・維持に重要な知見であり、加えて関連する知識も豊富であることから、学位授与に値すると判断した。　（主査：　和田孝一郎）

申請者はGnRH 産生ニューロンのモデル細胞株を用いて、キスペプチンはERK とcAMP/PKAの両経路を活性化し、GnRH受容体発現を促進することを明らかにするとともに、これによりGnRHに対する反応性を高め，更なるGnRH産生能を獲得している可能性を提起した。関連領域の知識も豊富で、学位授与に値すると判断した。　（副査：　杉本利嗣）

申請者はキスペプチンがGnRH産生ニューロンに対してERK及びcAMP/PKA経路の活性化によってGnRH受容体発現を増加させることをGT1-7細胞を用いて分子生物学的に明らかにした。これはGnRH分泌調節機構に新知見を与え、無月経の病態論に貢献しうる研究であることから学位授与に値すると判断した。　（副査：　内尾祐司）

（備考）要旨は、それぞれ４００字程度とする。