学 位 論 文 の 要 旨

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学 位 論 文 名　Induction of Dual-Specificity Phosphatase 1 (DUSP1) by Static and Pulsatile Gonadotropin-Releasing Hormone Stimulation: Role for Gonadotropin Subunit Expression in Mouse Pituitary LbetaT2 Cells

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論 文 内 容 の 要 旨

INTRODUCTION

The gonadotropins luteinizing-hormone (LH) and follicle-stimulating hormone (FSH) are synthesized and secreted by gonadotrophs under GnRH regulation. The mitogen-activated protein kinase 3/1 (MAPK3/1) pathway is an important mediator in the regulation of GnRH-induced gonadotropin gene expression. Dual-specificity phosphatase 1 (DUSP1) is a dephosphorylating enzyme that inactivates MAPK3/1 through a negative feedback control. GnRH is released from the hypothalamus in a pulsatile manner, and GnRH pulse frequency differentially regulates gonadotropin subunit gene expression. In mouse pituitary gonadotroph LβT2 cells, high frequency of GnRH pulses maximally stimulate gonadotropin *Lhb* subunit gene expression; whereas low frequency pulses preferentially stimulate *Fshb*. Previous study demonstrated that high frequency GnRH pulses transiently activated MAPK3/1; whereas low frequency activated MAPK3/1 more rapidly and sustainably. Based on the above, we have sought to investigate the expression of DUSP1 and the correlation with MAPK3/1 activation by GnRH stimulation. In addition, the relationship between DUSP1 and MAPK3/1 by pulsatile GnRH stimulation and the role for gonadotropin subunit gene expression were also examined.

MATERIALS AND METHODS

Cell culture and Perifusion system

LβT2 cells were preincubated with or without inhibitors for 30 min, and then GnRH was added to the culture dish. In perifusion experiments, LβT2 cells were plated in perifusion chambers and then mounted in perifusion system, and GnRH pulses were delivered by a set of peristaltic pumps controlled by a time controller in high frequency (one pulse, 5 min flow every 30 min) or low frequency (one pulse, 5 min flow every 2 h).

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 either DUSP1 or phosphorylated-MAPK3/1 antibody. For total MAPK3/1 determination, after strip washing, membranes were re-probed with anti-MAPK3/1 antibody.

Transfections and luciferase assays

The reporter constructs used were generated by fusing -797/+5 of the rat *Lhb* gene or -2000/+698 of the rat *Fshb* to firefly luciferase cDNA in pXP2. LβT2 cells were transiently transfected by electroporation together with pRL-TK vector containing renila luciferase. In some experiments, serum response factor *(Srf)*-luciferase in pXP2, DUSP1 expression vector in pWay21, or *Dusp1* si-RNA was cotransfected. Gonadotropin promoter activities were measured using luminometer and determined by the ratio of firefly to the Renilla luciferase activities.

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

DUSP1 expression was markedly increased 60 min after GnRH stimulation and MAPK3/1 activation, which was activated maximally 10 min after GnRH addition, was gradually decreased after 60 min, consistent with the function of DUSP1 as a negative feedback regulator in the MAPK pathway. MAP2K inhibitor, U0126, inhibited GnRH-increased MAPK3/1 phosphorylation completely and also decreased GnRH-induced DUSP1 expression partially, suggesting that DUSP1 induction by GnRH occurs via both MAPK3/1-dependent and -independent mechanisms. Diterpenioid triepoxide, a DUSP1 inhibitor, prevented GnRH-induced DUSP1 expression with concomitant increase in MAPK3/1 phosphorylation.

Both gonadotropin subunit promoter activities are reduced in the presence of U0126, suggesting that gonadotropin promoter activities were MAPK3/1 dependent; however, DUSP1 inhibitor strongly inhibited the effects of GnRH on gonadotropin as well as *Srf*-promoter activities despite an increase in MAPK3/1 phosphorylation. DUSP1 knockdown with si-RNA did not increase gonadotropin promoter activities even though MAPK3/1 phosphorylation was augmented. In particular, DUSP1 siRNA significantly reduced *Lhb* promoter activity, suggesting that *Lhb* was more sensitive to DUSP1. DUSP1 overexpression significantly prevented GnRH-induced MAPK3/1 phosphorylation and both *Lhb* and *Fshb* promoter activities were reduced. Taken together, we suggest that gonadotropin expression requires somehow a balance between MAPK3/1 phosphorylation and DUSP1 expression. Insulin-like growth factor 1 (IGF1)

increased MAPK3/1 phosphorylation; however, it did not increase DUSP1 as well as gonadotropin expression, suggesting that DUSP1 expression was not an automatic sequel of MAPK3/1 phosphorylation.

Next, we examined the DUSP1 expression by pulsatile GnRH stimulation in perifused cells. DUSP1 expression was increased more prominently following high frequency (every 30 min) GnRH pulse stimulation compared to that of low frequency (every 120 min) GnRH pulses. Under 10 nM GnRH pulses in high frequency, DUSP1 expression was increased 2 h after GnRH pulse initiation (4 GnRH pulses of 5 min). To adjust the amount of GnRH exposure to the cells in the low frequency group, we increased GnRH pulse concentration to 40 nM and 100 nM. DUSP1 expression, however, remained uninduced following low frequency GnRH pulses, indicating that both the number of GnRH pulses and the high frequency with which they are delivered are more important than concentration in terms of DUSP1 expression induction.

Under high frequency GnRH pulse stimulation, MAPK3/1 phosphorylation was observed 10 min after a single GnRH pulse, and decreased to the basal levels at 30 min. The pattern of MAPK3/1 phosphorylation induced by the GnRH pulse after 4 h stimulation was similar. Transient phosphorylation of MAPK3/1 occurred even though DUSP1 was expressed at 4h. Taken together, these suggest that DUSP1 modulates the pattern of MAPK3/1 phosphorylation following GnRH pulses, but did not dephosphorylate MAPK3/1 which was activated at each GnRH pulse. The repeated MAPK3/1 activation in high-frequency GnRH pulses might be necessary for DUSP1 induction in perifused cell setting, which ultimately forms the different pattern of MAPK3/1 activation by the different frequency of GnRH pulse.

Overexpression of MAP3K1, a kinase upstream of MAPK3/1, increased both *Lhb* and *Fshb* subunit promoter activity, which could be completely inhibited by co-transfection with DUSP1-expressing vectors. *Srf*- promoter activities induced by MAP3K1 were also prevented by DUSP1 overexpression. Both high and low frequency GnRH pulse stimulation failed to increase *Lhb* and *Fshb* subunit gonadotropin gene expression upon DUSP1 overexpression, confirming again the important role of MAPK3/1 in gonadotropin subunit gene expression.

CONCLUSION

DUSP1 may participate in the differential regulation of gonadotropin subunit expression directly and through its role as MAPK3/1 dephosphorylating enzyme. DUSP1 may be involved in *Lhb* transcription. Furthermore, DUSP1 expression and the pattern of MAPK3/1 dephosphorylation during high frequency GnRH pulse might prevent *Fshb* transcription. Even though MAPK3/1 pathway is important in the regulation of both gonadotropin subunits, *Fshb* subunit is probably more largely dependent to MAPK3/1. These findings may contribute to the elucidation of the mechanism of gonadotropin subunit regulation.

別紙

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論　　文　　名

1. Induction of Dual-Specificity Phosphatase 1 (DUSP1) by Gonadotropin-Releasing Hormone (GnRH) and the Role for Gonadotropin Subunit Gene Expression in Mouse Pituitary Gonadotroph LbetaT2 Cells

2. Induction of Dual-Specificity Phosphatase 1 (DUSP1) by Pulsatile Gonadotropin-Releasing Hormone Stimulation: Role for Gonadotropin Subunit Expression in Mouse Pituitary LbetaT2 Cells

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