主論文の要旨

Effect of the Neuropeptide Phoenixin and Its Receptor GPR173 During Folliculogenesis

（卵胞発育における神経ペプチド **Phoenixin** と
その受容体 **GPR173** の作用）

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【INTRODUCTION】

Folliculogenesis is a complex process, defined by the growth and development of follicles from the primordial population. Granulosa cells (GCs) play a vital role in every stage of follicular growth through proliferation, acquisition of gonadotropic responsiveness, steroidogenesis, and production of autocrine/paracrine factors.

Phoenixin, a highly conserved peptide among species, is expressed abundantly in hypothalamus. GPR173 is a member of a small subfamily of GPCRs called the Super Conserved Receptor Expressed in Brain (SREB) family. In humans, GPR173 is widely distributed in the hypothalamus, pituitary, and ovaries which share the identical HPG axis. Phoenixin acts through the orphan receptor GPR173 and activates the cAMP, protein kinase A (PKA) pathway to induce cAMP response element binding protein (CREB) phosphorylation.

Phoenixin induced GnRH receptor mRNA (GnRHR), increased GnRH and LH plasma levels. *In vitro* phoenixin increased the expression of GnRH and GnRHR mRNA as well as GnRH secretion.

In the present study, we examined the expression of phoenixin in human ovarian follicles and studied its function in GCs. We also analyzed the effects of phoenixin in folliculogenesis using murine ovarian tissue cultures.

【METHODS AND RESULTS】

Expression of phoenixin and GPR173 in human ovarian follicles

The expression levels and cellular distribution patterns of phoenixin and GPR173 in the human ovarian tissue were evaluated by immunohistochemistry. Phoenixin and GPR173 were expressed in the human ovarian follicle, with increased expression in GCs as the follicle grows. Both phoenixin and GPR173 immunostaining were prominently detected in oocytes in human ovary (Figures 1A and B, a, b, c). PCR results revealed that the mRNAs of SMIM20 (77 bp) and GPR173 (154 bp) were expressed in the HGrC1 cell line (Figure 1C).

Effect of phoenixin on GPR173 expression, CREB activation and GC steroidogenesis

Real-time PCR and western blot were used to examine the effect of phoenixin on GC steroidogenesis. Treatment with 100 nM phoenixin caused a significant increase in the expressions of GPR173 (2.13 fold, $p < 0.01$), CREB1 (1.44 fold, $p < 0.05$) and CYP19A1 (4.03 fold, $p < 0.01$) in HGrC1 cells (Figures 2A, B and C). Phoenixin at 10 nM and 100 nM concentrations increased the phosphorylation of CREB after 15 and 30 minutes of treatment (Figure 2D). Phoenixin induced E2 production in a concentration-dependent manner, especially with a significant induction at 100 nM (34.10 ± 1.60 vs. 24.90 ± 2.40 pg/mL respectively, $p < 0.05$) compared with the control group (Figure 2E).

Effect of phoenixin on the expression of follicle development-related genes

Phoenixin at a concentration of 100 nM significantly increased the mRNA levels of FSHR (5.78 fold, $p < 0.001$), LHR (4.89 fold, $p < 0.001$) and KITL (2.54 fold, $p < 0.05$), but decreased the mRNA levels of NPPC (0.54 fold, $p < 0.01$) in HGrC1 cells (Figures 3A, B, C, and D).

Effect of phoenixin on follicular growth

To evaluate follicular development when treated with phoenixin, mouse ovarian tissue culture method and time-lapse analysis were used. In the first week of ovarian tissue culture, the average area of follicles between the three treatment groups did not show significant difference. However, in the ovarian tissues induced with 100 nM of phoenixin, significant differences were observed from day 9 to the last day of culture. At 10 nM, phoenixin induced follicle growth, however, with slower and less effect (Figure 4A). After treatment with 100 nM of phoenixin for 2 days, the mean E2 production in the culture medium was significantly higher (298.00 ± 62.48 vs. 177.67 ± 23.68 pg/mL respectively, $p < 0.05$) in comparison to control (Figure 4B).

【DISCUSSION】

Our study is the first to show the correlation between the expression of phoenixin and GPR173 in each stage of follicular growth in human and murine ovarian follicles. Notably, phoenixin and GPR173 in GCs were detected in primary follicles, increased along with the stages of follicle development and showed the strongest expression in the secondary to antral follicle stages. The human GCs also expressed the mRNA of phoenixin precursor protein SMIM20 and its receptor, GPR173. Altogether, these data show that human GCs are both able to produce and respond to phoenixin, suggesting a paracrine or autocrine effect of phoenixin in human ovarian follicles.

Steroidogenesis is another critical role of GCs. Estradiol, which is mainly produced by the GCs, is converted from androgen by CYP19A1. In the peripheral tissues, phosphorylated CREB has been reported to activate the conserved cAMP response element (CRE) sequences present in the promoter of the *CYP19A1* gene and regulate the expression of aromatase activity in GCs. In HGrC1 cells, which have been shown to maintain non-luteinized GC characteristics and steroidogenesis, phoenixin increased phosphorylated CREB, up-regulated the expression of CYP19A1 mRNA and increased E2 production.

The expression of FHSR and LHR in GCs is essential for GC proliferation, differentiation, apoptosis, and hormone synthesis. KITL promotes primordial to primary follicle transition and increases the diameter of oocytes from growing primary follicles. C-type natriuretic peptide (CNP), encoded by the *NPPC* gene, is known to inhibit oocyte maturation. Our experiments demonstrated that phoenixin increased FSHR, LHR,

KITL mRNA, while it decreased NPPC mRNA in GCs. These findings suggest that phoenixin could act as an intraovarian factor to regulate follicle development-related gene expression.

In accordance with our molecular findings, phoenixin promoted follicle growth and oocyte maturity in our *ex vivo* studies with murine ovarian tissue culture. The average follicular area, the proportion of secondary and antral follicles, and E2 production were increased with phoenixin treatment in a concentration-dependent manner. In our study, follicle enlargement was seen in the later stages of follicular growth, which coincides with increased expression of the phoenixin receptor GPR173.

In summary, phoenixin is mainly expressed in the GCs and oocytes of the growing follicles in the ovary, and its expression in GCs increased with the stage of follicle development. *In vitro*, phoenixin accelerates GC proliferation and acts through GPR173 to stimulate E2 production via CREB signaling to increase the expression of CYP19A1 mRNA. Furthermore, phoenixin increases the expression of FSHR, LHR, KITL, and decreases CNP mRNA expression. Phoenixin treatment in murine ovarian tissue culture model increases the follicle area and oocyte maturation. Together, these findings suggest a potential role of phoenixin and GPR173 in promoting follicular growth.