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Control Mechanisms of Ovarian Follicle Development by Follicle Stimulating Hormone and Pituitary Adenylate Cyclase-activating Polypeptide

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난포자극호르몬과 Pituitary Adenylate Cyclase-activating Polypeptide에 의한 난소의 난포성장

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Objective: Pituitary adenylate cyclase-activating polypeptide (PACAP), a novel hypothalamic neuropeptide, has been suggested to play a role in ovarian folliculogenesis. The present study evaluated the effect of PACAP on the growth of preantral follicles.

Methods: Preantral follicles were mechanically isolated from ovaries of 21-day-old rats and cultured in groups for 3 days in serum-free medium in the absence or presence of PACAP-38 (10^{-6} M).

Results: Treatment with PACAP-38 resulted in an increase in follicle diameter by 75% whereas treatment with follicle stimulating hormone (FSH) increased follicle diameter by 65%. PACAP-38 treatment enhanced the granulosa cell proliferation as measured by thymidine incorporation analysis. Furthermore, the production of progesterone by cultured granulosa cells and GFSHR-17 cell line was stimulated by PACAP-38. Interestingly, PACAP enhanced FSH action on stimulation of SF-1 and aromatase gene expression.

Conclusion: The present results demonstrate that PACAP stimulated preantral follicle growth by potentiating proliferation and by stimulating steroidogenesis.

Key Words: Ovarian folliculogenesis, Follicle stimulating hormone, Pituitary adenylate cyclase-activating polypeptide (PACAP)

Mammalian ovaries consist of follicles as basic functional units. The fate of each follicles is controlled by endocrine as well as paracrine factors.[1] The follicles develop through primordial, primary, and secondary stages before acquiring an antral cavity. At the antral stage, most follicles undergo atretic degeneration, whereas a few of them, under the cyclic gonadotropin stimulation that occurs after puberty, reach the preovulatory stage.[2]

After initial recruitment, granulosa cells in primary follicles undergo profound changes, progressively acquiring the differentiated characteristics of epithelial cells found in secondary follicles. Treatment of dissociated ovarian cells from juvenile rats

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with activin and FSH enhances formation and growth of follicular structure.[3] Activin treatment also enhances FSH-stimulated inhibin production in dispersed ovarian cells from neonatal rats.[4] In mice, cultured preantral follicles secrete activin, and treatment with recombinant activin enhances FSH-stimulated inhibin and estrogen production.[5] High levels of IGF-I and IGF receptors have been found in postnatal rats during preantral follicle development.[6] However, follicles seem to develop relatively normally to the early antral stage in mutant mice lacking IGF-I, although numerical morphometrics were not performed.[7] Studies using these mutant mice further suggested that ovarian IGF-I expression serves to enhance granulosa cell FSH responsiveness by augmenting FSH receptor expression.[8] It is clear that paracrine growth factors are also involved in preantral follicle development. With recent advances in transgenic technology, more than 30 mouse models with ovarian defects at different stages of follicle development have been described.[9] Derivation of additional mutant mice with ovarian phenotypes will further enhance our understanding of early follicle development.

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide isolated from ovine hypothalamus and exist in two amidated forms, PACAP-38 and PACAP-27, sharing the same N-terminal 27 amino acids.[10] On the basis of sequence similarity, PACAP belongs to the vasoactive intestinal peptide (VIP)/secretin/glucagon/GH-releasing factor family of neuropeptides.[11] PACAP is a potent stimulator of cAMP formation and is known to stimulate cAMP and steroidogenesis in ovarian cells, an action it shares with the gonadotropins. Recent studies have shown that PACAP stimulates steroidogenesis and cAMP accumulation more potently than VIP in cultured rat granulosa cells. Furthermore, PACAP regulates meiotic maturation, mimicking the action of LH in rat oocytes, suggesting a role of PACAP in the regulation of ovarian function as a new local factor.

Ovarian folliculogenesis involves a complex series of events that is regulated by the hypothalamic-pituitary-gonadal axis in coordinated interaction with local regulators such as ovarian steroids, peptides, cytokines, growth factors, and eicosanoids. PACAP has recently been suggested as a new local ovarian regulator of gonadotropin actions based on the ability to stimulate cAMP formation and steroidogenesis in cultured granulosa cells. The present study was thus designed to examine the following subjects in the rat ovary:

1) Effect of PACAP on steroidogenesis and in vitro follicle growth

2) Effect of PACAP on granulosa cell proliferation

3) Identification of genes regulated by PACAP

MATERIALS AND METHODS

1. Hormones and reagents

Purified pituitary hFSH (ISIAFP-1; 8466 IU/mg) was obtained from the National Hormone and Pituitary Distribution Program, NIDDK, NIH (Baltimore, MD). Diethylstilbestrol (DES) was purchased from Sigma Chemical Co. (St. Louis, MO). PACAP-38 was obtained from Bachem (Torrance, CA). The progesterone antiserum was donated by Dr. Yong-Dal Yoon (Hanyang University, Seoul, Korea).

2. Animals

Immature female rats of the Sprague Dawley strain were purchased from Daehan Laboratories (Chungpuk, Korea). They were housed in groups in a room with controlled temperature and photoperiod (10-h dark, 14h-light, with lights on from 0600~2000 h). Animals were implanted with SILASTIC brand capsules (15 mm; dow Corning Corp, Midland, M1) containing DES at 21~24 days of age to stimulate the development of multiple

immature follicles.

3. Cell culture

The GFSHR-17 cells[12] were grown on 60-mm culture plates. Cells were cultured with Dulbecco modified Eagle medium Ham's F-12 (1:1 v:v; Biochrom KG, Berlin, Germany) containing 5% fetal calf serum (Biochrom KG, Berlin, Germany). Cells were maintained in culture for up to 25 passages.

4. Preantral follicle culture

Ovaries were collected from 21-day-old rats, and preantral follicles (200~220 μm in diameter) were dissected microscopically using fine needles. Follicles were cultured individually in 96-well dishes lined with polycarbonate membranes in 150 μl medium overlaid with 75 μl sterile mineral oils at 37℃ in a moist atmosphere of 5% CO_2 and 95% air. Basal (control) medium consisted of αMEM supplemented with 1% ITS (insulin, 10 ng/mL; transferrin, 5.5 ng/mL; selenium, 5 ng/mL) and Pen/strep (penicillin, 100 μ/mL; streptomycin, 100 mg/mL). Follicle diameters were measured daily as the average distance between the outer edges of the basement membrane in two perpendicular planes. At the end of the 72 h incubation, follicles were collected for further analysis.

5. Granulosa cell isolation and culture

Granulosa cells were collected from ovaries treated with DES for 3 days by the method of follicular puncture. Ovaries were incubated in DMEM/Ham's F-12 supplemented with antibiotics and 0.1% BSA containing 0.5 M sucrose and 10 mM EGTA at 37℃ for 30 min. Ovaries were washed three times in fresh DMEM/Ham's F-12, and individual follicles were punctured using 23-gauge needles under a dissection microscope. Cells were counted using trypan blue and cultured at a density of 1×10^6 cells/60-min dish in DMEM/Ham's F-12 supplemented with antibiotics and 0.1% BSA. Hormones were added at the beginning of culture, and cells were incubated at 37℃ in a humidified 95% air-5% CO_2 incubator.

6. Northern blot analysis

Total RNA from ovaries was isolated using Tri-Reagent solution (Sigma). Twenty micrograms of total RNA were fractionated by electrophoresis on a 1% agarose gel containing formaldehyde and transferred to nylon membranes by capillary blotting with 10 × sodium citrate-sodium chloride (SSC). After a UV cross-linking and prehybridization, membranes were hybridized overnight at 42℃ in a solution containing 50% formamide, 5 × SSC, 1 mM EDTA, 250 μg/ml denatured salmon sperm DNA, 500 μg/ml yeast transfer RNA, and a total of 1×10^7 cpm of a ^{32}P-labeled rat PACAP and PACAPR complementary DNA (cDNA) probe. After hybridization, membranes were washed twice for 5 min at room temperature in 2 × SSC and 0.1% SDS, followed by 1 h at 65℃ in 0.5 × SSC and 0.1% SDS. Membranes were then exposed using Kodak RX films (Eastman Kodak Co., Rochester, NY) for 7~10 days at -80℃. The band intensities were subsequently measured using a phosphoimager (Bio-Rad Laboratories, Inc. Hercules, CA), and the signals were normalized to the GAPDH RNA as an internal control.

7. [^{3}H]Thymidine incorporation

Thymidine incorporation into DNA was used to measure proliferation in granulosa cells according to a modification of the method of Geenwald and Roy.[13] One million viable cells from each groups were incubated in triplicate with 1.0 mCi of [methyl-^{3}H] thymidine in 1 mL krebs Ringer- bicarbonate buffer with 1% BSA and 5 mM Hepes (pH 7.0) for an optimum period of 24 h at 37℃. The cells were washed three times with ice-cold buffer to remove excess [^{3}H]thymidine, and the DNA was precipitated with the addition of ice-cold trichloroacetic

acid (final concentration 10% w:v) followed by centrifugation at 12,000 x g. After the pellet was washed twice with 10% trichloroacetic acid, the radioactivity in the DNA pellet was counted.

8. Radioimmunoassay

Progesterone concentrations in culture medium were measured by Radioimmunoassay (RIA). Culture media were assayed directly without further purification. General assay procedure was adapted from previously described method.[14] Labeled progesterone (1,2,6,7-3H-progesterone; 99 Ci/mM) was obtained from Amersham. The progesterone antiserum was developed in rabbit using progesterone (3-CO-carboxy methy oxine): BSA as a immunogen. The progesterone antiserum crossreacts 14% with 5α-dihydroprogesterone, 0.5% with 17α-hydroxyprogesterone, 0.2% with testosterone, 0.1% with cortisol and less than 0.001% with other steroid. Each sample was quantified for tritum using Parkard Tri-Carb 2300 liquid scintillation analyzer. Routinely, two sets of standard (12.5~200 pg) were included in each assay. Progesterone concentrations were calculated with secuRIA program (Packard) by personal computer. Between and within assay coefficients of variation for progesterone were 9.4% and 9.2%, respectively. The lower limits of assay sensitivity for progesterone were 6.5 pg.

9. Data analysis

Statistical differences were assessed by one-way analysis of variance (ANOVA), followed by Student's t test; and $p<0.05$ was considered to be statistically significant.

RESULTS

1. Effect of PACAP on progesterone production

Because PACAP is known to stimulate cAMP production, progesterone production by granulosa cells of diethylstilbestrol-treated ovaries was further tested during the 24 h of culture with PACAP-38. As shown in Figure 1, levels of progesterone were

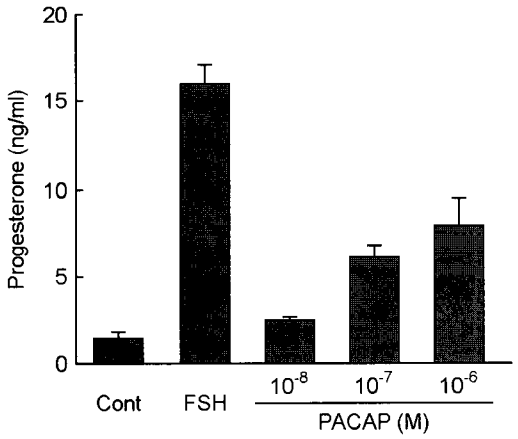

Figure 1. Stimulation of progesterone production by PACAP-38 in diethylstilbestrol-treated immature granulosa cells cultured *in vitro*. Granulosa cells collected from diethylstilbestrol-treated ovary were cultured in serum-free conditions for 24 h in the absence (Cont; control) or presence of FSH (200 ng/ml) or increasing concentration of PACAP-38. Steroid concentrations (mean ± SEM) from 6 different culture dishes in culture media were determined by RIA.

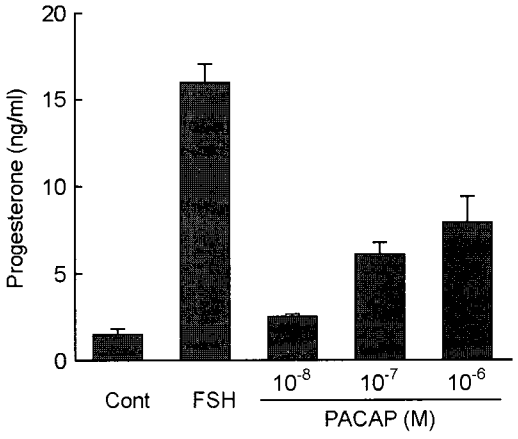

Figure 2. Stimulation of progesterone production by PACAP-38 in FSH-responsive granulosa cell line, GFSHR-17 cells. GFSHR-17 cells incubated in serum-deprived media for overnight were cultured in serum-free media for 24 h in the absence (Cont; control) or presence of FSH (200 ng/ml) or increasing concentration of PACAP-38. Steroid concentrations (mean ± SEM) from 4 different culture dishes in culture media were determined by RIA.

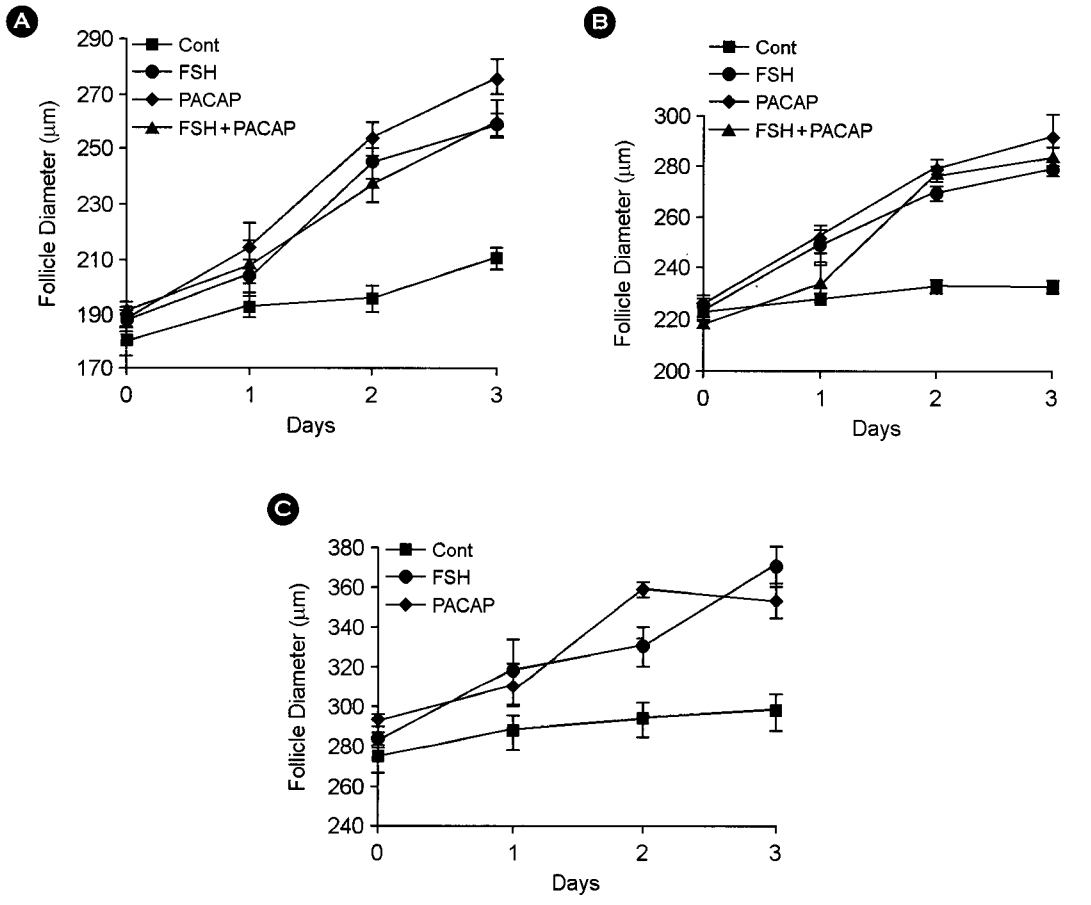

Figure 3. Stimulation of preantral follicle growth by PACAP-38 *in vitro*. Preantral follicles were cultured in serum-free media in the absence (control; Cont) or presence of PACAP (10^{-6} M) or FSH (100 ng/ml) for up to 3 days. Each value represents mean ± SEM of 60~180 follicles.

increased by FSH. Although less potent than FSH, treatment with PACAP-38 caused a dose-dependent increase in progesterone production (4-fold at 10^{-6} M compare to that of control).

To further examine the effect of PACAP on steroidogenesis, a rat granulosa cell line, GFSHR-17,[12] stably transformed to express FSH-receptors was used. Similar to that in primary granulosa cells, levels of progesterone were increased by FSH (Figure 2). In addition, treatment with PACAP-38 caused a dose-dependent increase in progesterone production, and co-treatment with FSH and PACAP further stimulated progesterone production.

2. Effect of PACAP on the growth of immature follicles

To determine the effect of PACAP on follicle growth, preantral follicles with different diameter were cultured in the presence of PACAP alone or in combination with FSH. Preantral follicles without hormone treatment showed minimal growth over the 3 days culture period (Figure 3). In contrast, treatment with PACAP (10^{-6} M) resulted in an increase in follicle diameter by 75%. Consistent with earlier findings,[15] treatment with FSH also resulted in an increase in follicle diameter by 65%. However, cotreatment with PACAP did not poten-

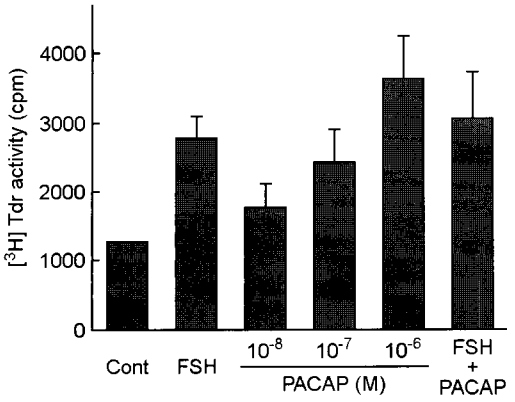

Figure 4. Effect of PACAP on the proliferation of granulosa cells. Granulosa cells collected from diethylstilbestrol-treated ovary were cultured in serum-free conditions for 48 h in the absence (Cont; control) or presence of FSH (200 ng/ml) or increasing concentration of PACAP-38. During the last 24 h of culture, [^{3}H] thymidine (1 μCi/well) was added to determine DNA synthesis. Values indicate the mean ± SEM from six replicates.

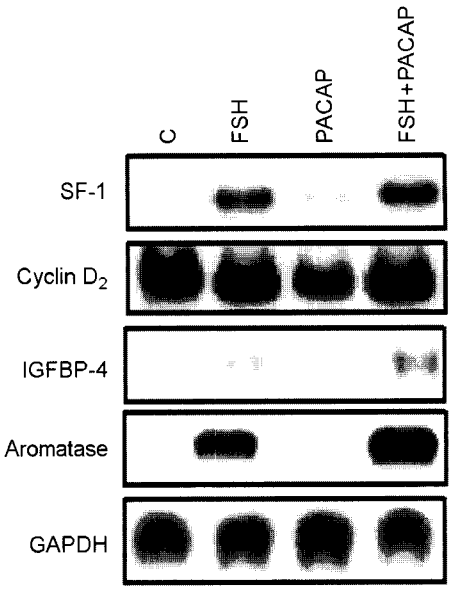

Figure 5. Changes in the expression of genes in growing follicles following PACAP-38 treatment. Growing follicles obtained from ovaries of diethylstilbestrol-treated immature rats were cultured in serum-free medium, under 5% CO_2-95% O_2 at 37℃, in the presence of PACAP (10^{-6} M) or FSH (100 ng/ml) for 48 h. Aliquots of total RNA (20 μg) isolated from follicles were assayed by Northern blotting using a rat cDNA probes. The expression of GAPDH was used as an internal standard. Data are representative of two separate experiments.

tiate the FSH action.

To test if the stimulatory effect of PACAP on follicle growth is due to an increase in proliferation, thymidine incorporation experiment was performed. As shown in Figure 4, PACAP increased DNA synthesis of preantral granulosa cells in a dose-dependent manner with the same potency with that of FSH. Cotreatment with PACAP and FSH did not have an additive effect.

Using culture of immature preantral follicles, genes known to be important for follicle growth were examined after PACAP treatment. The effect of PACAP on the expression of genes controlling *de novo* steroid synthesis was examined. Although treatment with PACAP alone had no effect, cotreatment with PACAP greatly enhanced the FSH action on stimulation of SF-1 and aromatase expression (Figure 5). In contrast, PACAP did not have an effect on the expression of cyclin D_2 and IGFBP-4.

DISCUSSION

The present study shows that PACAP stimulated preantral follicle growth by enhancing proliferation and by stimulating steroidogenesis. The previous study has been shown that granulosa cells of the large preantral follicles are the major cell type expressing high levels of PACAPR mRNA and PACAP is produced and secreted by theca cell of the large preantral follicles and/or granulosa cells of preovulatory follicles. Therefore, it is possible that PACAP is a autocrine/paracrine hormone within the ovarian follicle. It has been demonstrated that the addition of PACAP to culture medium resulted in an increase follicular diameter and granulosa cell proliferation as well as progesterone production.

A factor secreted by the granulosa cells of preantral follicles enhances theca cell differentiation before the expression of LH receptor,[16] whereas

coculture of theca and granulosa cells increases the proliferation and steroidogenesis of both cell types.[17] Thus, paracrine factors secreted by theca and granulosa cells likely play a critical role in preantral follicle development. Many studies have reported on the regulation of growth and differentiation of preantral follicles. FSH is a growth and differentiation factor for preantral follicles in rats.[18] FSH is indispensable for the survival of the rat preantral follicles in long-term culture in serum-free medium.[19] *In vivo*, folliculogenesis is known to be regulated by both endocrine and intraovarian autocrine and paracrine factors. There is growing evidence that endocrine and paracrine growth factors play key roles in follicular development and that they modulate survival, proliferation, and differentiation of follicular cells, acting in concert with gonadotropins. In the present study, treatment with PACAP stimulated follicle growth of rat preantral follicles. The observation that PACAP increases the total number of granulosa cells confirms that PACAP promotes cell proliferation.

Progesterone and estrogen are well-known endocrine and intrafollicular autocrine mitogenic compounds.[20] The present observation, demonstrating an increase in progesterone production by PACAP-38, implicates the presence of PACAP receptors in preantral follicles. Indeed, PACAP has been shown to be more potent than VIP in the stimulation of cAMP and steroidogenesis in cultured granulosa cells from estrogen-treated immature rats.[21] Since the acquisition of the responsiveness to FSH at an early stage of follicular development is considered essential for its subsequent follicular development,[1] it is possible that PACAP up-regulates the sensitivity to FSH in granulosa cells of immature rats.

It is known that ovarian follicular growth begins and proceeds to the late preantral stage independently of gonadotropin regulation.[22] Further development depends upon FSH acting upon its cognate receptor expressed by granulosa cells.[23] PACAP possibly exerts its stimulatory effect on follicle development by modulation of the FSH receptor expression in the preantral follicles. The present study demonstrated that cotreatment with PACAP greatly enhanced the FSH action on the stimulation of P450scc and SF-1 expression. It has been suggested that insulin-like growth factor I, expressed in granulosa cells of healthy growing follicles, may promote follicle growth by augmenting granulosa cell FSH receptor responsiveness,[8] and thereby amplifying FSH-induced aromatase expression and LH receptor induction.[24] Our findings confirm and extend those of previous studies regarding FSH/IGF-I action.[25]

In summary, the present study has demonstrated that PACAP promotes the growth of preantral follicles by enhancing cell proliferation and/or differentiation and stimulating steroidogenesis.

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= 국문초록 =

목 적: 본 연구는 흰쥐 난소를 실험모델로 하여 미성숙 전동 난포의 성장에 대한 pituitary adenylate cyclase-activating polypeptide (PACAP)의 영향을 알아보고자 하였다.

연구방법: 미성숙 전동 난포를 생후 21일된 흰쥐로부터 분리하여 PACAP을 첨가하거나 첨가하지 않은 무혈청 배양액에서 3일 동안 배양하고, 푸로게스테론 호르몬의 생성, 난포의 성장, 과립막세포의 증식 및 유전자의 동태 등을 관찰하였다. 증식의 정도는 thymidine incorporation 방법으로 검색하고 유전자의 변동은 Northern 분석을 이용하였다.

결 과: PACAP으로 처리한 군은 난포의 직경이 75% 증가한 반면, 난포자극호르몬인 FSH로 처리한 군은 65% 증가하였고, PACAP 처리는 과립막 세포의 증식을 강화시켰다. FSH와 PACAP 공히 배양된 흰쥐 난포의 과립막 세포와 FSH에 반응하는 세포주인 GFSHR-17에서의 프로게스테론 생성을 촉진시켰고, PACAP이 FSH의 작용을 증진시켜 SF-1과 아로마타제 유전자 발현을 촉진시켰다.

결 론: 본 연구는 PACAP이 과립막증식과 스테로이드합성을 통하여 전동 난포의 성장을 촉진함을 시사하였고, 또한, SF-1, 아로마타제 등에 대한 FSH의 작용을 도와주는 역할을 PACAP이 담당하므로 PACAP은 초기 난포성장에 필요한 난소국소인자임을 유추할 수 있었다.

중심단어: 난포 성장, 난포 자극호르몬, PACAP