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Influence of pretreatment of insulin on phosphorylation of extracellular receptor kinase by gonadotropin releasing hormone in cultured mouse granulosa gells

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Summary

Background: To investigate the influence of pretreatment of insulin on the phosphorylation of extracellular receptor kinase (ERK) by gonadotropin releasing hormone (GnRH) in cultured mouse granulosa cells. *Materials and Methods:* The granulosa cells from the mouse were collected simultaneously from the ovaries of gonadotropin-primed ICR female mice. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was used to examine the expression of the receptors of GnRH and insulin. The granulosa cells were cultured with ten nM of insulin, ten nM of GnRH, and ten nM of insulin pretreatment before ten nM of GnRH. Western blotting was used for analysis of phosphorylation of ERK. Student *t*-test and Dunnett's comparison test were used for statistical analysis and statistical significance was defined as $p < 0.05$. *Results:* The authors confirmed the presence of receptors of GnRH and insulin in the mouse granulosa cells by RT-PCR. Both insulin and GnRH could activate ERK phosphorylation. Pretreatment of insulin for 30 minutes before GnRH treatment inhibited ERK phosphorylation by GnRH. *Conclusion:* Insulin might have a negative effect of GnRH regulation of mouse ovarian physiology by inhibition of GnRH activation of ERK. Based on these study results, the authors demonstrated the role of insulin as a delicate modulator of reproductive function.

Key words: Extracellular receptor kinase; Gonadotropin releasing hormone; Insulin; Mouse granulosa cells.

Introduction

Reproductive function is regulated by various factors including hormones and cytokines. All factors affecting the hypothalamus-pituitary-ovarian (HPO) axis could be considered as having regulatory function. Among numerous factors affecting the process of reproductive function, extensive evidence has been shown that insulin plays a role in modulation of reproduction [1-4]. Many studies show that luteinizing hormone (LH) levels are negatively correlated to circulating insulin levels. These observations suggest that insulin could modulate pituitary LH secretion. However, other studies have suggested that insulin could potentiate gonadotropin release in response to gonadotropin releasing hormone (GnRH) stimulation [5-8]. Thus, the direct effect of insulin on gonadotropin production remains unresolved.

In humans, especially women with polycystic ovary syndrome (PCOS), it has been suggested that circulating LH level has negative correlation with hyperinsulinemia [9, 10]. It is well known that PCOS patients have reproductive disturbances such as ovulatory dysfunction and high circulating insulin levels.

The ovaries have insulin receptors, and insulin action is mediated by their receptors [11, 12]. It was reported that there are GnRH receptors in extra-pituitary tissues, and GnRH receptors are expressed in the human ovary. There is a physiological role of regulatory system involving GnRH and GnRH receptor in folliculogenesis and corpus luteal function [13]. Hence, it might be possible that there is some correlation between insulin and GnRH on reproductive function in ovary. However, there is no study assessing insulin action on GnRH in ovary. For this purpose, this study was performed to find insulin action on GnRH in the cultured mouse granulosa cells (GCs) by investigation of insulin action on the phosphorylation of extracellular receptor kinase (ERK) by GnRH.

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Materials and Methods

GCs from the mouse were collected simultaneously from the ovaries of gonadotropin-primed ICR female mice. Gonadotropin priming was achieved by intraperitoneal (i.p.) injection of five IU PMSG (pregnant mare's serum gonadotropin) per mouse. The mice were sacrificed by cervical dislocation 24 hours after gonadotropin priming. The ovaries were dissected and placed in HEPES-buffered medium supplemented with antibiotics (50 μg/ml penicillin and 75 μg/ml streptomycin). Under the view of a stereomicroscope, two- to three-layered follicles were released by manual puncture and dissection of a mixture of large and small antral follicles in HEPES-buffered medium supplemented with antibiotics. To remove RBC, collected GCs were centrifuged on ficoll gradients for 20 minutes in 600 g. After washing twice, GCs were then seeded within a four-well Nunclon dish, at a seeding density of $1x10^5$ vital cells per well (diameter of 10 mm) with 20% FBS medium 199 and then oocytes were removed from GC layers. Cultures were carried out in medium 199 supplemented with 10% FBS at 37°C in a humidified atmosphere of 5% CO_2 in air. After 24 hours, unattached and dead cells were removed by gently rinsing the wells with fresh culture medium. GCs monolayers were cultured for up to six to seven days after seeding, and culture medium was changed on every other day.

Total RNA was extracted from mouse GCs using an RNeasy mini kit according to the manufacturer's instructions. RNA quality was ensured by gel visualization and spectrophotometric analysis (OD260/280). First-strand cDNA was synthesized using avian myeloblastosis virus reverse transcriptase and random hexamers from one μg of each total RNA. cDNA samples were subjected to PCR amplification with specific primers (Table 1) under appropriate conditions. The mouse β-actin gene was used as an internal control. All PCR products were electrophoresed in 1.2% agarose gels and stained with ethidium bromide.

GCs were lysed un Triton X-100 lysis buffer (1% Triton X-100, 1 mM EDTA, 150 mM NaCl, 50 mM NaF and a protease inhibitor mixture, and insoluble materials were precipitated by centrifugation at 16,000 X g for ten minutes at 4°C. The supernatant was transferred to new tube and protein concentrations were determined by using the Bio-Rad protein assay dye reagent according to manufacturer's recommendations. Twelve μg of each GCs lysate was resolved by 12% SDS-PAGE and transferred to nitrocellulose membrane, and then immunoblotted with anti-β-actin, anti-GnRH receptor, anti-insulin α receptor, anti-insulin β receptor, anti-ERK1/2, or anti-p-ERK1/2 antibody. Binding of primary antibodies was detected by incubating blots with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibody, and blots were developed using enhanced chemiluminescence (ECL Western blotting detection reagents).

Student's *t*-test and Dunnett's comparison test were used for statistical analysis and statistical significance was defined as < 0.05. This study was approved by the Institutional Review Board for human investigation of Asan Medical Center.

Table 1. — *Primer sequences for RT-PCR.*

Genes	Forward and reverse primer sequences	Product size (bp)
GnRH receptor	F : 5'-cactggatgggagtggaata-3' R : 5'-ggtagcgaatgcgactgtca-3'	533
Insulin receptor	F : 5'-tttgtcatggatggaggcta-3' R : 5'-cctaatcttggggttgaact-3'	98
β-actin	F : 5'-tggccttagggttcaggggg-3' R : 5'-tcgtgggccgctctaggcac-3'	242

F: forward, R: reverse.

Results

The GCs were cultured under controlled conditions and viewed under the inverted microscope (Figure 1). To demonstrate the action of insulin and GnRH on the cultured mouse GCs, total RNA was extracted from mouse GCs, and first-strand cDNA was synthesized. The authors confirmed the presence of receptors of GnRH and insulin in the mouse GCs by RT-PCR (Figure 2).

The GCs were cultured with ten nM of insulin, ten nM of GnRH, and ten nM of insulin pretreatment before ten nM of GnRH. Western blotting was used for analysis of phosphorylation of ERK1/2. Treatment with ten nM of insulin for five and 30 minutes showed that insulin could activate phosphorylation of ERK (Figure 3). Time course of ERK1/2 phosphorylation in mouse GCs incubated with GnRH showed that treatment with ten nM of GnRH for five and 30 minutes could induce ERK1/2 phosphorylation (Figure 4). However, it was shown that pretreatment with ten nM of insulin for 30 minutes before GnRH treatment inhibited ERK phosphorylation that formerly stimulated by GnRH (Figure 5).

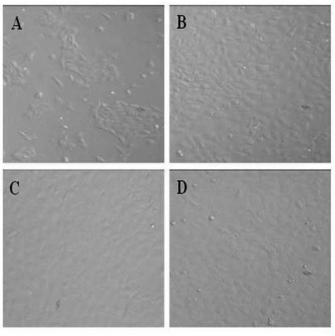

Figure 1. — In vitro culture of mouse granulosa cells. Photomicrographs of granulosa cells cultured for (A) one day, (B) two days, (C) three days or (D) four days under controlled conditions and viewed under the inverted microscope

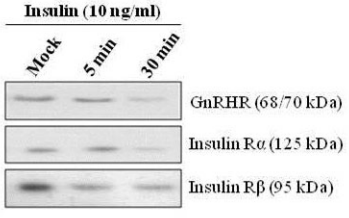

Figure 2. — Presence of GnRH receptor (68/70 kDa), insulin Rα (125kDa) and insulin Rβ (95kDa) in the mouse granulosa cells. GnRHR: gonadotropin releasing hormone receptor.

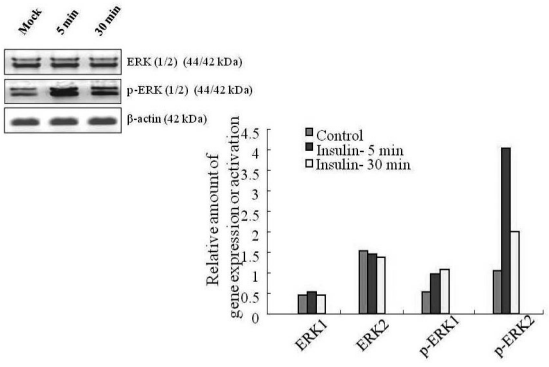

Figure 3. — Time course of ERK1/2 phosphorylation in the mouse granulosa cells incubated with insulin.
ERK: extracellular receptor kinase, p-ERK: phospholylated extracellular receptor kinase

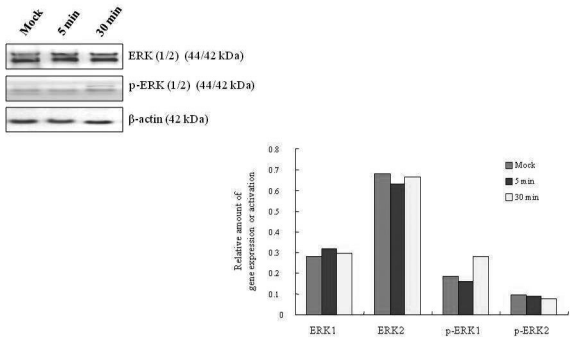

Figure 4. — Time course of ERK1/2 phosphorylation in the mouse granulosa cells incubated with GnRH.
GnRH: gonadotropin releasing hormone, ERK: extracellular receptor kinase, p-ERK: phospholylated extracellular receptor kinase.

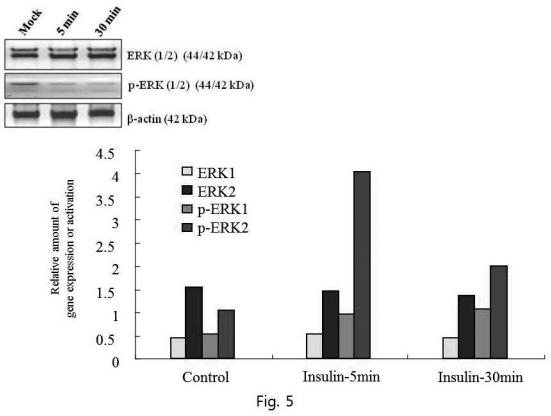

Figure 5. — Time course of ERK1/2 phosphorylation in the mouse granulosa cells incubated with GnRH after pretreatment of insulin.
GnRH: gonadotropin releasing hormone, ERK: extracellular receptor kinase, p-ERK: phospholylated extracellular receptor kinase.

Discussion

In the present study, the authors found that insulin might have a negative effect of GnRH regulation of mouse ovarian physiology by inhibition of GnRH activation of ERK1/2. Because both insulin and GnRH could act via ERK1/2 pathway and activate phosphorylation of ERK1/2, from this result, it is possible that insulin could attenuate GnRH action through modulation of ERK1/2 activation. In stead of an additive or synergistic action of insulin with GnRH, insulin pretreatment showed an antagonistic effect on GnRH-mediated ERK1/2 activation. This results indicated that although activation by GnRH is not completely blocked, it is significantly decreased by insulin pretreatment, and it is possible that insulin may alter the rate of ERK1/2 phosphorylation. Based on these study results, the authors demonstrated the role of insulin as a delicate modulator of reproductive function. For this purpose, we investigated the influence of insulin on the phosphorylation of ERK by GnRH in cultured mouse GCs. The mitogen activated protein kinase (MAPK) pathway plays an important role in the modulation of several physiological events, including cell-cycle regulation and proliferation, and apoptosis. The MAPK pathway is composed of three subgroups, the extracellular signal-regulated kinases 1 and 2 (ERK1/2), the p38 MAPK, and the stress-activated protein kinase or c-Jun N-terminal kinase (SAPK/JNK) [14, 15]. It can be activated by several growth factors such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), and hormones such as GnRH and insulin. Many signals initiate the MAPK and activated ERK phosphorylates a variety of target proteins and other protein kinases.

GnRH was originally thought as a hypothalamic hormone, however, GnRH receptors have been found in various extra-pituitary tissues and it has been reported that GnRH has extra-pituitary actions such as neuromodulation, immunomodulation, regulation of ovarian steroidogenesis, apoptosis in ovarian follicle and corpus luteum, and ovulation rather than gonadotropin secretion [16, 17]. The several actions of GnRH could be due to the divergence of signaling pathways by GnRH receptors [18]. Chakrabarti *et al.* measured the expression of mRNA and protein of GnRH and GnRH receptors in the monkey corpus luteum during different stages of the luteal phase of the menstrual cycle and they found that GnRH and their receptors might have a role on both luteinization and luteolysis in paracrine and autocrine manner [19]. It was suggested that GnRH has direct effects on the rat ovary and especially stimulates inositol phospholipid turnover in GCs. GnRH can regulate the aromatase response to FSH by biphasic actions [20]. In addition, GnRH has been shown to mimic the actions of LH on oocyte maturation and ovulation in rat ovary [21]. There was a report of direct ovarian stimulation by GnRH used for pituitary

desensitization during in vitro fertilization, although it was only observed in some patients [22]. GnRH agonist might have a direct effect on the development of the follicle in the human and rat ovary [23]. It was found that the expression of GnRH receptor in the rabbit GCs and theca cells increased in mature follicles and so these receptors play regulatory roles in follicular development [24]. GnRH could directly downregulate progesterone release of corpora lutea in buffalo ovary with the concomitant increases of several cytokines or growth factors [25]. As described above, the authors could surmise that GnRH might have a direct regulatory role on ovarian physiology.

In humans, insulin resistance and hyperinsulinemia could cause various pathophysiologic conditions, including altered reproductive physiology such as PCOS [26-28]. Insulin resistance is a component of PCOS, and this association suggests a potential correlation of insulin and reproductive function in this disorder. The failure of ovulation induction was reported to be related to increased fasting serum insulin levels and lower insulin sensitivity. This low responsiveness might support the hypothesis that gonadotropin release is inhibited by high insulin. This association of insulin and reproductive function may explain that insulin has a regulatory role on reproduction. Insulin receptor signaling may have been suggested to play a role in GnRH function of in obese women with hyperinsulinemia, and it was reported that GnRH neurons are sensitive to insulin and related to MAPK pathway [29]. Furthermore, there was an interesting report that genetic variant in GnRH receptor might relate to insulin secretion in PCOS [30]. Kim *et al*. suggested that insulin may regulate reproductive function by direct effects on the GnRH neurons [31]. However, there was no study about insulin action on GnRH function in the ovary itself.

The exact role of GnRH on reproductive function in ovary is still poorly understood, but in this study, the authors surmise that insulin could attenuate GnRH action in ovary. It could be possible that one of the mechanisms that has a negative impact on reproductive function of clinical hyperinsulinemia might be an attenuation of insulin to GnRH action in the ovary.

In conclusion, insulin might have a negative effect of GnRH regulation of mouse ovarian physiology by inhibition of GnRH activation of ERK. Based on this study results, the authors demonstrated the role of insulin as a delicate modulator of reproductive function.

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