Gonadotropin Regulation of Regulator of G Protein Signaling 2 (RGS-2) Expression in the Rat Ovary

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백서 난소에서 성선자극호르몬에 의한 RGS-2의 발현 조절

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목 적: 본 연구는 백서 난소에서 성선자극호르몬에 의한 RGS-2의 발현조절 양상을 알아보고자 하였다.

연구방법: 미성숙 백서 난소의 과배란 유도를 위해 PMSG를 주사하고, 배란을 위해서 hCG를 주입하였다. RGS-2의 유전자 발현양상을 조사하기 위하여는 Northern blot 분석과 *in situ* hybridization 분석을 시행하였다.

결 과: 미성숙 백서에 성선자극호르몬인 PMSG를 복강내 주사했을 때 RGS-2 mRNA 발현에 영향을 미치지 않음을 Northern blot analysis로 확인할 수 있었으나, hCG를 주입했을 때는 1시간에서 3시간 내에 발현이 증가됨을 알 수 있었다. *In situ* hybridization으로 살펴본 RGS-2 mRNA의 발현세포는 난포의 크기에 관계없이 난자였으나, hCG로 처리한 후에는 배란 전 난포와 성장중인 난포의 과립막 세포이었다. 그러나, RGS-2 단백의 발현은 hCG 처치와 관계없이 난포막 세포이었다. 상기 생체 실험과 마찬가지로 시험관에서도 배란 전 난포의 과립막 세포에 대한 LH 처리는 RGS-2 유전자 발현을 1시간 내에 촉진하였다. 또한, 성선자극호르몬 분비호르몬 2 길항제도 이러한 LH의 촉진작용을 증진시켰다.

결 론: 본 연구로 배란 전 과립막 세포에서 성선자극호르몬인 LH/hCG와 성선자극호르몬 분비호르몬 길항제에 의해 RGS-2의 발현이 증진되는 양상으로 보아 RGS-2가 배란과정 동안에 Gq protein 신호전달을 조절할 것으로 추정된다.

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중심단어: RGS-2, 배란, 배란전 난포, 유전자 발현

A variety of hormone and neurotransmitter receptors transduce signals through heterotrimeric G proteins. G proteins exist as heterotrimers consisting of α, β and γ subunits with guanine diphosphate bound to Gα.[1] Upon agonist occupation, the receptors promotes guanine diphosphate/guanine triphosphate (GDP/GTP) exchange, and activate GTP-bound Gα subunit and Gβγ dissociate to interact with target effector proteins. Signaling is terminated by the hydrolysis of GTP to GDP and the subsequent formation of the heterotrimer. Recently, regulator of G protein signaling (RGS) proteins have been shown to interact directly with Gα subunits to decrease the lifetime of the active GTP-bound complex.[2,3] RGS proteins attenuate heterotrimeric G protein signaling by functioning as both GTPase-activating proteins (GAPs) and inhibitors of G protein/effector interaction.[4,5] Among the well-studied RGS proteins,

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RGS-2 is the one first identified by screening cDNA libraries prepared from activated human monocytes,[6] and has been shown to mediate its GAP activity.[7] RGS-2 potently and selectively interferes with signaling through receptors that couple to Gqα.[5]

It is known that LH receptor activates Gqα as well as Gsα protein. LH/hCG has been shown to stimulate phospholipase C, leading to inositol phosphate breakdown and the consequent production of inositol triphosphate (IP_3) and diacylglycerol (DAG).[8,9] Our preliminary microarray data using mouse preovulatory follicles with Affimatrix chips show that, among RGS family, RGS-2 expression is stimulated by LH/hCG. It is thus to plausible to hypothesize that RGS-2 may be expressed in ovarian cells and play a role during the ovulatory process. In the present study, therefore, the regulation of RGS-2 expression by LH/hCG was examined.

MATERIALS AND METHOD

1. Hormones and animals

Ovine LH (LH-S-26; 2,300 IU/mg) was obtained from the National Hormone and Pituitary Distribution Program, NIDDK, NIH (Baltimore, MD). Human chorionic gonadotropin (hCG), pregnant mare's serum gonadotropin (PMSG) were purchased from Sigma (St. Louis, MO). GnRH agonist and antagonist were provided by Dr. JY Sung (Korea University, Seoul, Korea).

Immature female rats of the Sprague Dawley strain were purchased from Daehan Laboratories (Chungbuk, Korea) and maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. They were housed in groups in a room with controlled temperature and photoperiod (10-h dark, 14-h light, with lights on from 0600~2000 h). The animals at 26-day-old age (body weight, 60~65 g) were injected subcutaneously with 10 IU PMSG to induce multiple follicle growth. Some rats received a single intraperitoneal injection of 10 IU hCG to induce ovulation, and ovaries were obtained at different time intervals for the analysis.

2. Granulosa cell culture

Ovaries were isolated and incubated in Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F-12 containing 0.5 M sucrose and 10 mM ethylenediamine tetraacetic acid (EDTA) at 37℃ for 30 min. Granulosa cells of preovulatory follicles were collected by the method of follicular puncture using 23-gauge needles under a dissection microscope as previously described.[10] Viable and non-viable cells were counted using 0.5% trypan blue and cultured at a density of 1×10^6 cells/tube in 300 μl DMEM/Ham's F-12 supplemented with antibiotics and 0.1% bovine serum albumin. Hormones and chemicals were added at the beginning of culture, and cells were incubated at 37℃ in a humidified 95% O_2-5% CO_2 incubator.

3. Northern blot analysis

Total RNA from ovaries and cultured granulosa cells were isolated using Tri-Reagent solution (Molecular Research Center, Inc., Cincinnati, OH). Twenty micrograms of total RNA were fractionated by electrophoresis on 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, 1mM EDTA, 250 μg/ml denatures salmon sperm DNA, 500 μg/ml yeast transfer RNA, and a total of 1×10^7 cpm of a ^{32}P-labeled rat RGS-2 complementary DNA (cDNA) probe cloned by RT-PCR. After hybridization, membranes were washed twice for 5 min at room temperature in 2 × SSC and 0.1% sodium dodecyl sulfate-polyacryamide (SDS), followed by 5 min at 65℃ in 0.5 × SSC and

0.1% SDS. Membranes were then exposed using Kodak RX films (Eastman Kodak Co., Rochester, NY) for 1 day at -70℃. The signals were normalized to the 28S ribosomal RNA as an internal control.

4. *In situ* hybridization

Ovaries were fixed at 4℃ for 6 h in 4% paraformaldehyde in phosphate buffered saline (PBS), followed by immersion in 0.5 M sucrose in PBS overnight. Cryostat sections (14 μm thick) were mounted on poly-L-lysine (Sigma Chemical Co.)-coated microscope slides, fixed in 4% paraformaldehyde in PBS, and stored at -70℃ until analyzed. The hybridization procedure was essentially the same as previously described.[11] In brief, sections were pretreated serially with 0.2 M HCl, 2 × SSC, pronase E (0.125 μg/ml), 4% paraformaldehyde, and acetic anhydride in triethanolamine. Hybridization was carried out at 52~55℃ overnight in the mixture containing ^{35}S-labeled rat RGS-2 complementary RNA (cRNA) probe (2×10^8 cpm/ml), 50% formamide, 0.3 M NaCl, 10 mM Tris-HCl, 5 mM EDTA, 1 × Denhardt's solution, 10% dextran sulfate, 1 μg/ml carrier transfer RNA, and 10 mM dithiothreitol. Posthybridization washing was performed under stringent conditions that included ribonuclease A (25 μg/ml) treatment at 37℃ for 30 min and a final stringency of 0.1 × SSC. Slides were dipped into NTB-2 emulsion (Eastman Kodak Co.), exposed at 4℃ and developed after 2 weeks. The slides were stained with hematoxylin and eosin and examined under the light microscope with bright- and dark-field illumination.

5. Immunohistochemistry

Ovaries were dissected out, fixed in Bouin's solution and embedded in paraffin blocks. The sections (3 μm thick) were deparaffinized in Histoclear and rehydrated according to standard procedure. After blocking with rabbit serum, the sections were incubated with rabbit anti-RGS-2 antibody (1:100, Santa Cruz Biotechnology) for overnight at 4℃ and followed by biotinylated goat anti-rabbit IgG (Zymed, LAB-SA kit) for 30 min. The sections were then sequentially incubated with streptavidin peroxidase for 30 min. Finally, the sections were again rinsed in PBS and developed using the AEC substrate kit for approximately 5 min or until adequate signal was seen. Slides were then washed in distilled water, mounted with GVA mounting solution, and observed under light microscope with bright-field illumination.

6. Data analysis

Statistical differences were assessed by one-way

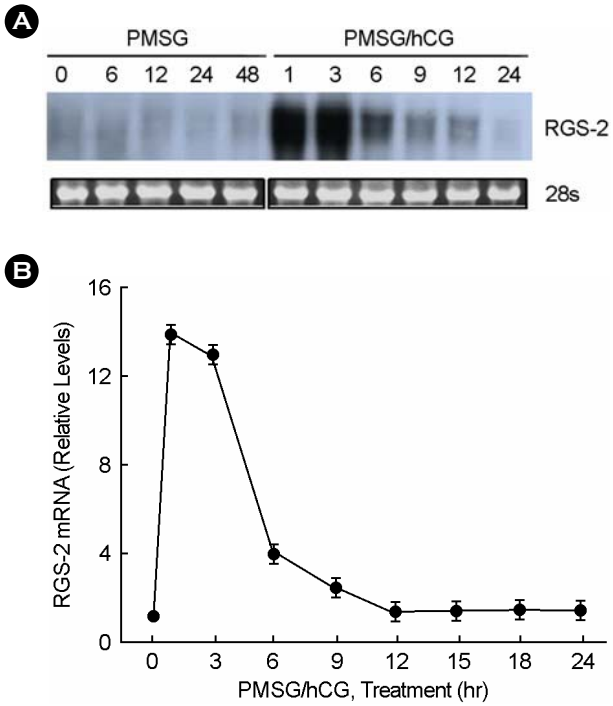

Figure 1. RGS-2 gene expression in the rat ovary after gonadotrophin stimulation. **A**, Twenty micrograms of ovarian total RNA were analyzed by Northern blotting using rat RGS-2 cDNA probe. Expression of 28S ribosomal RNA was used as an internal standard. **B**, Quantitative estimation of data in A. RGS-2 transcript was quantified using a phosphorinager and normalized for 28S RNA levels in each sample. Results are expressed relative to ovarian RGS-2 levels found before hCG treatment (0 h). Each data point represents the mean ± SEM from three independent experiments.

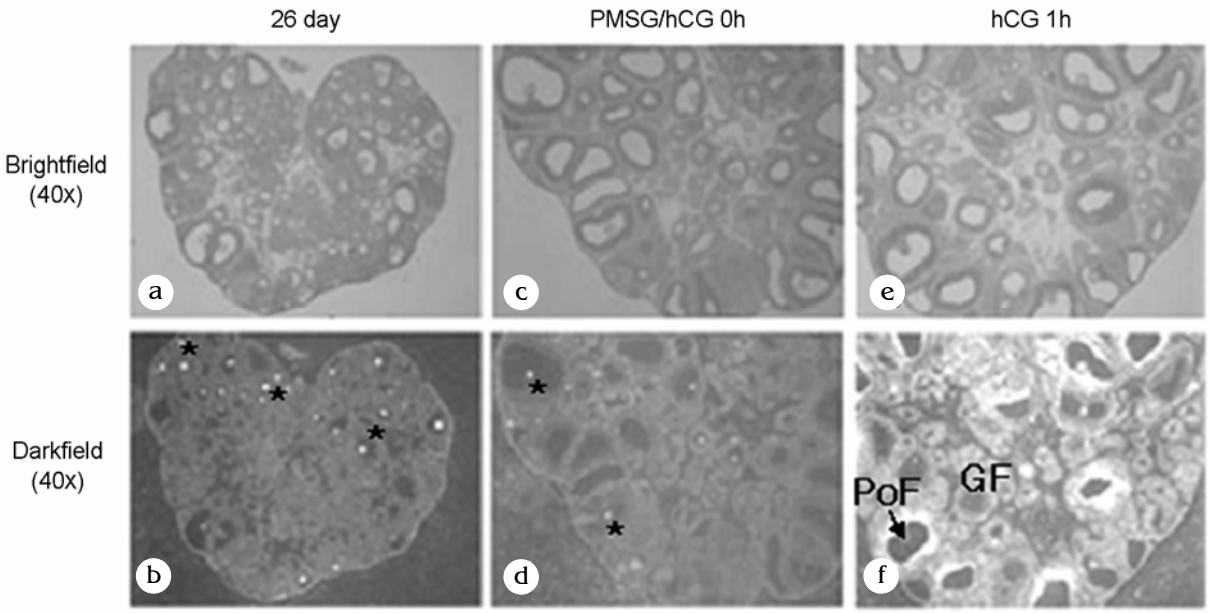

Figure 2. *In situ* localization of RGS-2 gene in the rat ovary after gonadotrophin stimulation. Ovarian sections were hybridized with ^{35}S-labeled rat RGS-2 cRNA probes. Note that RGS-2 expression in oocyte (asterisk) regardless of follicle size. Photomicrograph were taken under bright and darkfield illumination. GF, growing follicle; POF, preovulatory follicle.

analysis ofμvariance (ANOVA), followed by Student's *t* test; and $p<0.05$ was considered to be statistically significant.

RESULTS

1. Cell-type specific regulation of RGS-2 by LH/hCG *in vivo*

To study regulation of RGS-2 gene expression in the ovary, total RNA extracted from ovaries at different times after gonadotropin treatment was analyzed by Northern blotting. As shown in Figure 1A, PMSG treatment of 26-day-old immature rats did not affect RGS-2 mRNA levels. However, treatment of PMSG-primed rats with hCG resulted in an increase in RGS-2 expression within 1~3 h. The levels of RGS-2 mRNA declined to control levels by 6 h after treatment. Quantitative analysis revealed the 14-fold stimulation at 1 h (Figure 1B; $p<0.05$).

To determine the cell types expressing RGS-2 mRNA, antisense and sense cRNA probe for RGS-2 were generated for *in situ* hybridization analysis. RGS-2 mRNA was localized in oocytes of 26-day-old immature rat ovary (Figure 2, a and b). In ovaries of PMSG-primed immature rats, RGS-2 mRNA was also localized in oocytes (Figure 2, c and d). Interestingly, hCG treatment for 1 h caused the stimulation of RGS-2 gene expression in granulosa cells of both preovulatory and growing follicles (Figure 2, e and f). No specific signal was detected in ovaries hybridized with RGS-2 sense probe (data not shown).

Immunohistochemical study revealed that cell types expressing RGS-2 protein were theca cells regardless of hCG treatment (Figure 3). Granulosa cells of ovaries treated with hCG for 1 h were devoid of RGS-2 signal although the increased signal was observed in theca cells.

2. Regulation of RGS-2 expression by LH and GnRH in cultured preovulatory granulosa cells

To determine whether LH also stimulates RGS-2

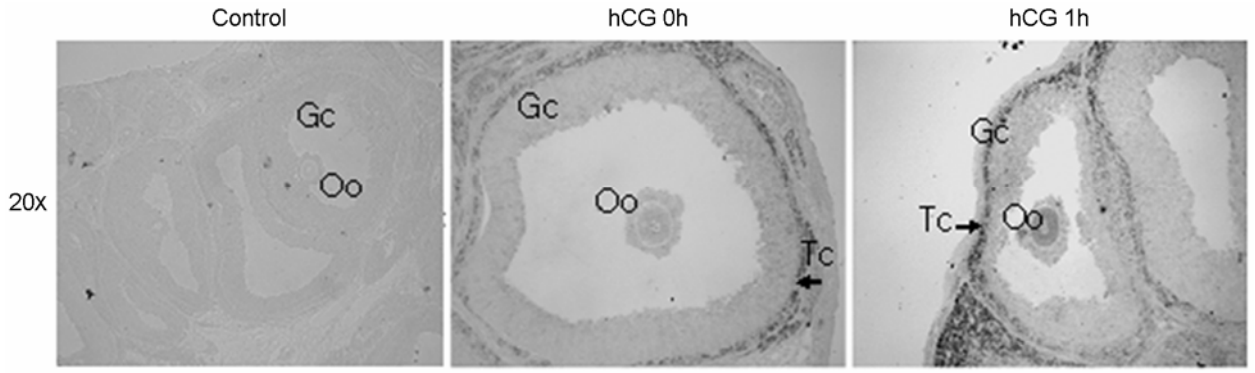

Figure 3. Immunohistochemical analysis of RGS-2 in hCG-stimulated ovary. Ovarian sections were incubated with rabbit anti-RGS-2 antibody followed by biotinylated goat anti-rabbit IgG. Oo, oocyte; GC, granulosa cell; TC, theca cell.

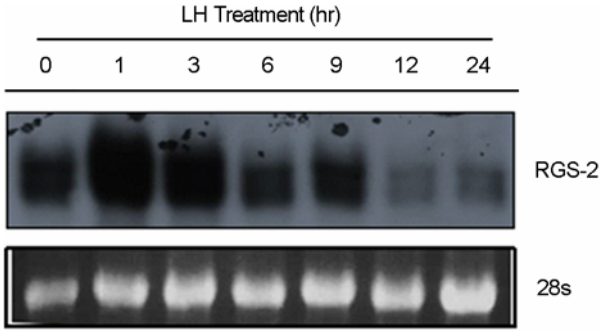

Figure 4. Stimulation of RGS-2 expression in granulosa cells cultured *in vitro*. Preovulatory granulosa cells collected from PMSG-treated ovaries were cultured in serum-free media under 5% CO_2-95% O_2 at 37℃ in the presence of LH (200 ng/ml). Twenty micrograms of total RNA were analyzed for RGS-2 mRNA levels by Northern blotting using a rat RGS-2 cDNA probe. Expression of 28S ribosomal RNA was used as an internal standard. Data are representative of three independent experiments.

mRNA expression *in vitro*, granulosa cells of preovulatory follicles obtained from ovarie treated with PMSG for 2 days were cultured in serum-free conditions. Similar to *in vivo*, LH treatment caused a rapid and transient stimulation of RGS-2 m RNA levels within 1 h (Figure 4). The increased levels were sustained until 3 h and declined to control levels 6 h after treatment. Because it is known that the action of GnRH mimicks that of LH,[12] the effect of GnRH on the RGS-2 expression was tested in cultured granulosa cells using GnRH agonist or antagonist. Treatment with either GnRH I or II agonist did not affect RGS-2 expression (Figure 5A). Interestingly, however, addition of GnRH II antagonist, but not GnRH I antagonist, further enhanced the stimulatory action of LH on RGS-2 expression (Figure 5B).

DISCUSSION

The present study was to investigate expression of regulator of G protein signaling 2 in the rat ovary during ovulation. Our results show that LH/hCG treatment resulted in the rapid and transient stimulation of RGS-2 gene expression in granulosa cells of preovulatory follicles. Ujioka et al.[13] also reported the transient stimulation of RGS-2 gene expression by LH/hCG in the same experimental system. However, the peak time for the RGS-2 expression was earlier in our study than in Ujioka et al.'s report (1 hr vs. 4 hr). In addtion, Ujioka et al. could not find the expression of RGS-2 in the oocytes. The reason for the differences could not be presumed at the moment. Furthermore, GnRH II antagonist also enhanced the action of LH on the stimulation of RGS-2 mRNA levels.

The present observations demonstrating the stimulation of RGS-2 by LH/hCG may imply its role in the

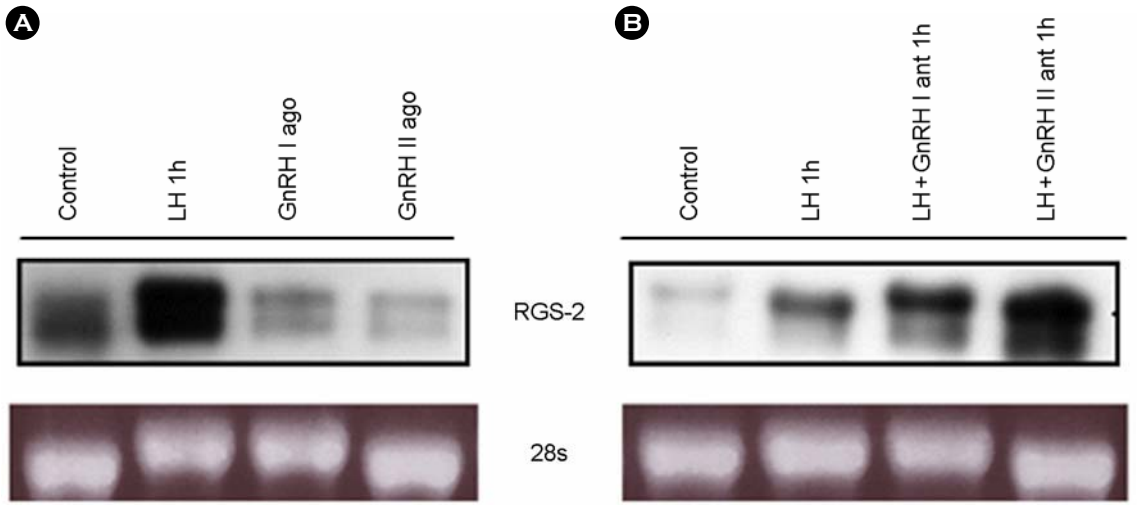

Figure 5. Effect of GnRH on RGS-2 mRNA expression in granulosa cells of preovulatory follicle. Granulosa cells of preovulatory follicles were cultured in serum-free conditions in the absence (control) or presence of GnRH-I or II agonist (10^{-7} M) and GnRH I (cetrolix) or II (trytorelix) (10^{-6} M) antagonist with or without LH (200 ng/ml) for 6 h. Total RNA was analyzed for RGS-2 mRNA levels by Northern blotting using a rat RGS-2 cDNA probe. The expression of 28S RNA was used as an internal standard. Data are representative of three independent experiments.

ovulatory process by regulating LH receptor signal pathways. RGS family proteins sharing a homologous 120~125 amino acid domain (sometimes referred to as the RGS box) react directly with the GTP-bound α subunit to facilitate GTPase activity and promote the hydrolysis of α-GTP to α-GDP and reformation of the αβγ-GDP trimer to attenuate signaling. RGS-2, selectively regulates Gq more than Gα-proteins such as Gs or Gi.5 Especially, Gq is activated by calcium-mobilizing hormones and stimulates phospholipase C-β to produce the interacellular messengers, inositol triphosphate and diacylglycerol. LH/hCG has been shown to stimulate phospholipase C, leading to inositol phosphate breakdown and the consequent production of inositol triphosphate and diacylglycerol.[8,9] GnRH receptors have been identified in granulosa and luteal cells in the ovary[13~15] and binding of GnRH to the receptor activates phospholipase C via Gq.[16,17] Threrfore, it is likely that the transient stimulation of RGS-2 in preovulatory granulosa cells may desensitize and thus culminate the LH or GnRH receptor mediated protein kinase C signals, resulting in the transition of granulosa cells from proliferation to differentiation into corpus luteum.

RGS-2 in the oocyte may play a role in oocyte development or/and maturation. Oocytes within mammalian ovarian follicles begin meiosis during embryogenesis but then arrest at prophase of meiosis I until luteinizing hormone acts on the follicle to cause meiosis to resume.[18] The maintenance of prophase arrest in oocytes within antral follicles requires the activity of signaling molecules within the oocyte. One of the most important molecules believed to be responsible for maintaining meiotic arrest is cAMP.[19] Recent report demonstrates that adenyyll cyclase 3 present in the oocyte is responsible for cAMP accumulation in the oocytes.[20] It is demonstrated that, in addition to Gq protein, RGS-2 inhibits the activity of several adenylyl cyclase isoforms and regulate cAMP accumulation.[21] Our result showing that RGS-2 expression in the oocytes may therefore regulate cAMP by direct interaction with adenylyl cyclase and thus may play a role in the oocyte development or/and maturation. However, protein expression of RGS-2 in the oocyte could not be observed in the present study. We can not speculate the reason for

the absence of RGS-2 protein expression in the oocytes; RGS-2 gene in the oocytes may be a masked mRNA, or the sensitivity of immunocytochemistry assay used in this study may not be suitable to detect oocyte protein expression of RGS-2.

GnRH antagonizes the action of FSH that promotes follicle development, and it has been suggested that GnRH may act primarily as an atretogenic factor for the majority of follicles that do not ovulate.[22] Support for this concept comes from studies showing that GnRH directly promotes apoptotic cell death in the ovary.[23] By contrast, however, GnRH exerts its positive action in preovulatory follicles. GnRH has been shown to cause follicle rupture and oocyte maturation[24,25] and to induce *in vitro* luteinization of preovulatory granulosa cells.[26] Stimulation of RGS-2 expression by GnRH-II antagonist implies that the positive action of GnRH may be through the activation of RGS-2. The presence of specific GnRH-II receptors in the ovarian cells remains to be elucidated.

In summary, the present study shows cell-type dependent activation of RGS-2 by LH in preovulatory follicles. Further studies are needed to identify RGS-2 interacting protein(s) during ovulation and to clarify the function of RGS-2 in the oocyte during the follicle development.

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= **Abstract** =

Objective: The purpose of the present study was to examine the hormonal regulation of RGS-2 in the rat ovary.
Methods: Immature rats were injected with 10 IU of PMSG to induce multiple growth of preovulatory follicles and 10 IU of hCG to induce ovulation. Northern blot analysis performed for gene expression and in situ hybridization performed for mRNA localization.
Results: Northern blot analysis revealed that pregnant mare's serum gonadotropin (PMSG) treatment did not affect RGS-2 mRNA levels. In contrast, human chorionic gonadotropin (hCG) treatment of PMSG-primed rats resulted in an increase in RGS-2 expression within 1~3 h. The major cell-types expressing RGS-2 mRNA were oocytes regardless of follicle size. Interestingly, hCG treatment caused the stimulation of RGS-2 gene expression in granulosa cells of preovulatory and growing follicles. In contrast, cell types expressing RGS-2 protein were theca cells regardless of hCG treatment. Like *in vivo*, treatment of preovulatory granulosa cells with LH *in vitro* stimulated RGS-2 levels within 1 h. Interestingly, GnRH antagonist II enhanced the stimulatory action of LH.
Conclusion: The present study demonstrates the LH/hCG induction of RGS-2 in preovulatory granulosa cells and suggests a role of RGS-2 in Gq protein signaling pathway during ovulation.

Key Words: RGS-2, Ovulation, Preovulatory follicle, Gene expression