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Growth-Regulated α Expression in Human Preovulatory Follicles and Ovarian Cells

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PROBLEM: Around the time of ovulation the number of neutrophils increases in the theca of the leading follicle. We hypothesized that growth-regulated α (GROα), a neutrophil chemoattractant/activating factor, may be a modulator of periovulatory neutrophil chemotaxis.
METHOD: GROα levels were measured in follicular fluids (n = 61). Granulosa-lutein and ovarian stromal cells were also cultured. After experimental paradigms, GROα mRNA was evaluated by Northern analysis, GROα in follicular fluids, and culture supernatants were quantified using ELISA.
RESULTS: In follicular fluids the mean pre-human chorionic gonadotropin (hCG) GROα level was 51 ± 24 (±SEM) pg/ml, post-hCG it was 210 ± 20 pg/ml ($P = 0.04$). GROα was produced constitutively by ovarian stromal and granulosa-lutein cells. Interleukin-α (IL-1α) and tumor necrosis factor-α (TNF-α) further stimulated GROα production. Treatment of stromal cells with hCG also stimulated GROα production.
CONCLUSION: GROα is a constituent of periovulatory follicular fluid. Ovarian stromal and granulosa-lutein cells express the GROα mRNA and produce the protein. The regulation of GROα by cytokines and hCG suggests that GROα may play a role in the process of ovulation.

Key words:
Chemokines, cytokines, follicular fluid, GROα, human ovary, ovulation

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INTRODUCTION

Ovulation is a complex process involving not only gonadotropins and steroid hormones but also mediators common to inflammatory reactions, including cytokines, prostaglandins, plasminogen activators, histamine, and bradykinin.[1] It is becoming increasingly evident that immune-ovarian cell interactions are essential components of the ovarian cycle including ovulation.[2] Indeed, the number and type of leukocytes in the human ovary varies in a predictable manner throughout the ovulatory cycle, and a preovulatory increase in the neutrophil density has been observed in the theca concomitant with the luteinizing hormone (LH) surge.[3] Once in and around the mature follicle, neutrophils may play a crucial role in timely follicular rupture by secreting specific proteolytic enzymes, prostaglandins, and other paracrine factors. Leukocytes (especially neutrophils and monocytes/macrophages) have been shown to enhance the LH-induced ovulation rate of in vitro-perfused rat ovaries,[4] and their depletion from the peripheral blood is associated with a decreased ovulation rate.[5]

Although it has been shown that human follicular fluid contains chemotactic activity toward neutrophils,[6,7] specific chemotactic factors responsible for the recruit-

ment and activation of neutrophils in and around the periovulatory follicles are not fully identified. Several structurally related peptides capable of inducing neutrophil chemotaxis have been described.[8] Recently, we have identified interleukin-8 (IL-8), a proinflammatory chemoattractant cytokine for neutrophils, in the follicular fluid of women undergoing ovulation stimulation.[9] Another candidate, reported to be 10-fold more potent than IL-8 as a neutrophil chemoattractant, is growth-regulated α (GROα).[10] GROα was originally described as "melanocyte growth stimulating activity," a factor capable of stimulating the growth of human melanoma cells in culture, and later it was found to possess chemotactic and activating properties toward neutrophils.[11] GROα gene expression has been demonstrated in fibroblasts, endothelial cells, and activated neutrophils and monocytes.[8,12] Two other subtypes of the GRO family, GROβ and GROγ, share, respectively, 90 and 86% of their nucleotide sequences with GROα. All three gene products have similar neutrophil-activating properties.[13]

We postulated that GROα may be an important modulator of periovulatory events by attracting and activating neutrophils that would play a role in timely follicular rupture. In the present study, we have investigated GROα concentrations in human follicular fluid before and after human chorionic gonadotropin (hCG) administration. Using ovarian stromal and granulosa-lutein cells in culture we also evaluated the regulation of GROα gene expression by cytokines and hormones.

MATERIAL AND METHODS

Follicular Fluid and Tissue Collection

Follicular fluids and cells for granulosa-lutein cell culture were obtained from 24–43-year-old women (n = 57; 61 cycles) undergoing in vitro fertilization and embryo transfer (IVF-ET) therapy at the Yale University IVF Program. Ovarian tissue was obtained from women of reproductive age undergoing hysterectomy with oophorectomy for reasons other than ovarian disease. Written informed consent was obtained from each woman before the procedure; consent forms and protocols were approved by the Human Investigation Committee of this university. A standard IVF protocol described previously was used.[14] After removal of the cumulus-oocyte complexes, follicular fluids were centrifuged at 600*g* for 20 min and aliquots of cell-free supernatants were stored at –80°C until assayed. Cell pellets were used for culture of granulosa-lutein cells. The four samples of follicular fluid collected before hCG administration were obtained from King Faisal Specialist Hospital and Research Center, Riyadh, Kingdom of Saudi Arabia and were shipped on dry ice and stored at –80°C until assayed together with other samples. Consent forms and protocols were approved by the Human Investigation Committee of that institution. These samples were obtained from women undergoing controlled ovulation stimulation with gonadotropin-releasing hormone (GnRH) agonist and human menopausal gonadotropin (hMG) for timed intrauterine insemination who underwent ultrasound guided follicular reduction (of follicles 16 mm or larger in diameter) just before hCG administration to prevent ovarian hyperstimulation syndrome and/or multiple gestation.

Blood samples were collected from six women on the day of oocyte retrieval. The blood was centrifuged to separate serum and was stored at –80°C until assayed.

Isolation and Culture of Ovarian Stromal and Granulosa-Lutein Cells

Ovarian stromal cells and granulosa-lutein cells were cultured as described previously[9] and were plated in Ham F12/Dulbecco's minimal essential medium (DMEM) (1:1, v/v) that contained antibiotics-antimycotics (1%, v/v) and fetal bovine serum (FBS) (10%, v/v). Cells were plated in culture dishes (35 or 100 mm diameter, or 24-well plates), as appropriate for the experimental design, and were allowed to replicate to confluence. Experiments were commenced 1–3 days after confluence was attained. Confluent stromal cells were treated with serum-free media for 24 hr before treatment with test agents was initiated. Granulosa-lutein cells were incubated for 1–6 days at 37°C in a humidified atmosphere of 5% CO_2 in air with daily replacement of medium. In some experiments, granulosa-lutein cells were treated with the monoclonal anti-CD45 antibody coupled with magnetic immunobeads (Amac, Westbrook, ME) to remove white blood cells.

At the end of each experiment, the culture media were collected and frozen at –80°C for quantification of GROα by enzyme-linked immunosorbent assay (ELISA). Cells were used for quantification of total protein or for isolation of RNA. Similar experiments were conducted on at least three different occasions.

Immunocytochemical analyses of granulosa-lutein and ovarian stromal cells were conducted using factor VIII as a marker of endothelial cells, low molecular weight cytokeratin as a marker of epithelial cells, vimentin as a marker of mesenchymal cells, aromatase as a marker of estrogen-producing cells, and HAM 56 as a marker of monocyte/macrophages (specific for CD56). In the granulosa-lutein cell cultures, CD56-marker-positive cells, endothelial cells, and cytokeratin-positive cells accounted for 6–9, 1–2, and 0%, respectively. Nearly all the cells (95 to 100%) were positive for vimentin. CD56-marker-positive cells represented less than 1% after treatment with anti-CD45 immunobeads. In confluent ovarian stromal cell cultures after first passage, CD56-marker-positive cells, endothelial cells, and cytokeratin-positive cells accounted for 2–4, 1–2, and 5–7%, respectively. Nearly all the cells (95–100%) were positive for vimentin. Approximately 10–15% of the cells were positive for aromatase. These data were compiled from preparations of cells from three different samples.

Preparation of Total RNA and Northern Analysis

Total RNA from cells in culture was extracted using Trizol (GIBCO BRL, Life Technologies, Grand Island, NY).[15] Hybridizations were conducted for 16 hr at 65°C in a buffer that contained GROα-specific oligonucleotide probe (5′-GGC ATG TTG CAG GTC CCT CA-3′) end-labeled with [γ-^{32}P]ATP; the sequence of this probe corresponds to the portion between bases 714 and 695 of the GROα gene.[16] Thereafter, the blots were washed with 6× standard saline citrate (SSC) and sodium dodecyl sulfate (SDS; 0.1%, w/v) for 15 min at room temperature, once with 2× SSC and SDS (0.1%, w/v) for 15 min at room temperature, and once for 20 min at 65°C. Autoradiography of the membranes was performed at –70°C using Kodak (Rochester, NY) X-Omat AR film. The amount of RNA in each lane was normalized by the analysis of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA, using a cDNA probe (Clontech Laboratories, Palo Alto, CA) radiolabeled with [α-^{32}P]dCTP by random hexamer priming. The autoradiographic bands were quantified by using a laser densitometer (Molecular Dynamics, Sunnyvale, CA). GROα in each band was normalized by using the value for the corresponding G3PDH mRNA, thus correcting for any variation in amounts of RNA applied to each lane. Similar experiments were conducted on three different occasions using cells obtained from three different patients.

GROα Immunoassay

Immunoreactive GROα in follicular fluid samples as well as culture supernatant from ovarian stromal and granulosa-lutein cells were quantified using an ELISA (R&D Systems, Minneapolis, MN). According to the manufacturer, there is no measurable cross-reactivity with other known cytokines in this assay. The sensitivity for GROα was 5 pg/ml in the sample. All follicular fluid samples were evaluated in a duplicate assay. Validation of its use for human follicular fluid was also performed: Recombinant GROα was diluted in an assay buffer and pooled follicular fluid, and parallelism was observed between the standard curve of the buffer and follicular fluid dilutions. Each experiment was done using three replicate wells for each condition, and supernatant from each well is tested in a single ELISA assay. Each experimental setup was repeated on at least three occasions using cells obtained from three different patients. The intraassay and interassay coefficients of variation were 4.4 and 8.1%, respectively.

Statistical Analyses

Because the levels of GROα in the follicular fluid were not normally distributed, they were analyzed with nonparametric analysis of variance by ranks (Kruskal-Wallis test) and within group differences by post hoc analysis using the Mann-Whitney test. Data from ELISA assays of cell culture supernatants were normally distributed and evaluated by analysis of variance with Bonferroni correction for multiple comparisons. Correlation analysis by ranks was calculated with the Pearson or Spearman correlation coefficient. P value < 0.05 was considered significant. The statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS) Version 6.0 for Windows (SPSS Inc., Chicago, IL).

Reagents

Culture media, antibiotics-antimycotics, estradiol, progesterone, human recombinant hCG, and FBS were from Sigma Chemical Co. (St. Louis, MO). IL-1α, tumor necrosis factor-α (TNF-α) (both recombinant) were from the R&D Systems. Human LH and follicle-stimulating hormone (FSH) were obtained from the National Hormone and Pituitary Program, NIH.

RESULTS

Immunoreactive GROα in Follicular Fluid

The mean concentration of GROα in follicular fluid samples obtained from women (n = 4) before the administration of hCG and before any detectable LH surge was 51 ± 24 pg/ml (±SEM). In follicular fluid samples obtained from women (n = 57) 34 hr after the hCG administration, the concentration of GROα was 210 ± 20 pg/ml, which was significantly higher than pre-hCG levels (P = 0.039) (Fig. 1). When we measured GROα levels in individual follicles according to the size of the follicle (between 15–25 mm diameter) from 6 women 34 hr after the hCG administration, the mean concentration of GROα from these follicles did not differ significantly.

In six women from whom blood samples were taken on the day of follicular aspiration we observed substantially higher (mean: 5.1-fold) follicular fluid GROα levels (202 ± 26 pg/ml) than serum GROα levels (40 ± 8 pg/ml) (P = 0.001).

Cytokine Modulation of GROα Production and mRNA Expression in Ovarian Stromal and Granulosa-Lutein Cells

Ovarian stromal cells secreted GROα (840 ± 190 pg/mg of total protein by 8 hr). Treatment of ovarian stromal cells with TNF-α (10 ng/ml) resulted in increased accumulation of GROα in the media (2,019 ± 120 pg/mg of total protein by 8 hr) (P = 0.001). Similar but to a lesser degree, increases in GROα levels were observed after treatment with IL-1α (0.1 and 1 U/ml) and lower concentrations of TNF-α (0.1 and 1 ng/ml) by 8 hr (data not shown). The increase of GROα levels was evident by 1 h, and high levels of GROα were present by 4 to 6 hr after treatment of TNF-α (10 ng/ml) (Fig. 2). The level of GROα mRNA was low but detectable in stromal cells maintained in serum-free medium. The GROα mRNA level increased markedly in response to IL-1α (10 U/ml) and TNF-α (10 ng/ml)

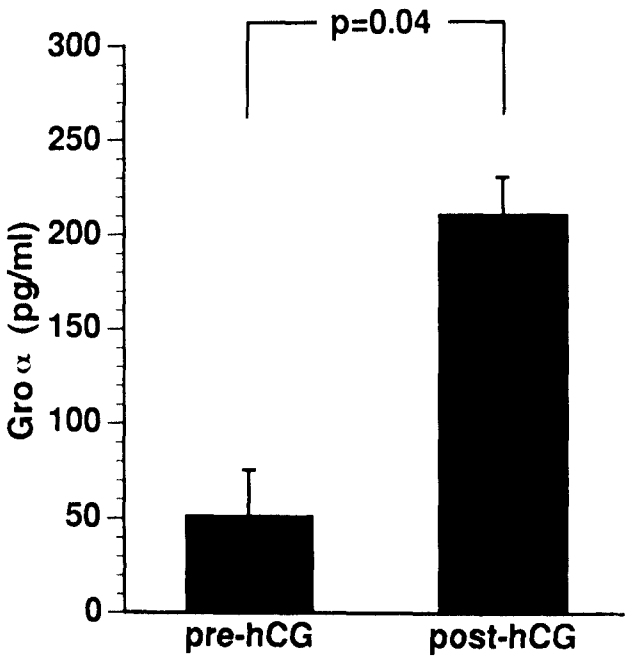

Fig. 1. Immunoreactive GROα concentrations in follicular fluid obtained before (n = 4) and 34 hr after (n = 57) hCG administration. Values are the mean ± SEM.

treatment alone or in combination (Fig. 3). This increase was evident by 1 hr, and high levels of GROα mRNA were present by 4 to 6 hr after commencement of either treatment, after which they began to decrease (Fig. 4). The increase in the levels of GROα mRNA in stromal cells was dependent upon the concentration of IL-1α (0.01–100 U/ml) (Fig. 5) or TNF-α (.01 to 10 ng/ml).

In granulosa-lutein cell cultures performed after purification with anti-CD45 to eliminate leukocytes, granulosa-lutein cells secreted GROα (22 ± 4 pg/ml) into the culture medium. Granulosa-lutein cells treated with IL-1α (10 U/ml) and TNF-α (10 ng/ml) produced higher level of GROα than untreated cells by 24 hr (71 ± 14 pg/ml and 47 ± 2 pg/ml, respectively; $P = 0.001$ and $P = 0.03$, respectively).

Hormone Modulation of GROα Production in Ovarian Stromal Cells

The treatment of ovarian stromal cells with hCG (1 to 100 mIU/ml) for 8 hr led to increases of GROα protein secretion into the media that was not concentration-dependent (Fig. 6). Treatment of stromal cells with FSH, estrogen, and progesterone did not affect GROα protein production (data not shown).

DISCUSSION

The importance of the role of leukocytes in ovulation has been established.[4] The observation that there is an infiltration of granulocytes into the area surrounding the leading follicle in rat[17] and human[3] ovaries just before ovulation suggests a role for these leukocytes in periovulatory events. Although the regulation of the traffic of granulocytes in and around the periovulatory follicles is not clearly understood, the temporal and spatial distribution would point to a hormonally dependent event.

In the human, preovulatory follicular fluid has been shown to contain neutrophil chemotactic activity[6,7] that is higher in conceptual cycles than in non-conceptual cycles. Possible candidates for this activity are chemokines. They are a recently described family of inflammatory proteins related on the basis of their primary structure, notably the conservation of a four-cysteine residue motif.[10] Two chemokine families exist: The C-C family, in which the first two cysteine residue are adjacent to one another, and the C-X-C family, in which there is an intervening amino acid between the first two cysteine residues. The C-X-C family, which includes IL-8, GROα, neutrophil-activating protein 2, platelet factor-4, and γ-interferon inducible protein-10, is predominantly chemotactic for neutrophils. We have recently shown that one member of this family, IL-8, is found in the follicular fluid of women undergoing ovulation stimulation.[9]

GROα, a chemokine of 73 amino acid residues, was originally described as a mitogen for human melanoma cells (termed melanoma growth stimulatory activity) and subsequently was found to have proinflammatory activity leading to neutrophil recruitment and activation.[18,19] GROα is produced by a variety of cells such as monocytes, endothelial cells, fibroblasts, and synovial cells, and its synthesis can be induced by a variety of inflammatory

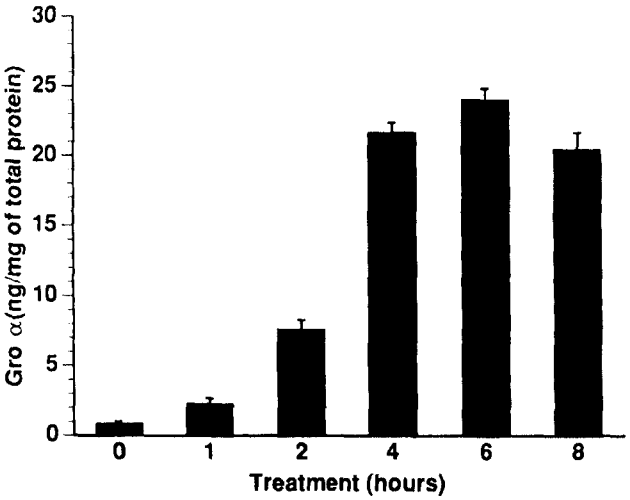

Fig. 2. Immunoreactive GROα concentrations in supernatants of ovarian stromal cell cultures treated with TNF-α. Confluent ovarian stromal cells were incubated with TNF-α (10 ng/ml) for 0 to 8 hr. The culture media were collected, and GROα was quantified by ELISA. Data are the mean ± SEM for three replicates.

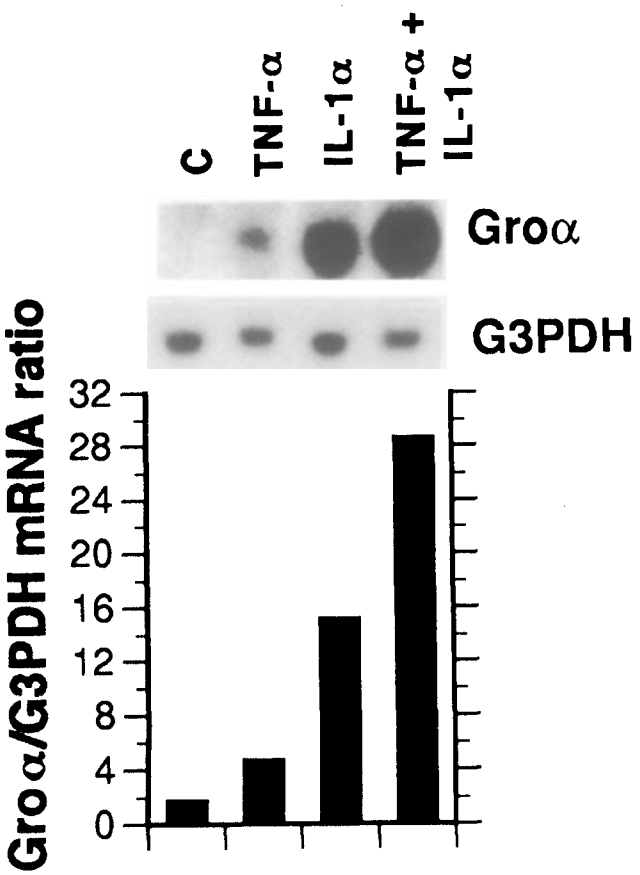

Fig. 3. Northern analysis of GROα mRNA in ovarian stromal cells treated with IL-1α and TNF-α. Confluent ovarian stromal cells were incubated for 8 hr with TNF-α (10 ng/ml), IL-1α (10 U/ml), or both. Total RNA (10 μg per lane) was evaluated. C denotes control.

mediators, including TNF-α and IL-1.[20] IL-8 and GROα exert their biological activities by binding to specific cell receptors. The type A IL-8 receptor binds IL-8 with high affinity, but GROα with low affinity, whereas the type B IL-8 receptor binds both IL-8 and GROα with high affinity. Hammond et al.[21] used antibodies to assess the role of each receptor in the chemotactic response of neutrophils to GROα and found that GROα stimulates chemotaxis exclusively through the type B receptor.

We postulated that GROα may be involved in periovulatory events attracting and activating neutrophils that would play a role in timely follicular rupture. If GROα is truly involved in the periovulatory events, the concentration of GROα should increase in follicular fluids obtained after the LH surge (or after the administration of hCG) when compared to presurge follicular fluids. This is precisely the finding of this study: GROα is found in greater quantities in follicles after the hCG administration.

If GROα is a participant in the chemoattraction of granulocytes in and around the periovulatory follicle, then where does this cytokine originate? Peripheral blood contamination is one potential source, but the GROα concentrations observed in follicular fluid are 5-fold higher than in serum. Therefore, the markedly elevated GROα concentration should reflect the true follicular fluid level and not contamination from peripheral blood. Although granulosa-lutein cells predominate in follicular fluid, resident macrophages and monocytes comprise 5–15% of human follicular tissue cells,[22] and these cells are a known source of GROα.[8] We found that after purification of granulosa-lutein cells by the use of anti-CD45 immunobeads the level of GROα production decreased but was still present. Thus, both granulosa cells and macrophages may contribute to the follicular fluid GROα pool. Finally, another potential source is the theca cell from the ovarian stroma. We found that cultured ovarian stromal cells express GROα mRNA and secrete GROα protein. Thus, sources of GROα are abundant in and around the follicle.

Another question we asked was how GROα secretion might be regulated. This study presents evidence that the expression of this chemoattractant cytokine from ovarian stromal and granulosa-lutein cells is modulated by other cytokines such as IL-1 and TNF-α. An intraovarian IL-1 system complete with ligands, receptors, and a receptor antagonist in humans is now well established.[23] In addition, human follicular fluid was reported to contain considerable IL-1 activity.[24] TNF-α also is found in the preovulatory follicular fluid at concentrations used in the

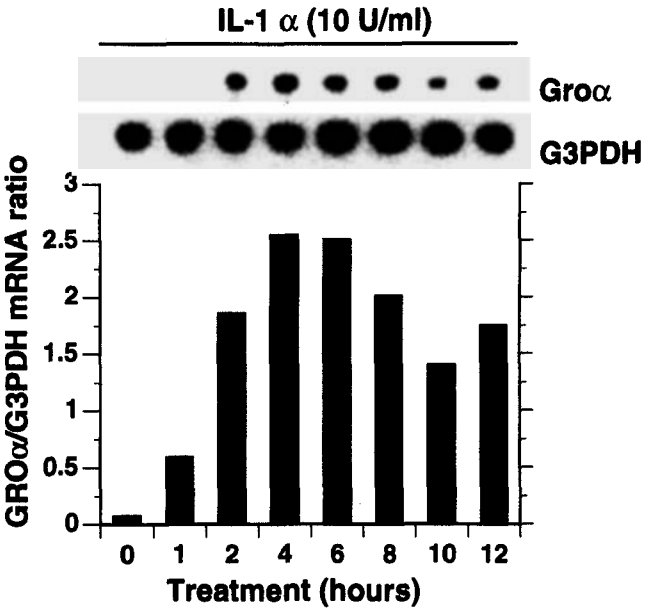

Fig. 4. IL-1α-mediated increase in GROα mRNA levels. Confluent ovarian stromal cells were placed in serum-free medium 24 hr before incubation for 6 hr in a culture medium containing IL-1α (10 U/ml) for 0 to 12 hr. GROα mRNA was evaluated by Northern analysis of total RNA (10 μg per lane).

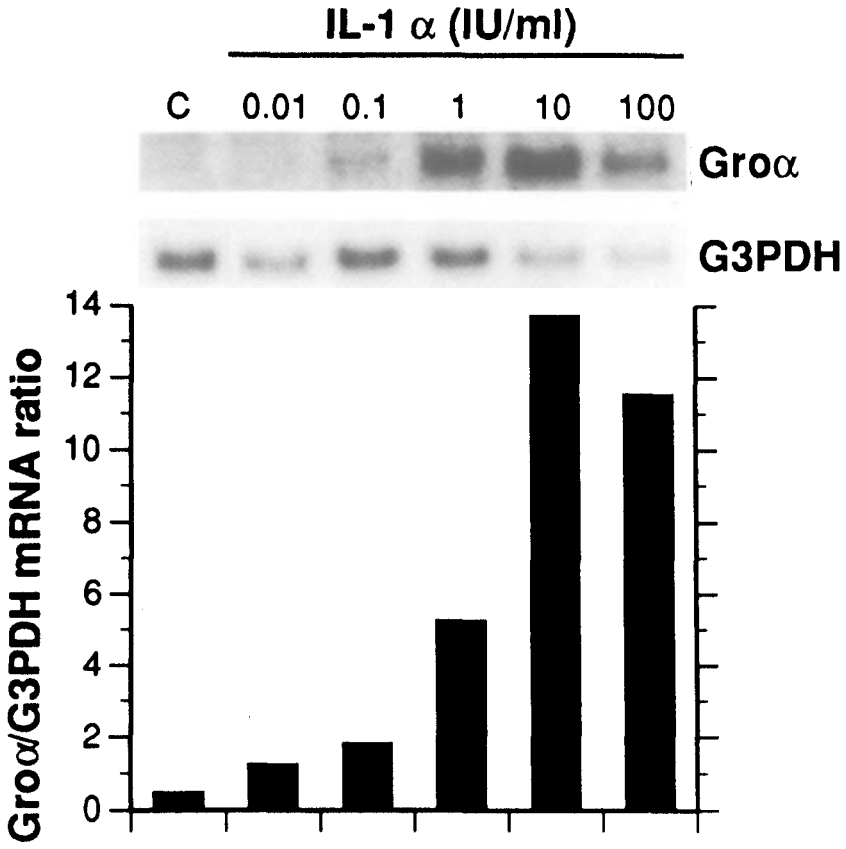

Fig. 5. Induction of GROα mRNA in ovarian stromal cells by IL-1α: Dose response. Confluent ovarian stromal cells were placed in serum-free medium 24 hr before incubation for 6 hr in a culture medium containing various concentrations of IL-1α (.01–100 U/ml). At the end of the incubation period, total RNA was prepared from the cells. GROα mRNA was evaluated by Northern analysis of total RNA (10 μg per lane).

present study.[25] This study presents evidence that the expression of GROα from ovarian stromal and granulosa-lutein cells is modulated by other cytokines such as IL-1 and TNF-α. Thus, both cytokines may play some role in the constitutive secretion of GROα as well as in stimulating enhanced production and secretion.

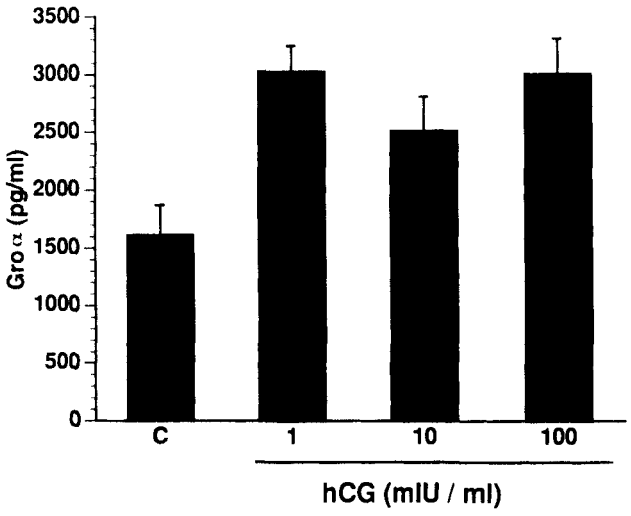

Fig. 6. hCG stimulation of immunoreactive GROα production by ovarian stromal cells in culture. Confluent ovarian stromal cells were treated with serum-free medium alone (C, control) or containing hCG for 8 hr. The culture media were collected, and GROα was quantified by ELISA. Data are the mean ± SEM for four replicates.

As our hypothesis is that granulocyte migration in and around the periovulatory follicle is hormonally regulated and that regulation occurs through modulation of the expression of GROα, we also investigated the effect of hormones on the expression of GROα. We have found that hCG stimulates GROα protein production from ovarian stromal cells and granulosa-lutein cells within 6 to 8 hr. This finding is consistent with existing ovarian physiology: Granulocytes should arrive in and around the preovulatory follicle in response to the LH surge to release their secretory products such as histamine, bradykinin, prostaglandins, serotonin, and cytokines, all of which have a well-documented role in the biochemistry of ovulation.[26]

In summary, we find that GROα levels are elevated in periovulatory follicular fluid and that ovarian stromal cells express the mRNA and produce the protein. Modulation of GROα in these cell cultures by steroid and trophic hormones suggests that GROα may play an important role in the physiology of ovulation, such as in aiding follicular rupture.

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