Tumor necrosis factor-α activates nuclear factor-κB but does not regulate progesterone production in cultured human granulosa luteal cells

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Abstract

Background. The role of tumor necrosis factor-α (TNF-α) in granulosa luteal cell function and steroidogenesis is still controversial. Our aim was to examine the steroidogenic response, together with the simultaneous expression and activation of nuclear factor-κB (NF-κB), in cultured human granulosa luteal cells (GLCs) following administration of TNF-α.

Materials and methods. This prospective controlled study was conducted in the Human Reproduction Division at the Institute of Maternal and Child Research, Faculty of Medicine, University of Chile and the San Borja Arriarán Hospital, National Health Service, Santiago, Chile. GLCs were obtained from aspirates of follicles from women undergoing *in vitro* fertilization (IVF). Thirty-two women undergoing IVF for tubal-factor and/or male-factor infertility participated in this study. Protein levels of NF-κB, the NF-κB inhibitor IκBα and steroidogenic acute regulatory protein (StAR) were determined by Western blot and localization of NF-κB was studied by indirect immunofluorescence. Progesterone production was determined by radioimmunoassay.

Results. TNF-α did not affect the expression of StAR protein or the synthesis of progesterone. NF-κB was expressed in the GLCs and activated by TNF-α, resulting in degradation of IκBα and mobilization of the p65 NF-κB subunit into the nucleus.

Conclusions. These results indicate that TNF-α did not modulate steroidogenesis in cultured human GLCs. However, NF-κB was activated by TNF-α. Therefore the activation of NF-κB via the TNF-α pathway is likely associated with other preovulatory granulosa cell processes important for human ovarian function.

Keywords: *Steroidogenesis, granulosa luteal cells, TNF-α, NF-κB, IκBα*

Introduction

Cholesterol is a key precursor for the ovarian synthesis of estrogens, androgens and progesterone [1]. The rate-limiting step in steroidogenesis is the translocation of cholesterol from the outer to the inner mitochondrial membrane. The sterol movement is governed by the steroidogenic acute regulatory protein (StAR), a phosphoprotein expressed primarily in steroidogenic cells [2–4]. In humans, StAR is expressed in the ovary, testis, adrenal cortex and brain [4]. Intracellular or extracellular signals such as cyclic AMP [1,5], gonadotropins and growth factors [5,6], prostaglandins and cytokines [7,8] regulate StAR expression in the steroidogenic cells of many species.

The cytokine tumor necrosis factor-α (TNF-α) regulates numerous cellular process including immune and inflammatory responses, differentiation, proliferation and cell death [9]. A number of studies suggest that TNF-α modulates several biological processes in the mammalian ovary including granulosa cell proliferation [10], follicular development, ovulation and luteolysis [11], steroidogenesis [12–15], and prostaglandin and proteoglycan biosynthesis [16,17]. TNF-α and its specific receptors have been identified in the ovarian cells of many mammals [18].

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Moreover, variations in peripheral blood levels of immunoreactive TNF-α throughout the menstrual cycle and secretion of TNF-α from the human corpus luteum have been described [19]. These data suggest that ovarian cells are a site of TNF-α expression and action. However, *in vitro* studies have shown that TNF-α stimulates [20,21], inhibits [22–24] or does not affect [25] steroid hormone secretion in mammal ovarian cells.

Most of the effects of TNF-α are mediated by the transcription factor nuclear factor-κB (NF-κB) [26]. There are five members of the NF-κB family: Rel A (p65), c-Rel, Rel B, NF-κB1 (p105/p50) and NF-κB2 (p100/p52) [27]. NF-κB/Rel proteins exist as homo- or heterodimers and possess a conserved Rel homology domain that mediates dimerization as well as binding to DNA [28]. In most resting cells, NF-κB is bound to the cytoplasmic inhibitory proteins IκB, and remains in the cytoplasm as a latent transcription factor. Upon stimulation, the IκB kinase complex is activated and in turn phosphorylates IκBα and IκBβ proteins [28]. Phosphorylation triggers ubiquitin-dependent degradation of IκB proteins by the 26S proteosome, resulting in the release of NF-κB [27,28]. Subsequently, NF-κB translocates into the nucleus and activates transcription of specific target genes. Although NF-κB is expressed in the human ovary [29], its role is not well understood. Vaskivuo and colleagues have shown that NF-κB is important in human corpus luteum regression [29] and Fujii and associates suggested that NF-κB mediates the action of interleukin (IL)-1β in normal human ovarian cells and a granulosa tumor cell line [30]. There are controversial reports on the role of TNF-α in granulosa luteal cell function and steroidogenesis. Therefore, our aim in the present study was to investigate whether TNF-α regulates steroidogenesis and activates NF-κB in cultured human granulosa luteal cells (GLCs).

Materials and methods

Aspiration of granulosa cells of follicular fluid

Primary cultures of human GLCs were prepared from cells collected from patients undergoing oocyte retrieval following standard follicular hyperstimulation, due to tubal- or male-factor infertility, in the Department of Obstetrics and Gynecology, Human Reproduction Division at the Institute of Maternal and Child Research, Faculty of Medicine, University of Chile and the San Borja Arriarán Hospital, National Health Service, Santiago, Chile. Patients received a gonadotropin-releasing hormone agonist, leuprolide acetate (Lupron®; Abbot Laboratories, Abbot Park, Illinois, USA), for pituitary suppression and recombinant follicle-stimulating hormone (FSH) (Puregon®; NV Organon Oss, The Netherlands) and human menopausal gonadotropin (HMG Menogon®; Ferring, Kiel, Germany) for follicular recruitment, followed by a single dose of 10 000 IU human chorionic gonadotropin (hCG) (Pregnyl®; Organon Lab) 36 h before oocyte retrieval.

Granulosa luteal cells isolation

Granulosa cells from individual patients were isolated from aspirated follicular fluid after ovum pick-up as previously reported [5]. The follicular fluid was centrifuged at 400***g*** for 5 min, and the cell pellets were dispersed with 1 mM ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid (EDTA) in phosphate-buffered saline (PBS) for 10 min and centrifuged at 400***g*** for 5 min. The cell pellets were re-suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with $NaHCO_3$ 1.2 g/l, penicillin 100 IU/ml, streptomycin 100 μg/ml and fungizone 25 ng/l. The contaminating red blood cells were removed using a 50% Ficoll–Histopaque 1077 (Sigma Chemical Co., St. Louis, MO, USA) column and centrifuged at 400***g*** for 30 min. The GLCs were colleted by aspiration at the interface and re-suspended in basic medium. The cell pellet was centrifuged at 400***g*** for 5 min and re-suspended in growth medium consisting of DMEM supplemented with 10% fetal bovine serum, 20 nM insulin, 20 nM selenium and 1 μM vitamin E, and the cells were plated in 60-cm^2 dishes for 20 min at 37°C. This procedure removed macrophages (adherent cells), and was followed by aspiration of the non-adherent cells, centrifugation at 400***g*** for 5 min at room temperature and re-suspension of the pellet in growth medium.

Granulosa luteal cell treatment

The cells were cultured for 48 h in growth medium and for 72 h in serum-free DMEM supplemented with 0.1% bovine serum albumin (BSA), 20 nM insulin, 20 nM selenium, 20 nM apo-transferrin and 1 μM vitamin E. Cultured cells were maintained at 37°C in a humidified atmosphere of 95% air − 5% CO_2. Medium was removed and replaced with fresh culture medium every 24 h. After 24 h of incubation in the presence and absence of recombinant human TNF-α, the medium was collected for progesterone determination and the cells were processed for Western blot analysis or NF-κB immunofluorescence.

Viability of cultured granulosa luteal cells

Viability of the GLCs was determined spectrophotometrically by measuring the incorporation of crystal violet. Cells were treated with various concentrations of TNF-α (0–20 ng/ml) for 24 h. After the cells were washed with PBS and incubated for 5 min with crystal violet solution (0.4% v/v) at room

temperature, 25% ethanol in 50 mM NaH_2PO_4 (pH 4.5) was added for 5 min at room temperature. The absorbance was measured at 600 nm with a microplate reader.

Assay for progesterone

The concentration of progesterone in the supernatant of cultured GLCs was determined by radioimmunoassay in duplicate as previously reported [5].

Western blotting

We determined the protein levels of StAR, NF-κB and IκBα by Western blot analysis as previously described [6,31]. After each treatment, the GLCs were solubilized in lysis buffer (sucrose 0.25 M, Tris–HCl 10 mM, EDTA 10 mM, aprotinin 10 μg/ml) and the homogenates were centrifuged at 400***g*** for 10 min. The total protein concentration in the pellet was measured by the Bradford method and stored at −80°C. Samples of 10 μg of protein for StAR and 40 μg for NF-κB and IκBα were loaded onto different sodium dodecyl sulfate–polyacrylamide gels (10%) for electrophoresis. The separated proteins were transferred to a polyvinylnylidene difluoride membrane (Amersham Biosciences, Piscataway, NJ, USA). Immunoreactive proteins were detected using a chemiluminescence kit. The densitometry analysis was performed using Image-ProPlus program (version 4.5.1.22; Media Cybernetics Inc., Silver Spring, MD, USA). The applied antibodies were: anti-StAR (donated by Dr J. Strauss III, Virginia Commonwealth University School of Medicine, Richmond, VA, USA), anti-p65 NF-κB (Santa Cruz Biotechnology, Palo Alto, CA, USA) and anti-IκBα (Cell Signaling Technology, Danvers, MA, USA).

Immunofluorescence

The cellular distribution of NF-κB in cultured GLCs was determined by indirect immunofluorescence using the methodology described elsewhere [31]. After each treatment, the cells were washed with PBS at 4°C, fixed in methanol at −20°C for 15 min and permeabilized in 0.3% Triton X-100 in PBS at 4°C. Non-specific binding was blocked with 3% BSA in PBS at room temperature for 1 h followed by washing with PBS and then overnight incubation at 4°C with anti-NF-κB (anti-p65) at a 1:300 dilution in PBS containing 1% BSA. Subsequently, cells were washed with PBS and incubated for 1 h at 37°C with fluorescein-conjugated anti-rabbit immunoglobulin G (Sigma Chemical Co.) at a 1:5000 dilution in PBS with 1% BSA, then the cells were washed with PBS and incubated with 0.05% Tween-20 in PBS for 5 min. Coverslips were mounted on glass slides with DAKO® Fluorescent Mounting Medium (DAKO Corporation, Carpinteria, CA, USA). The samples were analyzed by fluorescence microscopy. Treatment of the sample with a non-immune serum or without primary antibody was used as a negative control for antibody specificity.

Statistical analyses

All experiments were performed in duplicate and repeated at least three times. The experimental data are presented as mean ± standard error of the mean and the number of experiments is indicated in the figure legends as n. Data were analyzed by analysis of variance and the Tukey test for comparison between control and treated conditions; $p < 0.05$ was considered statistically significant.

Results

Effect of tumor necrosis factor-α on cell morphology and viability of cultured granulosa luteal cells

To determine the cytotoxic effects of TNF-α on cultured GLCs, we examined the cell morphology by phase contrast microscopy. Figure 1A shows that treatment with TNF-α did not change the morphological characteristics of the GLCs. Moreover, cell viability was evaluated by crystal violet assay. The GLCs were cultured for 24 h in the absence or presence of increasing concentrations of TNF-α. Figure 1B shows that the addition of different concentrations of TNF-α (2.5 to 20 ng/ml) did not decrease GLC viability.

Tumor necrosis factor-α did not alter progesterone production or steroidogenic acute regulatory protein levels in cultured granulosa luteal cells

Figure 2A depicts progesterone production by GLCs cultured for 24 h in the presence or absence of TNF-α (10 ng/ml). No differences were observed between basal progesterone production and that in TNF-α-treated GLCs ($p > 0.05$). hCG stimulated progesterone accumulation (threefold over control) in cultured GLCs. The hCG-stimulated progesterone production was not inhibited by TNF-α. These findings suggest that TNF-α at this dose did not change progesterone production in cultured GLCs. The same result was found for StAR protein expression levels. As expected, hCG significantly increased StAR protein levels (fourfold over control). The hCG dose was chosen based on previously dose–response studies [5]. As depicted in Figure 2B, no differences were observed in the abundance of StAR protein between the control group and the groups treated for 24 h with TNF-α (10 ng/ml) or with hCG plus TNF-α.

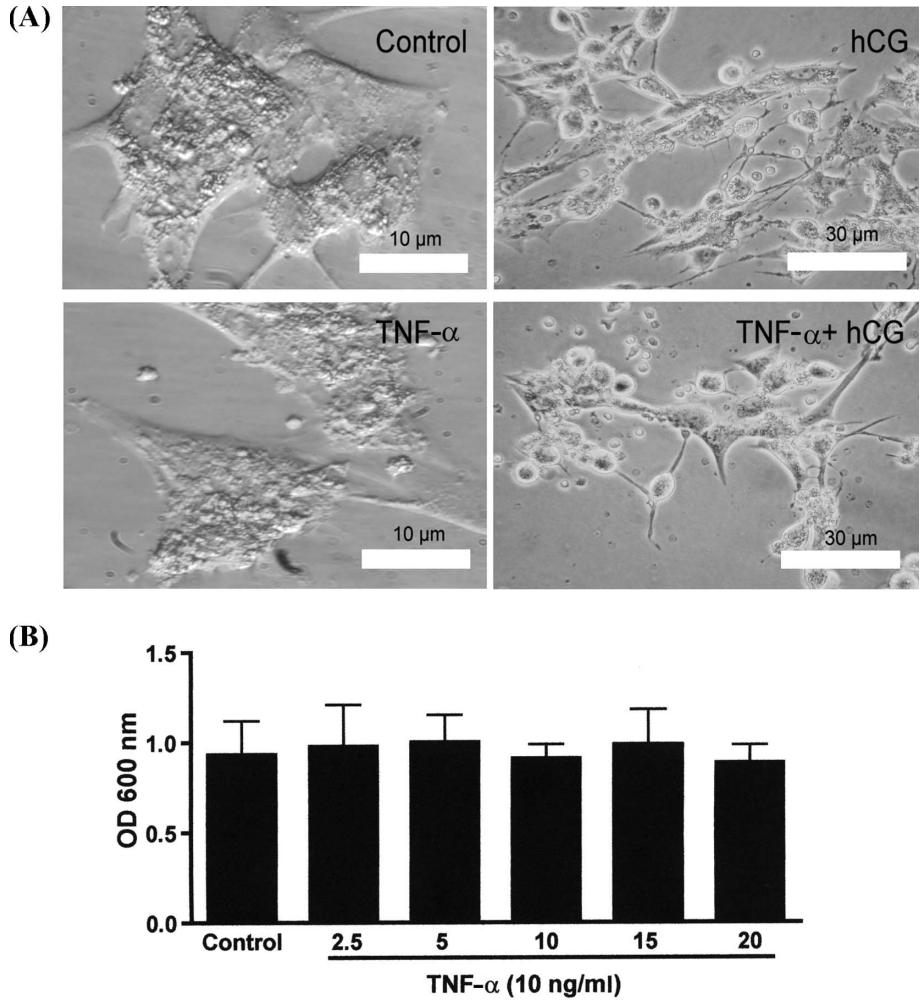

Figure 1. Effect of tumor necrosis factor-α (TNF-α) on cultured granulosa luteal cells (GLCs). (A) Cultured GLCs were incubated for 24 h under different conditions: untreated (control), human chorionic gonadotropin (hCG, 10 IU/ml), TNF-α (10 ng/ml), hCG plus TNF-α. The fields were visualized by phase contrast microscopy. (B) Cell viability of adherent cells was measured by crystal violet assay. GLCs were incubated with increasing concentrations of TNF-α for 24 h in serum-free medium. Values are expressed as optical density (OD) at 600 nm of viable cells compared with untreated controls (80 000 cells). Results are mean ± standard error of the mean ($n = 3$ independent experiments, each performed in duplicate).

Expression of nuclear factor-κB in cultured granulosa luteal cells

NF-κB expression in GLCs from three different patients was evaluated by Western blot and immunocytochemistry analysis of the NF-κB p65 isoform (Figure 3). In all cases, NF-κB p65 isoform was found in total extracts from GLC cultures, with both cytosolic and nuclear distribution. Treatment of the sample with a non-immune serum or without primary antibody was the negative control (data not shown).

Tumor necrosis factor-α activates nuclear factor-κB in cultured granulosa luteal cells

IκBα degradation is a critical step in activation of NF-κB by proinflammatory cytokines. Figure 4 shows the activation of NF-κB by TNF-α in a time-dependent manner. The distribution NF-κB p65 isoform and the time-course of IκBα levels were assessed by immunohistochemestry and Western blot, respectively, in cultured GLCs exposed to TNF-α (10 ng/ml) for 0, 30, 60 and 90 min. TNF-α stimulated a transient nuclear accumulation of NFκB p65 isoform between 60 and 90 min of exposure (Figure 4A). TNF-α induced IκBα degradation after 60 min of exposure, resulting in a progressive decrease in its levels compared with control values (31% to 64%) at 60 and 120 min, respectively (Figure 4B).

Discussion

The present study addresses the concept of whether TNF-α is a molecular modulator of hCG-stimulated production of progesterone by cultured preovulatory human GLCs. Our main findings were: (1) TNF-α affected neither the expression of StAR protein nor

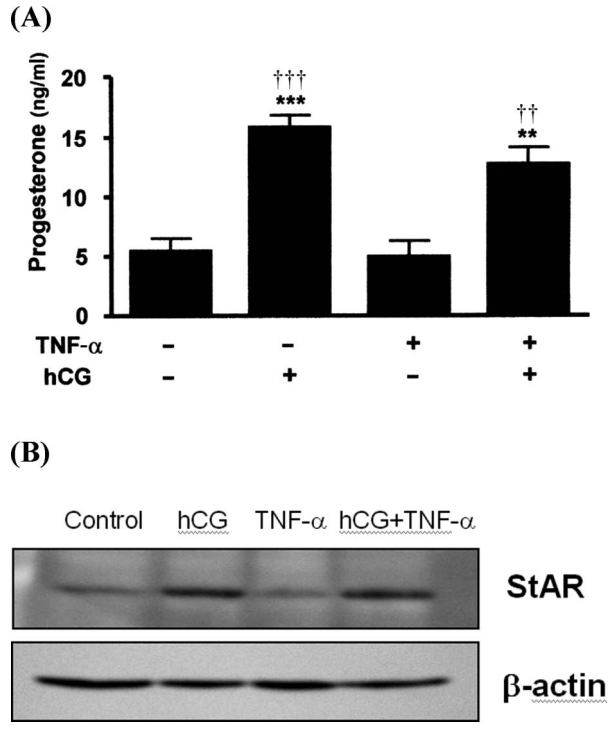

Figure 2. Tumor necrosis factor-α (TNF-α) did not regulate steroidogenesis in cultured granulosa luteal cells (GLCs). GLCs were cultured in serum-free medium for 24 h in the absence or presence of human chorionic gonadotropin (hCG, 10 UI/ml), and with or without TNF-α (10 ng/ml). (A) Progesterone accumulation. Results are mean ± standard error of the mean (SEM) ($n = 7$ independent experiments); $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. control, $^{\dagger\dagger}p < 0.01$ and $^{\dagger\dagger\dagger}p < 0.001$ vs. TNF-α; control progesterone = 5.41 ± 1.10 ng/ml. (B) Representative Western blot for steroidogenic acute regulatory (StAR) protein (band at 30 kDa) and quantification of the StAR protein by densitometric analysis. Results are mean ± SEM ($n = 6$ independent experiments); $^{***}p < 0.001$ vs. control, $^{\dagger\dagger\dagger}p < 0.001$ vs. TNF-α; control = 5.8 ± 0.9 AU StAR/β-actin.

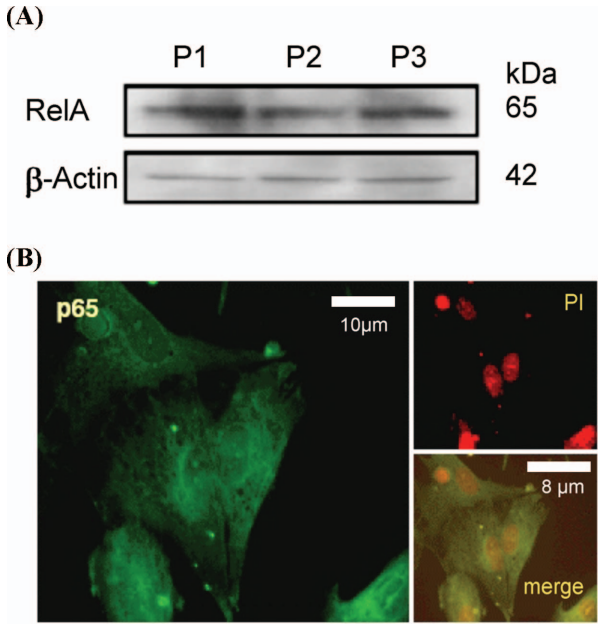

Figure 3. Expression and subcellular localization of nuclear factor-κB (NF-κB) in granulosa luteal cells (GLCs). (A) Western blot for p65 isoform of NF-κB in cultured GLCs from three different patients (P1–P3). (B) Immunocytochemistry of p65 NF-κB in GLCs was performed using, as primary and secondary antibodies, anti-p65 NF-κB and goat anti-rabbit immunoglobulin G conjugated with fluorescein, respectively. Nuclei were stained with propidium iodide. Photomicrographs were obtained using a fluorescence microscope at 40×. Images are representative of preparations from three different patients.

progesterone synthesis; and (2) the p65 isoform of NF-κB was expressed in GLCs and was activated by TNF-α, resulting in the degradation of IκBα and mobilization of the NF-κB p65 subunit to the nucleus. These results collectively suggest that TNF-α does not modulate steroidogenesis in human preovulatory GLCs and that the TNF-α/NF-κB signaling pathway is presumably associated with other cellular processes in these cells.

The importance of the immune–endocrine crosstalk between ovarian cells has been recognized, particularly in the area of cell proliferation and steroidogenesis [32]. A number of studies have suggested a role for intraovarian TNF-α on follicular development, ovulation and corpus luteum function in humans [11,20,21]. Human preovulatory granulosa cells, granulosa lutein cells as well as theca cells and the follicular fluid of antral follicles are sources of immunoreactive TNF-α [17,19]. Additionally, TNF-α inhibits FSH-induced estradiol biosynthesis in cultured human granulosa cells [33] and the TNF-α receptor has been detected in ovarian cells of several mammals [17,18,34]. Several studies report differential effects of TNF-α on human ovarian cell steroidogenesis [12,17,20,23]. This discrepancy may be based on different cell culture systems, including distinct types and species of ovarian cells, the presence or absence of leukocytes and serum, the exposure time and/or concentration of stimulating peptides (FSH, luteinizing hormone/hCG) and dose of TNF-α, encompassing diverse steroids determined in the culture media [21,35,36]. Wang and colleagues demonstrated that TNF-α increases the inhibitory effect of interferon-γ on progesterone production by hCG-stimulated granulosa cells, suggesting that cytokines modulates *in vitro* luteal steroidogenesis [24]. Our data clearly showed that TNF-α did not change the expression of StAR protein or the synthesis of progesterone in cultured preovulatory human GLCs. These results are in contrast to those of Xiong and Hong, who reported that TNF-α decreased the expression of P450 scc, P450c17 and 3β-hydroxysteroid dehydrogenase in primary cultures of mouse Ledyig cells, and down-regulated StAR levels in primary cultures of testicular steroidogenic cells, respectively [36,37]. As described above, this divergence could be related to

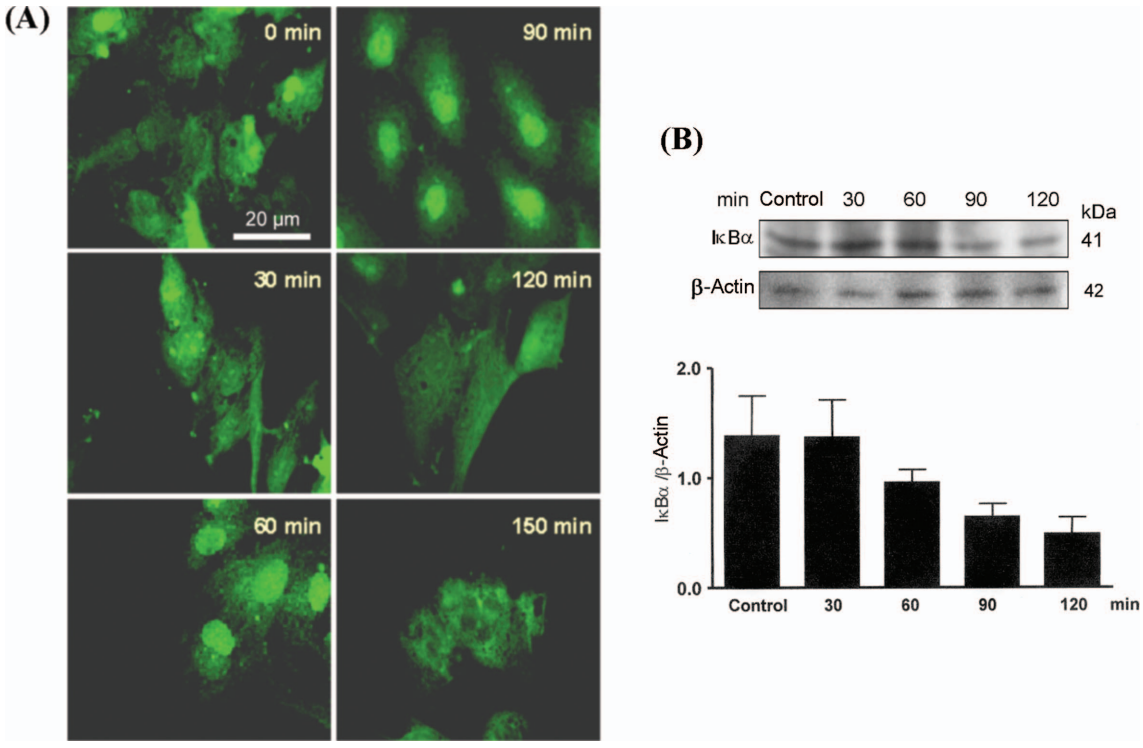

Figure 4. Tumor necrosis factor-α (TNF-α) activates nuclear factor-κB (NF-κB) in granulosa luteal cells (GLCs). GLCs were cultured in serum-free medium for 0, 30, 60, 90 and 120 min with or without TNF-α (10 ng/ml). (A) Immunofluorescence for NF-κB in GLCs untreated or treated with TNF-α for 30, 60, 90, 120 and 150 min. Images are representative of three independent experiments. (B) Western blots for IκBα and β-actin protein levels and quantification plot. Values are mean ± standard error of the mean ($n = 3$ independent experiments); control = 1.4 ± 0.4 AU IκBα/β-actin.

distinct types of cells, different experimental conditions and different species used by the research groups. It is important to highlight that, in this work, cultured primary monolayer GLCs were obtained from women enrolled in our *in vitro* fertilization program, indicating that all patients were previously treated with 10 000 IU of hCG. Thus, luteinization was pharmacologically induced. The majority of the GLCs were luteinized and differentiated, exhibiting non-proliferative morphological and biochemical characteristics. In addition, the concentration of TNF-α used in our experiments did not affect cell viability; thus it is unlikely that the number of cultured cells has an effect on progesterone production. Differential expression of specific genes and proteins has been demonstrated in granulosa cells at the time of ovulation and during corpus luteum formation in several species [38]. Therefore, our data do not necessary imply that preovulatory GLCs will exhibit the same steroidogenic response to TNF-α as corpus luteum granulosa lutein cells.

In numerous types of cells, TNF-α activates NF-κB, a pivotal transcription factor that governs the expression of early-response genes [39]. The presence of NF-κB in granulosa cells has been described in mice, rats and man [30,40,41]. Even though the activation of NF-κB by IL-1β has been described in GLCs [30], the effect of TNF-α on this pathway has not been previously investigated. Our results demonstrated the presence of the p65 NF-κB subunit in cultured human GLCs by Western blot and immunofluorescence. The p65 NF-κB subunit was localized in the cytoplasm under non-stimulated conditions (control), presumably sequestered in the cytoplasm by its association with IκBα. In our model, TNF-α stimulates degradation of IκBα and translocation of the p65 NF-κB subunit to the nucleus. These findings did not match with those determined in mouse Ledyig cells, in which NF-κB translocation to the nucleus resulted in inhibition of the orphan nuclear receptor SF-1 and testicular steroidogenesis [37]. Additionally, in primary cultures of Leyding cells, TNF-α inhibits StAR expression [37]. In our experimental system, although TNF-α did not affect StAR expression in both basal and hCG-stimulated conditions, its bioactivity was demonstrated by activation of NF-κB.

In summary, our results indicate that the TNF-α-induced NF-κB pathway is present and active in human granulosa luteal cells but presumably is not involved in luteal steroidogenesis. Further work should clarify the role of TNF-α and NF-κB in other cellular processes related to the function of preovulatory granulosa cells, such as cell differentiation or the inflammatory response process associated with ovulation or cell survival [38,41].

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