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# Induction of apoptosis in granulosa cells by TNFα and its attenuation by glucocorticoids involve modulation of Bcl-2

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**Abstract**

Tumor necrosis factor α (TNFα) plays a role in mammalian ovarian follicular development, steroidogenesis, ovulation, luteolysis, and atresia, but the exact mechanism of TNFα action is not completely understood. Induction of apoptosis and suppression of steroidogenesis by TNFα in primary preovulatory rat and human granulosa cells, as well as, in human granulosa cells immortalized by mutated p53, were characterized in the present work. Dexamethasone (Dex) and hydrocortisone efficiently suppressed TNFα-induced apoptosis in granulosa cells. TNFα dramatically reduced intracellular levels of Bcl-2, while Dex abrogated this reduction. TNFα reduced considerably intracellular levels of StAR protein, a key regulating factor in steroidogenesis. This reduction can be explained only in part by elimination of cells through apoptosis, since loss of steroidogenic capacity was much higher and faster than the rate and extent of loss of cell viability induced by TNFα, suggesting independent mechanisms for TNFα-induction of apoptosis and TNFα-suppression of steroidogenesis. 

*Keywords:* Tumor necrosis factor α (TNFα); Dexamethasone (Dex); Apoptosis; Steroidogenesis; Granulosa cells

TNFα is one of the most potent modulators of apoptosis in rat and bovine ovary, [1] and in cultured mouse, hen, and human granulosa cells [2–4]. Many of the biological effects of soluble TNFα are mediated via type 1 TNFα receptor, which contains a cytoplasmic death domain involved in signal transduction and apoptosis [5,6]. Expression of type 1 TNFα receptor was recently characterized in bovine and porcine corpora lutea and bovine and hen granulosa cells [4,7–9]. The exact mechanism of how TNFα affects apoptosis in the ovary is only partially characterized. One possible mechanism is through the induction of rapid hydrolysis of sphingomyelin to ceramide, which was shown to play as an important mediator in the signal transduction mechanism leading to apoptosis in several cell lines [10,11]. Coupling of TNFα signaling to the sphingomyelinase pathway was suggested in granulosa cells [12]. It was also documented that TNFα induces apoptosis by activating apoptosis executors, such as interleukin-1β (IL-1β)-converting enzyme (ICE) and ICE like cystein proteases [13], but the possible involvement of survival and death genes in TNFα induced apoptosis in granulosa–lutein cells is still obscure. It is believed that TNFα is released from macrophages during the process of ovulation and follicular rupture, which leads to inflammation [14,15]. It was also suggested that TNFα could be synthesized by granulosa–lutein cells [16]. However, healing of the follicular wall during the luteinization and termination of the inflammatory process during the estrous/menstrous cycle is not completely understood. TNFα is a modulator of steroid hormone production induced by gonadotropins in bovine, rat, porcine, and human granulosa cells [12,17–20]. TNFα enhances production of progesterone in cultured intact follicles [7]. In recent studies it was suggested that the luteolitic effect of TNFα may be mediated by inhibition of the steroidogenic acute regulatory protein (StAR) expression, a key regulatory protein in inducible steroidogenesis [21], or by indirectly decreasing luteinizing hormone (LH) receptor expression in addition to stimulation of the luteolitic prostaglandin PGF2α production

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[21,22]. TNFα type 1 receptor knockout mice strongly suggest that the mechanism of TNFα mediated modulation of steroidogenesis is most likely via TNFα type 1 receptor [7]. However, the exact mechanism by which TNFα exerts its effect on ovarian steroidogenesis is not completely understood.

We have recently demonstrated in a human granulosa cell line which keeps its high potential of steroidogenesis, that Bcl-2 plays an important role in the regulation of apoptosis. We demonstrated that glucocorticoids protect against serum starvation-, p53-, and cAMP-induced apoptosis [23]. In the present work we demonstrate for the first time that TNFα induction of apoptosis in both primary and immortalized human granulosa cells involves attenuation of intracellular levels of Bcl-2. Moreover, attenuation of steroid production by TNFα, which involves down regulation of StAR, precedes TNFα-induced apoptosis, and thus the two processes may be controlled independently.

## Materials and methods

*Antibodies*. Antibody against progesterone was a gift of Dr. F. Kohen (Department of Biological Regulation, Weizmann Institute of Science, Rehovot, Israel). Polyclonal rabbit anti-human Bcl-2 was purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Goat anti-rabbit or anti-mouse IgG coupled to horseradish peroxidase (HRP) was obtained from Biomakor (Rehovot, Israel). Anti-human StAR antibodies were generously provided by Dr. J.F. Strauss III (University of Pennsylvania Medical Center, Philadelphia, PA, and USA). Anti-mouse β-tubulin was purchased from Sigma–Aldrich (Rehovot, Israel).

*Reagents*. Reagents for TUNEL staining were purchased from Boehringer Mannheim, Germany. Forskolin (FK, a potent activator of adenylate cyclase), dexamethasone, and 4′,6-diamido-2-phenylindole hydrochloride (DAPI, for DNA staining) were purchased from Sigma Chemical Co. Specific inhibitors of MAPK activity, PD 98059, and UO126 were purchased from Calbiochem (San Diego). Recombinant TNFα was generously denoted by Dr. D. Wallach, Department of Biological Chemistry, Weizmann Institute of Science ($1\,\mu g = 6 \times 10^7$ IU). Human lutenized hormone (hLH) and human chorionic gonadotropin (hCG) were kindly provided by the NIH and Dr. Parlow.

*Cells and treatments*. Primary human granulosa cells were obtained from women undergoing in vitro fertilization (IVF) at Sheba Medical Center, Tel-Hashomer, Israel. Patients received gonadotropin-releasing hormone agonist (GnRH-a, Decapeptyl) in combination with follicle-stimulating hormone (FSH) or human menopausal gonadotropin (hMG), followed by administration of hCG. Granulosa cells were isolated from aspirated follicular fluid after ovum retrieval. For each set of experiments luteinizing granulosa cells from three women were pooled together. Each set of experiments was repeated twice in triplicate plates (total number of patients = 6). The follicular fluid was centrifuged at 300*g* for 5 min to separate granulosa cells from red blood cells. The resulting pellet was re-suspended in 10 mM Tris, 0.84% $NH_4Cl$, pH 7.4, to lyse red blood cells (15 min shaking at 37 °C). Elimination of the cell debris was achieved by several washings in phosphate-buffered saline (PBS). Cells were plated in Dulbecco's modified Eagle's medium (DMEM/Ham's F12 (1:1)), supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml), and 5% fetal calf serum (FCS) for 7 days, to release them from desensitization to gonadotropin stimulation [24]. Estimation of purity of cell culture, achieved by staining the cells with DAPI for general nuclear DNA and with anti-StAR antibodies, was 93% (data are not shown). Cells were maintained at 37 °C in a $CO_2$ incubator with 95% humidified air and 5% $CO_2$. For stimulation, cells were washed on day 8 of culture with PBS, and incubated in serum-free medium, containing the different stimulants [24].

Primary rat granulosa cells of preovulatory follicles were obtained from 25- day-old female rats treated with 15 IU PMSG for 48 h. Cells were plated on 35-mm plastic dishes in Dulbeco's modified Eagle's medium (DMEM):F12 (1:1) containing 5% fetal calf serum. After 24 h cells were washed three times in PBS and incubated at 37 °C in serum-free (DMEM):F12 medium containing the desired stimulants [25].

Human granulosa cell line HO-23 was established by triple transfection of primary human granulosa cells, with SV40 DNA, Ha-ras, and a temperature-sensitive (Ts) mutant of p53 (p53val135) [26]. Shifting the temperature to 32 °C leads to manifestation of wild-type p53 activity [27,28].

*Biochemical assays*. Progesterone was determined by RIA at the end of stimulation [27,29]. Protein was quantified by the Bradford method [30].

*Western blot analysis*. Cells were washed with cold PBS and harvested in lysis buffer containing 50 mM HEPES (pH 7.2), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, 1% Triton X-100, 10 μg/ml leupeptin, 10% glycerol, 30 mM NaF, 30 mM Na-pyrophosphate, 1 mM orthovanadate, and 5 μg/ml aprotinin. Lysates were boiled in sample buffer for 10 min. Samples containing equal amounts of protein (30 μg) were separated by 12% (to detect Bcl-2 and StAR) SDS–PAGE and transferred to nitrocellulose membranes. The blots were blocked using 5% milk powder in PBS plus 0.05% Tween 20 and reacted (overnight, 4 °C) with anti-Bcl-2, anti-StAR, or anti-β-tubulin followed by 1 h incubation at room temperature with goat anti-rabbit or anti-mouse IgG conjugated to HRP. Detection was carried out using enhanced chemiluminescence [26].

*Fluorescent microscopy*. Staining for DAPI and TUNEL was performed on cells grown on glass coverslips. At the end of culture period, medium was aspirated and stored at −20 °C for progesterone measurement. Cells were washed with PBS, fixed with 4% paraformaldehyde for 30 min in 24 °C, and permeabilized for 4 min with 1% Triton X-100 in PBS at 4 °C. Fixed cells on coverslips were incubated with TUNEL reaction mixture (fluorescein-dUTP and terminal deoxynucleotidyl transferase (TdT)) for 60 min at 37 °C in a humidified atmosphere in the dark. Cells were washed with PBS and stained with DAPI for 30 min at 24 °C. Cells were gently washed (×3) with PBS, mounted in Mowiol and analyzed by Zeiss Axioskop Microscope (Carl Zeiss, Oberkochen, Germany) in both phase and fluorescent modes.

For quantifying apoptotic events, photographs of 5–10 random fields of each treated culture were taken through 40× objective lens. Apoptotic and total cell nuclei were counted on 400× magnified images scoring 194–238 nuclei at each treatment, and the percentages of the apoptotic nuclei in the total nuclei scored were calculated. Data from three different experiments were combined and expressed as means ± SEM. This method of in situ DNA staining allows visualization of chromatin condensation and fragmentation as typical pattern of apoptosis [24].

*Fluorescence-activated cell sorting (FACS) analysis of DNA content*. Floating and trypsinized cells, which were attached to the bottom of the culture dishes from each treatment, were collected and combined to ensure a complete recovery of the cell population. Cells were washed with cold PBS and fixed in cold methanol (−20 °C) for 1 h. Subsequently, cells were centrifuged, re-suspended in 0.5 ml cold PBS, and stained for 15 min with 50 μg/ml propidium iodide in the presence of RNase A (100 μg/ml). Cells were analyzed in the fluorescence-activated cell sorter. Five thousand events from the gated sub-population of each treatment were recorded separately [23,31].

*Statistical analysis*. Analysis of progesterone production and densitometer tracing on Western blots autoradiograms (means ± SEM) were performed using ANOVA followed by Fishers' LSD test. Differences between treatment groups were considered statistically significant at $P < 0.05$.

## Results

### *TNFα induces apoptosis and blocks steroidogenesis*

TNFα (100 IU/ml) induces massive apoptosis in primary cultures of rat granulosa cells obtained from preovulatory follicles during 24 h incubation at 37 °C in serum-free medium. This phenomenon was verified by morphological examination of the cultures, by DAPI staining and by the TUNEL method. Incubation of the cells with Dex (100 nM) for 24 h at 37 °C abolished this phenomenon almost completely (Fig. 1). For quantification of this phenomenon, incidence of apoptosis was scored in cells stained with TUNEL, which is one of the most sensitive methods in detecting apoptosis. We observed excellent correlations among all three methods of apoptosis detection. For each treatment 194–238 cell nuclei were scored, and progesterone released in the culture was assayed in parallel (Fig. 2). TNFα increased apoptosis by 208% and by 232% in the presence of 100 and 300 IU/ml of TNFα, respectively. Interestingly, blockage of steroidogenesis was pronounced (20% of control) only at 100 IU/ml of TNFα with no significant change at 300 IU/ml of TNFα. While Dex reduced dramatically TNFα-induced apoptosis, which was even considerably lower than controls (to 7.7% of TNFα-stimulated cells), it was not able to restore steroidogenesis. hCG (1 IU/ml) moderately reduced apoptosis (to 74% of non-stimulated cells) and blocked in part TNFα-induced apoptosis (to 76% of TNFα-stimulated cells). TNFα attenuated steroidogenesis induced either by hCG alone (to 62% of hCG-stimulated cells) or by co-stimulation with Dex + hCG (to 25% of hCG + Dex-stimulated cells). Using hLH 1 IU/ml instead of hCG gave essentially similar results (not shown). Dex and hydrocortisone (100 nM) attenuated TNFα-induced apoptosis to 6.6% and to 46%, respectively (Fig. 2). Dex or hydrocortisone alone were also able to attenuate apoptosis (to 72% and 75% of control, respectively). Interestingly, the suppression of apoptosis by Dex and TNFα was much more pronounced compared to treatment with Dex alone. In addition, Dex or hydrocortisone enhanced steroidogenesis by 187% (not shown for hydrocortisone in Fig. 2). Collectively, these data suggest that TNFα considerably induces apoptosis and attenuates steroidogenesis while Dex reduces the anti-steroidogenic effect of TNFα in non-stimulated cells and completely abolished it in hCG-stimulated cells.

To examine the effect of TNFα on steroidogenesis and apoptosis in human granulosa cells, primary cultures obtained from women who underwent IVF procedures were stimulated for 24 h at 37 °C with hLH (1 IU/ml), TNFα (300 IU/ml), TNFα (300 IU/ml) + LH (1 IU/ml), and TNFα (300 IU/ml) + DEX (100 nM). LH augmented steroidogenesis by 640% with a very modest elevation of apoptosis (120%) (Fig. 3). TNFα dramatically suppressed LH-stimulated progesterone levels (22.5% of LH-stimulated cells) even below basal levels (12.5% of basal), concomitant with elevation of the incidence of apoptosis in non-stimulated cells by 230% and by 132% in LH-stimulated cells. The remarkable TNFα induced lowering of progesterone production is due to stimulation of apoptosis, as well as to marked attenuation in StAR protein level (Fig. 4). Dex abolished the inhibitory effect of TNFα on the basal level of progesterone production and reduced the incidence of apoptosis to 32% of TNFα-stimulated cells (Fig. 3). Apoptosis was scored on DAPI stained cultures, and the experiment was repeated essentially with similar results with granulosa cells obtained from six women. These data suggest that TNFα induces apoptosis and attenuates steroidogenesis with different proportions.

### *Expression of StAR in primary rat preovulatory granulosa cells*

To verify whether reduction in steroidogenesis by TNFα involved changes in the intracellular level of StAR, we examined the expression of StAR by Western blot analysis (Fig. 4). While in the controls, the levels of StAR were almost undetectable, hLH (1 IU/ml) for 24 h at 37 °C elevated StAR expression dramatically by 1060%, which was slightly lower in the presence of DEX. TNFα reduced StAR expression induced by LH to 77% and 43% of LH-stimulated cells in the presence of 100 and 300 IU/ml of TNFα, respectively. Reduction of StAR expression by TNFα in the presence of LH and Dex was less pronounced. In contrast to the sharp modulation of StAR content, only modest elevation of adrenodoxin, which is an integral part of the cytochrome P450 side cleavage enzyme system (P450scc) [20], was recorded following LH stimulation (by 114%), and no significant changes in adrenodoxin was detected either by TNFα or Dex (data not shown).

### *TNFα induces apoptosis and attenuates steroidogenesis in HO-23 immortalized human granulosa cells*

To gain a deeper insight into the mechanism of TNFα action we utilized HO-23 cells that keep the steroidogenic capacity of primary cells [26]. FACS analysis of propidium iodide labeled cells demonstrated that 300 IU/ml of TNFα induced apoptosis already after 8 h of treatment (from 1.94% in control cultures to 7.50% in stimulated cultures). Further enhancement of apoptosis to 9.74% was observed following co-stimulation with 20 μM FK and 300 IU/ml of TNFα. This enhancement of apoptosis by co-stimulation with TNFα and FK was completely blocked in the presence of 100 nM of Dex which yield only 1.2% of apoptosis. FACS analysis also demonstrated that there was no dramatic changes in the cell cycle following TNFα or forskolin treatments in the

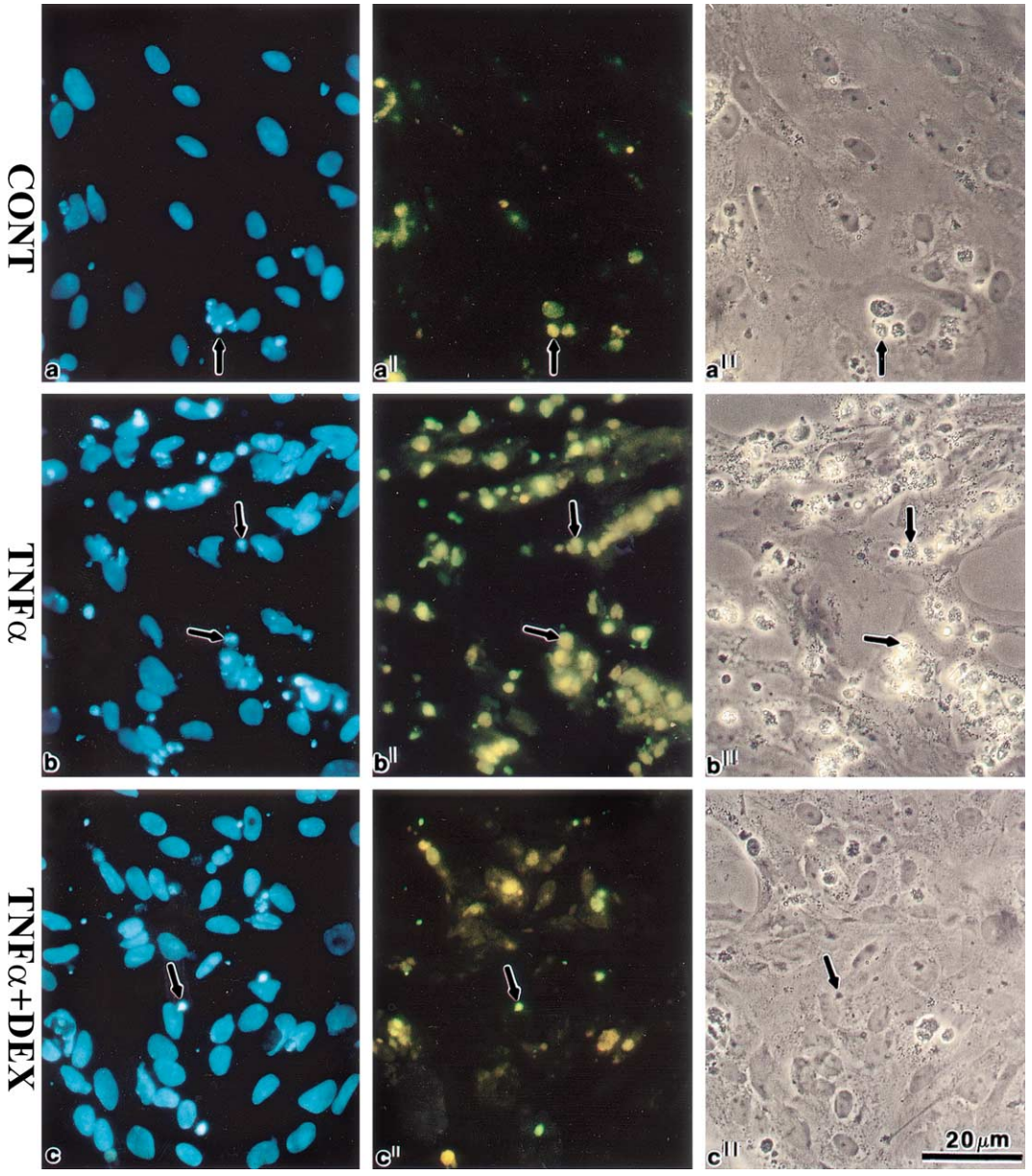


Fig. 1. Attenuation of TNFα-induced apoptosis by dexamethasone in rat preovulatory granulosa cells. Primary cultures were obtained from PMSG-induced immature female rats. Cells were incubated with no stimulants in DMEM/F12 serum-free medium (CONT a, a′, a″), TNFα (300 IU/ml) (b, b′, b″), or with TNFα + 100 nM of dexamethasone (DEX) (c, c′, c″) for 24 h at 37 °C. At the end of the incubation period cells were fixed with 4% paraformaldehyde and stained with DAPI (a,b,c) and TUNEL (a′, b′, c′). The same fields were also visualized by phase contrast microscopy (a″, b″, c″). Arrows are indicating apoptotic nuclei.

presence or absence of Dex during the 8 h of treatment with TNFα, with no significant changes in the G2M fraction (not shown). In a dose-dependence study, we discovered that the effect of TNFα on apoptosis is biphasic (Fig. 5). It increases apoptosis linearly at 10–100 IU/ml of TNFα while its effect decreases gradually at 300–1000 IU/ml of TNFα. Dex completely blocked TNFα-induced apoptosis at a range of 300–1000 IU/ml. Similarly to our observation in primary rat and human cells, TNFα attenuated basal and FK-induced steroidogenesis (to 50% of non-stimulated cells and to 36% of FK-stimulated cells), while DEX restores progesterone production to a large extent (to 82% of FK-stimulated cells) (Fig. 6A). It should be noted that Dex alone did not stimulate steroidogenesis in the HO-23 cells by itself since these cells in contrast to primary granulosa cells do not express the steroidogenic factors StAR and the P450scc without stimulation by cyclic AMP elevating substances, e.g., FK or 8Br-cAMP which lead to de-novo synthesis of these proteins [23,26].

We further examined whether TNFα affects steroidogenesis possibly by activation of a MAPK cascade.

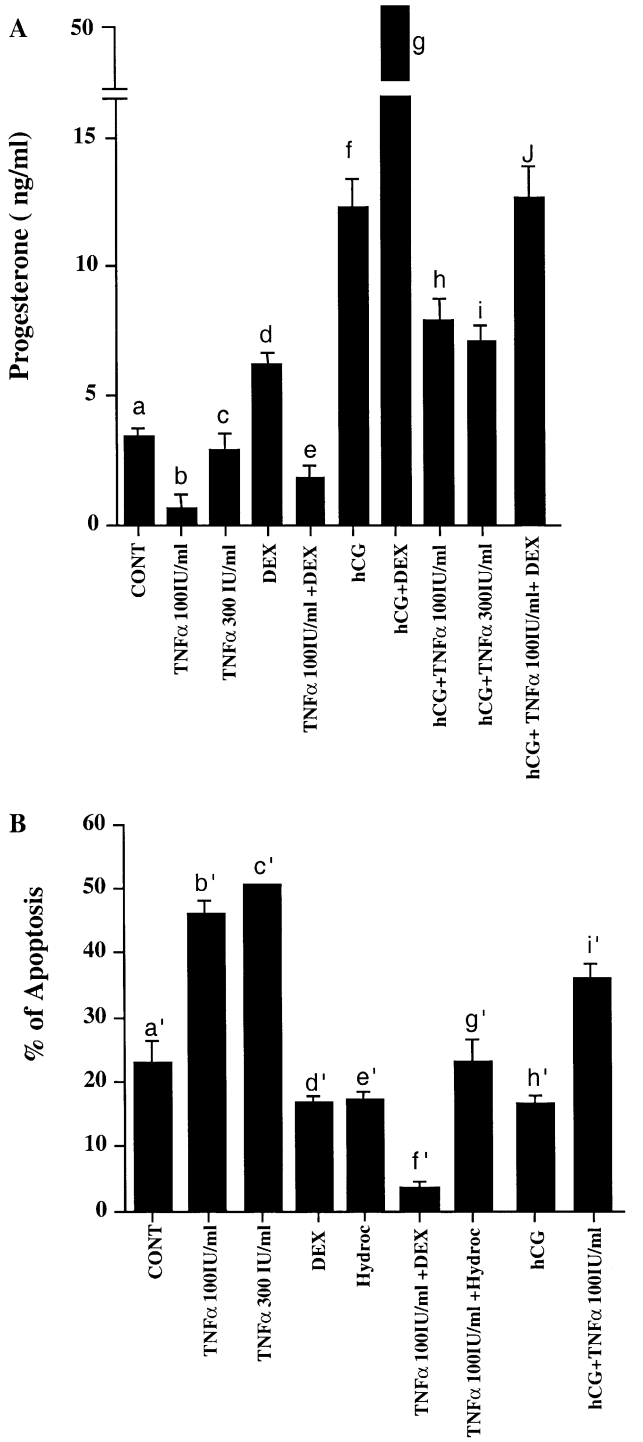


Fig. 2. Steroidogenesis and apoptosis in primary cultures of preovulatory rat granulosa cells. Progesterone released to the culture medium was assayed by RIA. Percentage of apoptosis was determined as % of positively stained nuclei by TUNEL per total nuclei stained by DAPI. Cells were incubated with the different stimulants for 24 h at 37 °C. Data are mean of triplicate plates ± SEM. a is different from b, d, e, f g, h, i, and j $P < 0.05$. a′ is different from b′, c′, d′, e′, f′, h′, and i′ $P < 0.05$.

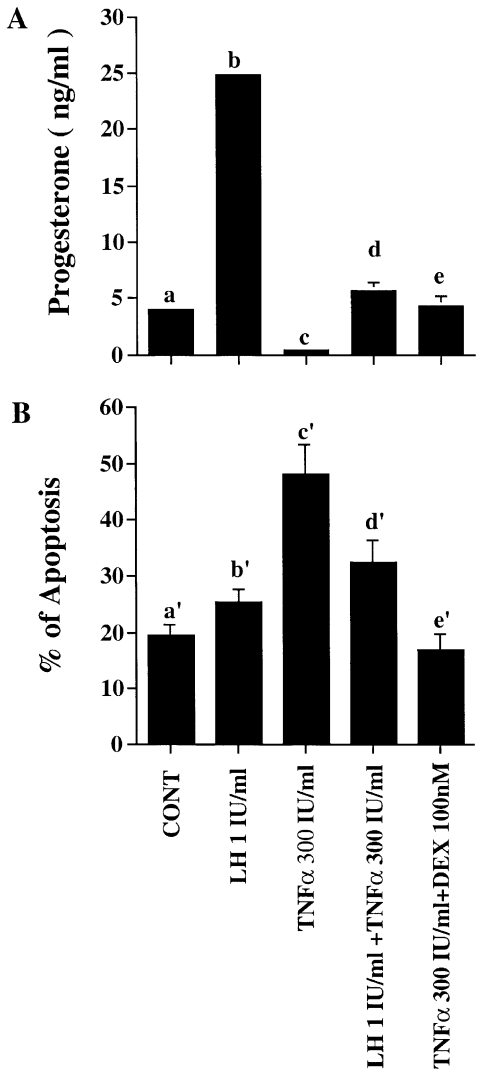


Fig. 3. Steroidogenesis and apoptosis in primary human granulosa cells obtained from an IVF program. Primary cultures were incubated with DMEM/F12 medium containing 5% fetal calf serum for 7 days to release them from desensitization [24] and subsequently in serum-free medium with the indicated hormones for 24 h at 37 °C. Progesterone was determined in triplicate plates. Percentage of apoptosis was determined after staining the cellular DNA by DAPI (see Materials and methods). Data are means of three triplicate plates obtained from two independent pools of cells ± SEM (see Materials and methods). Essentially similar results were obtained from pools of six patients. a is different from c and b is different from d $P < 0.05$. a′ is different from b′, c′ and c′ is different from d′ and e′ $P < 0.05$.

Specific inhibitors of the extracellular signal-regulated kinases (ERK-1 and 2), such as UO 3 μM and PD 8 μM, could completely block the anti-steroidogenic effect of TNFα (Fig. 6B). To examine whether apoptosis by TNFα involves reduction in intracellular levels of survival signals we performed Western blot analysis of homogenates using specific antibodies to Bcl-2. While TNFα dramatically reduced Bcl-2 levels, Dex almost completely protected against such a reduction in HO-23 cells (Fig. 7). The same tendency was also evident although less dramatic in primary human granulosa cells (Fig. 7).

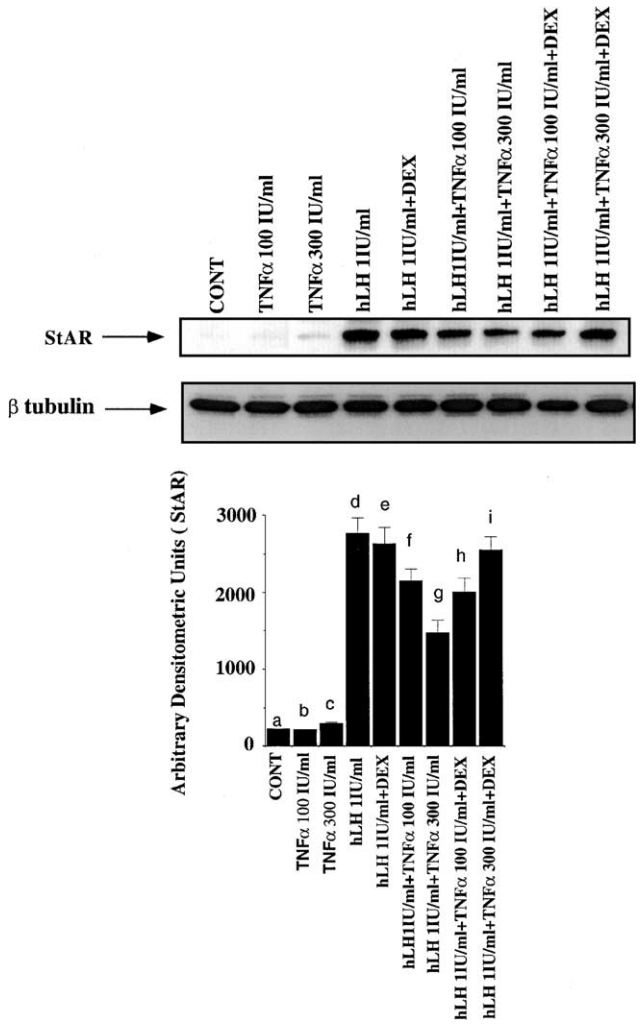


Fig. 4. StAR expression in TNFα treated primary rat preovulatory granulosa cells. Primary cultures were prepared as indicated in Materials and methods. Cells were incubated with the indicated hormones for 24 h in serum-free medium. Cell lysates were processed for Western blot analysis, using specific polyclonal antibodies to StAR and β-tubulin. a–c is different from d–i. d is different from f, g, h $P < 0.05$.

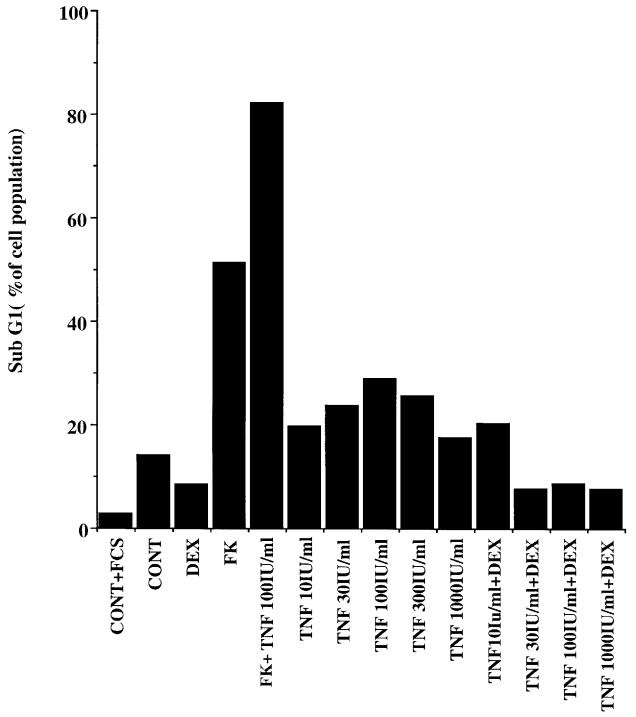


Fig. 5. Dose response of TNFα-induction of apoptosis in the absence and presence of dexamethasone in immortalized human granulosa cells derived from preovulatory follicle (HO-23). Cells were incubated with the indicated hormones for 24 h in serum-free medium. Incidence of apoptosis was quantified by flow cytometry (FACS) subsequent to labeling of cellular DNA by propidium iodide (see Materials and methods).

## Discussion

In the present work we demonstrated that TNFα-induced apoptosis in granulosa cells can be blocked by glucocorticoids. Moreover TNFα-induced apoptosis involves reduction in the intracellular levels of the survival gene Bcl-2, while dexamethasone abrogates this phenomenon. TNFα induction of apoptosis in granulosa–lutein cells involves stimulation of synthesis of PGF2α [32] and ceramide production [1,4]. It was recently reported that FSH, hCG, transforming growth factor-β1 (TGFβ1), and macrophage colony-stimulating factor (MCF) may negate the TNFα-induction of apoptosis in primary human granulosa cells [3]. However, we demonstrate here for the first time that glucocorticoids can attenuate this phenomenon in human and rat granulosa–lutein cells, and therefore may play important roles in the healing process of the ovarian follicular tissue following ovulation, follicular rupture, and corpus luteum formation. There are considerable differences in reports regarding the effect of TNFα on ovarian cell death, depending on the stage of follicular maturation [4], which may contain different densities of the TNFα receptor. Moreover, in preovulatory swine follicle it was recently suggested that TNFα leads to increased proliferation and at the same time induces apoptosis [33]. In human granulosa cells it was suggested that TNFα in follicular fluid enhances proliferation of granulosa–lutein cells [34]. Our results indicate that both in human and rat preovulatory granulosa cells TNFα induces apoptosis and there was no increase in cell proliferation as judged from the fraction of cells in G2M phase. However, it is possible that these different reports emerged from different culturing conditions and different experimental designs. It is generally accepted that preovulatory granulosa cells from rat, chicken, and humans do not proliferate in vitro, and we and others [35] could not find any mitotic figures in the primary cultures used for the present experiments whether treated or not with TNFα, as revealed by general morphology and DAPI staining (our unpublished observation). However it should be noted that swine and bovine granulosa cells maintain their potential to

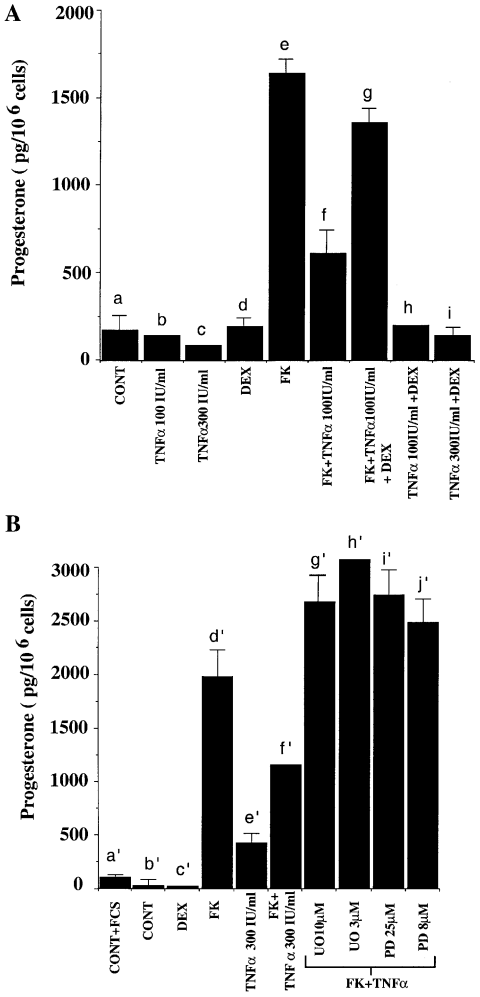


Fig. 6. Steroidogenesis and apoptosis in immortalized human granulosa cells (HO-23). HO-23 cells were incubated for 24 h at 37 °C in DMEM/Ham's F-12 medium supplemented with 5% FCS. The culture medium was replaced with serum-free medium containing the different stimulants: 20 μM forskolin (FK), 100 nM dexamethasone IU/ml TNFα, 300 IU/ml TNFα (A); 3 and 10 μM UO126, 8 and 25 μM PD 98059 (B) and the different combinations for 24 h. Progesterone production was measured by RIA. Data are the means ± SEM of triplicate culture plates. a–d<e $P < 0.05$; f, h, i < e $P < 0.05$; f′ < d′, g′–i′ $P < 0.005$.

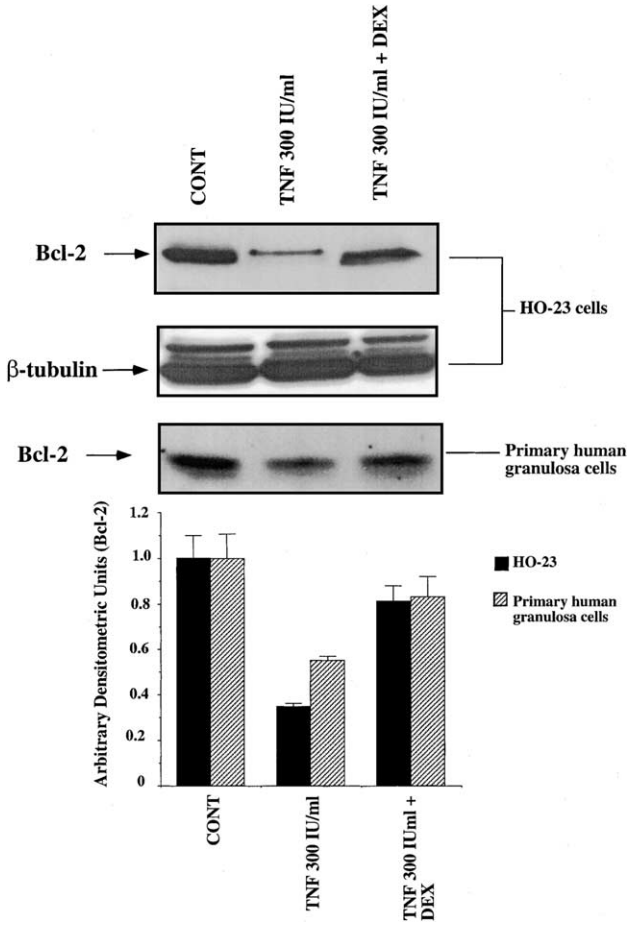


Fig. 7. Modulation of Bcl-2 levels by TNFα and dexamethasone in primary human granulosa cells and in HO-23 cell line. Primary cultures were incubated with DMEM/F12 medium containing 5% fetal calf serum for 7 days to release them from desensitization [24], and subsequently in serum-free medium with the indicated stimulants for 24 h at 37 °C. HO-23 cells were cultured for 24 h in medium containing 5% serum at 37 °C for 24 h. The culture medium was replaced with serum-free medium in the absence of stimulants (CONT), or in the presence of 300 IU/ml TNFα alone or with 100 nM of dexamethasone (DEX) for 24 h. Cell lysates were prepared, and Western blot analysis was performed using specific antibodies to Bcl-2 and β-tubulin. Lower part densitometer tracing of Bcl-2 in arbitrary units.

proliferate in vitro [36,37]. Adashi [38] reported no attenuation in cell numbers in TNFα-treated rat preantral granulosa cells. This may suggest that preovulatory granulosa cells are more sensitive to TNFα signals for apoptosis as indicated in the present work compared to rat preantral granulosa cells.

The inhibition of TNFα-induced apoptosis by dexamethasone was recently demonstrated in bovine glomerular endothelial cells and MCF-7 cells. Interestingly, this effect was unique to glucocorticoids while β-estradiol and testosterone had no effect [39,40]. The mechanism of action of Bcl-2 on protection against apoptosis was demonstrated in several systems such as pro B lymphocyte and oligodendrocyte cell line (OLN-93) [40,41]. However, the possible cross-talk between TNFα and Dex in modulation of apoptosis via the control of Bcl-2 levels is demonstrated for the first time in rat and human granulosa cells in the present work. Interestingly, it was demonstrated that overexpression of Bcl-2 in a leukemia cell line [4,42] blocks ceramide-induced cell death and constitutively elevates levels of Bcl-x long mRNA, providing resistance to TNFα-induced cell death in avian follicular granulosa cells [4,43]. Association of increased Bcl-2 expression with rescue from TNFα-induced cell death in the oligodendrocyte cell line OLN-93 was also demonstrated recently [41]. Taken together, these data suggest a central role to the modulation of TNFα-induced apoptosis by the different members of the Bcl-2 family [44]. It should be noted that Matsubara et al.

report an absence of Bcl-2 in human luteinized granulosa untreated or treated with TNFα, as revealed by immunocytochemistry. Most likely their detection assay was not sensitive enough to reveal small quantities of the Bcl-2 protein, as can be achieved by Western blot analysis. Moreover, we clearly find Bcl-2 mRNA expression in human granulosa–luteal cells using RT-PCR and Northern blots (Sasson R and Amsterdam A, unpublished results).

TNFα-mediated suppression of steroidogenesis was recently implicated with reduction in StAR intracellular levels in cultured porcine Leydig cells [45] and rat corpora lutea [21]. However, no studies on the correlation between TNFα induction of apotosis and TNFα attenuation of steroidogenesis have yet been reported. Our data suggest that both in human and rat granulosa cells, reduction in steroidogenesis and induction of apoptosis can be tracked in the same cell culture. We could not find a quantitative correlation between loss of steroidogenesis and increased incidence of apoptosis both in human primary and immortalized human granulosa cells. Namely, loss of steroidogenic activity was much more severe than increasing apoptosis. Since inactivation of MAPK activity could block the TNF inhibition of FK-induced steroidogenesis in immortalized human granulosa cells, it raises the possibility that TNFα exerts its anti-steroidogenic activity via activation of MAPK, which is in line with our recent observations [46] on the role of ERK1 and ERK2 phosphorylation on modulation of steroidogenesis in immortalized rat granulosa cells, exerted by lowering the intracellular level of StAR protein. Moreover, Glucocorticoids, which protect against TNFα-induced apoptosis, could not fully restore the steroidogenic activity attenuated by TNFα in human primary granulosa cells. These data suggest that TNFα-induced apoptosis, and reduction in steroidogenesis may involve different mechanisms.

Ovulation appears to be a cytokine-regulated process of inflammation, mediated by IL-6 and IL-7, followed by anti-inflammatory reactions mediated by an IL-1-receptor antagonist [47]. We suggest that TNFα may play an important role in this process, followed by anti-inflammatory action mediated by glucocorticoids. Indeed it was recently found that TNFα could up-regulate 11β HSD1, leading to the active follicular cortisol level [48]. Thus, ovarian glucocorticoid synthesis induced by TNFα may play an important role in the termination of the inflammation reaction evoked during ovulation and follicular rupture.

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