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17β-Estradiol Inhibits Tumor Necrosis Factor-α-Induced Nuclear Factor-κB Activation by Increasing Nuclear Factor-κB p105 Level in MCF-7 Breast Cancer Cells

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Tumor necrosis factor-α (TNF-α) exerts many cytological effects on a wide range of cells. TNF-α can activate nuclear factor-κB (NF-κB). Activation of NF-κB by TNF-α mediates many functions of TNF-α. The NF-κB inhibitor, IκBα, negatively regulates the activity of NF-κB. In MCF-7 cells (an estrogen and TNF-α receptor positive cell line), treatment with 17β-estradiol (E_2) inhibited TNF-α-induced NF-κB DNA binding activity in the gel retardation assays. But, the level of the IκBα and the TNF-α receptor, TNF-R1, were not obviously affected. The NF-κB precursor, NF-κB p105, has been shown to be associated with NF-κB in the cytoplasm and efficiently blocks its nuclear translocation and activation. Treatment of MCF-7 cells with E_2 increased the level of NF-κB p105 protein. The anti-estrogen, 4OH-tamoxifen, treatment inhibited E_2-induced NF-κB p105 expression. Our findings indicate that NF-κB p105 plays a role in modulating the functions of TNF-α in the estrogen receptor positive breast cancer cells.

Key Words: **17β-estradiol; TNF-α; IκBα; NF-κB p105; NF-κB; nuclear translocation; 4OH-tamoxifen; MCF-7 breast cancer cells.**

The growth of cells is influenced by cytokines produced by various host cell types. Cytokines surrounding a cell establish a complex microenvironment that has multiple effects on the cell. TNF-α is a polypeptide lymphoid cytokine that exerts cytological effects on a wide range of cells through binding to their cell surface receptor (1). TNF-α can activate NF-κB, activation of NF-κB mediates many cytological functions of TNF-α (2). For example, in regard to the suppression of tumor progression, NF-κB plays a role in the mediation of TNF-α-triggered activation of genes of many cytokines and factors that make up the immune system's response to cancer (3). Two TNF-α receptors, TNF-R1 (apparent molecular size 55–60 kDa) and TNF-R2 (apparent molecular size 75–80 kDa), have been identified (4). TNF-α-induced NF-κB activation primarily involves the TNF-R1 receptor (5).

NF-κB is a family of dimers composed of members of Rel/NF-κB proto-oncogene products present in the cytosol with the inhibitor factor, IκBα, as an inactive complex (6, 7). NF-κB activation requires the dissociation of IκBα and the subsequent nuclear translocation of NF-κB, where NF-κB presents as a primary p65/p50 heterodimer or a p50/p50 homodimer and binds directly to its cognate DNA sequence (8, 9). The p50 subunit of NF-κB is derived from the N-terminus of a precursor, NF-κB p105 (10–12). The C-terminus of NF-κB p105 is structurally and functionally similar to IκBα. The interaction of NF-κB p105 with NF-κB p50 or NF-κB p65 in the cytoplasm can also inhibit the nuclear transfer and activation of NF-κB (13–17).

Estrogen may promote the progression of a diverse of tumors, both within and outside the reproductive system. A cumulative analysis of tumor biopsies has shown estrogen receptors to be present in about 60% of primary breast tumors where they permit the mitogenic effect of estrogen (18). The cellular signaling transduction pathways are complex; multiple interactions among different signaling pathways may interfere with the function of one signaling inducer. We investigated whether estrogen could modulate TNF-α-induced NF-κB activation. We report here that 17β-estradiol (E_2) inhibited TNF-α-induced NF-κB activation in MCF-7 cells through inhibition of NF-κB nuclear translation. E_2 treatment increased the level of NF-κB precursor, NF-κB p105, but did not obviously affect TNF-α-triggered IκBα degradation. Our studies indicate that estrogen modulates the cytological functions of TNF-α by increasing NF-κB p105 level in the estrogen receptor positive cells.

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MATERIALS AND METHODS

Materials. 17β-Estradiol, 4OH-tamoxifen, and dextran-coated charcoal were from Sigma (St. Louis, MO). Human TNF-α was from Pepro Tech (Rocky Hill, NJ). Double-stranded NF-κB consensus binding oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′), mutant oligonucleotide (5′-AGTTGAGGCGACTTTCCCAGGC-3′), antibodies against NF-κB p65 (C-20), NF-κB p105 and NF-κB p50 (NLS), and IκBα/MAD-3 (C-21) were from Santa Cruz (Santa Cruz, CA). Antibody against TNF-α R1 receptor (MAB225) was from R&D (McKinley Place, MN). Avidin-biotin-peroxidase complex kit was from Vector Labs (Burlingame, CA). BCA protein assay kit was from Pierce (Rockford, IL).

Cell culture and treatment. The cell line MCF-7 is available from American Type Culture Collection (Manassas, VA). Dulbecco's modified Eagle medium (DMEM), fetal calf serum (FBS), penicillin, and streptomycin were from Gibco BRL (Life Technologies, Grand Island, NY). MCF-7 cells were maintained in DMEM media supplemented with 10% FBS, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in a cell culture incubator containing 5% CO_2 at 37°C. In every experiment, unless otherwise described, the cells (2×10^6) were grown in media with complete serum for 3 days, then they were washed two times with phosphate buffered saline (PBS) and were cultured overnight in DMEM media containing charcoal-pretreated FBS supplemented with or without 1 nM E_2. Charcoal treatment can remove the estrogen contained in FBS. The charcoal-pretreated serum was made by incubation of FBS with 0.5% dextran-coated charcoal at 37°C for 30 min, then filtered to remove the charcoal.

Gel retardation. At the time of harvest, cells were washed with ice-cold PBS. The nuclear proteins were extracted by adding 300 μl of cold hypotonic buffer (10 mM NaH_2PO_4, pH 7.0; 10 mM NaF; 5 mM $MgCl_2$; 1 mM EDTA; 1 mM PMSF; 1 mM DTT; 10 μg/ml leupeptin; 1 μg/ml aprotinin; and 1% NP-40) to the cell pellets and incubating the mixture on ice for 30 min. The resuspension was then centrifuged at 3000*g* for 30 min at 4°C, and the pellets were resuspended in 50 μl of ice-cold hypertonic buffer (10 mM NaH_2PO_4, pH 7.0; 10 mM NaF; 5 mM $MgCl_2$; 1 mM EDTA; 1 mM phenylmethanesulfonyl fluoride; 1 mM DTT; 10 μg/ml leupeptin; 1 μg/ml aprotinin; 1% NP-40; and 0.5 M NaCl). Cell debris was removed by centrifugation at 12,000*g* at 4°C for 30 min. The supernatant containing nuclear protein was stored at −70°C. Protein concentrations were determined with a BCA protein assay kit (Pierce, Rockford, IL). Equal amounts of protein (10 μg) were applied in the binding reaction. The binding reaction mixture (20 μl) containing 0.5 ng ^{32}P-labeled NF-κB oligonucleotide probe, 10 μg protein extract, 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol, and 1 μg poly(dI-dC) as nonspecific competitor DNA was incubated at room temperature for 20 min. The DNA–protein complex was resolved by 4% nondenatured PAGE. The gels were then dried, and the retarded signals were detected by X-ray film. In some reactions, 50-fold unlabeled NF-κB consensus or mutated oligonucleotides were added to verify the specificity of NF-κB complex formation.

Isolation of cytosol and nuclei-containing cell fractions. Cytosol and nuclei-containing cell extracts were separated by incubating cells in 200 μl hypotonic buffer (10 mM NaH_2PO_4, pH 7.5; 10 mM NaF; 5 mM $MgCl_2$; 1 mM EDTA; 1 mM PMSF; 10 μg/ml leupeptin; 1 μg/ml aprotinin; and 1% NP40) for 15 min at 4°C. Lysates were centrifuged at 1000*g* for 10 min at 4°C, the cytosol-containing supernatants were separated from the nuclei-containing pellet. The pellet was resuspended in 200 μl hypertonic buffer (i.e., hypotonic buffer containing 0.5 M NaCl) and incubated for 30 min at 4°C. The resuspension was centrifuged at 1000*g* for 10 min at 4°C, and the supernatant was nuclear extract.

Western blotting. Cells were harvested with a rubber policeman, washed with ice-cold PBS, and lysed in ice-cold RIPA buffer (25 mM Tris-HCl, pH 7.2, 0.1% SDS, 0.1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM phenylmethanesulfonyl fluoride, 10 μg/ml aporotinin, and 5 μg/ml leupeptin). The protein concentrations were determined by BCA protein assay kit (Pierce). In the Western blotting assay, 50 μg of each protein sample was loaded onto SDS-polyacrylamide gel. Western blot analysis was performed as described (19), and the levels of protein were detected by enhanced chemiluminescence with an ECL Western blotting detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK) or by the NBT-BCIP color development system.

Immunostaining. Cells were grown on glass slides. The samples were fixed in methanol and acetone (1:1) at −20°C for 10 min. Samples were blocked in PBS containing non-immune horse serum for 1 h. Samples were then incubated with anti-NF-κB antibody for 1 h at room temperature. The samples were subsequently incubated with biotin-labeled horse anti-mouse IgG (1:2,500) and then with avidin-biotin-peroxidase complex (Vector Labs, Burlingame, CA) for 30 min. Each incubation was followed by a 5-min wash in PBS. The samples were finally incubated with peroxidase substrate solution containing 0.05% diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide. The distribution of NF-κB was examined under a microscope.

RESULTS

17β-Estradiol Inhibits TNF-α-Induced NF-κB Activation in MCF-7 Cells

First we examined the effects of E_2 on TNF-α-induced NF-κB activity in MCF-7 cells cultured in estrogen-stripped serum (charcoal-treated serum) or in stripped serum supplemented with 1 nM E_2. The NF-κB activity was determined by gel retardation assay. MCF-7 cells expressed very low levels of endogenous NF-κB binding activity (Fig. 1A, lane 1), and 10 ng/ml TNF-α treatment induced a dense shifted NF-κB binding band (Fig. 1A, lane 2). But in the presence of E_2, TNF-α treatment only induced a faint shifted NF-κB binding band (Fig. 1A, lane 3). The shifted band formed by TNF-α treatment was competed with by the addition of wild type NF-κB consensus oligonucleotide (Fig. 1B, lane 2) but not by mutated NF-κB binding oligonucleotide (Fig. 1B, lane 1), indicating that the binding activity is specific to NF-κB. These results indicate that E_2 can inhibit TNF-α-induced NF-κB activation in MCF-7 cells.

TNF-α-Induced NF-κB Activation Is Accompanied by IκBα Degradation, and 17β-Estradiol Treatment Do Not Obviously Affect TNF-α-Induced IκBα Degradation

We further studied the mechanism by which E_2 regulated TNF-α-induced NF-κB activation. It is known that IκBα binds to NF-κB in the cytoplasm and plays a major role in the inhibition of NF-κB activation. We examined whether E_2 could affect TNF-α-induced IκBα degradation. The result in Fig. 2 shows that MCF-7 cells expressed an amount of IκBα, and it was rapidly degraded 3 h post-TNF-α treatment (Fig. 2A). E_2 treat-

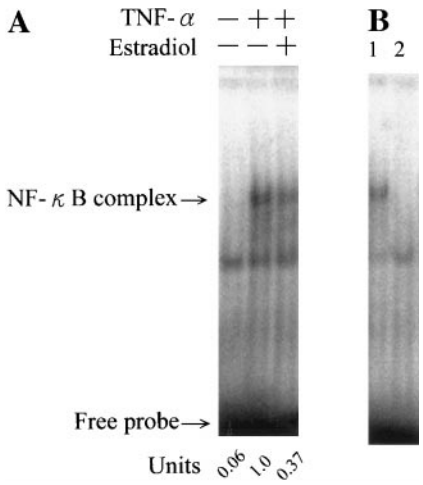

FIG. 1. 17β-Estradiol inhibits TNF-α-induced NF-κB DNA binding activity in MCF-7 cells. (A) Gel retardation assays of 10 μg nuclear protein extracted from MCF-7 cells treated with TNF-α (10 ng/ml) or 17β-estradiol (1 nM) for 3 h as indicated. The band intensities were quantified by densitometry. (B) The specificity of NF-κB complex formation was verified in TNF-α only samples by displacement with a 50-fold excess of the unlabeled mutated oligonucleotide (lane 1), and a 50-fold excess of the unlabeled consensus oligonucleotide (lane 2).

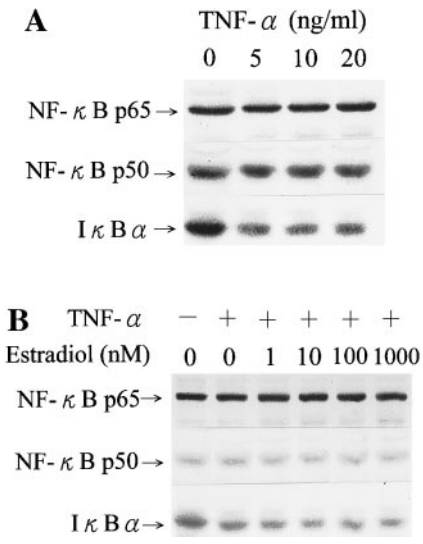

FIG. 2. Effects of 17β-estradiol on TNF-α-induced IκBα degradation. Western blot analysis of NF-κB p65, NF-κB p50, and IκBα protein level in MCF-7 cells treated with TNF-α (A), or TNF-α (10 ng/ml) plus 17β-estradiol (B) for 3 h as indicated.

ment did not obviously affect TNF-α-induced IκBα degradation at doses ranging from 1 to 1000 nM (Fig. 2B). Thus, the inhibition of TNF-α-induced NF-κB activation by E_2 is not through modulating the degradation of IκBα.

17β-Estradiol Treatment Do Not Decrease the Level of TNF-R1 Receptor in MCF-7 Cells

There is a possibility that E_2 might affect the expression of TNF-α receptor and thus inhibit TNF-α-induced NF-κB activation. The TNF-R1 receptor plays a primary role in TNF-α-induced NF-κB activation. We therefore assayed the effect of E_2 on expression of the TNF-R1 receptor in cells. As the data show in Fig. 3, MCF-7 cells expressed an amount of TNF-R1 receptor, and E_2 treatment did not decrease the level of TNF-R1 receptor in cells at doses ranging from 1 to 100 nM (Fig. 3).

17β-Estradiol Treatment Inhibits TNF-α-Induced NF-κB Nuclear Translocation

IκBα binds with NF-κB in the cytoplasm and inhibits NF-κB activation. After the cells are stimulated with a stimulator such as TNF-α, IκBα is degraded and NF-κB is subsequently transferred to the nucleus and activates its target genes. Therefore, we studied whether E_2 could affect NF-κB nuclear translocation. The distributions of NF-κB in MCF-7 cells treated with E_2 or TNF-α were analyzed. Both NF-κB p65 and NF-κB p50 are located primarily in the cytoplasm of MCF-7 cells before TNF-α stimulation (Fig. 4A, lanes 1 and 2). NF-κB p65 and NF-κB p50 were translocated to the nucleus 3 h post-TNF-α treatment (Fig. 4A, lanes 3 and 4). But when cells were co-treated with E_2, the TNF-α-stimulated nuclear translocations of NF-κB p65 and NF-κB p50 were attenuated (Fig. 4A, lanes 5 and 6). In the immunostaining assays, heavy staining of NF-κB p65 in the cytoplasm with unstained nucleus was observed in untreated MCF-7 cells (Fig. 4B). Nuclear staining of NF-κB p65 was observed in cells 3 h post-treatment with 10 ng/ml TNF-α (Fig. 4C). But in the presence of E_2, the staining of NF-κB p65 was primarily localized in the cytoplasm of TNF-α-treated cells (Fig. 4D).

Effects of 17β-Estradiol and 4OH-Tamoxifen on NF-κB p105 Protein Level in MCF-7 Cells

Our data show that E_2 inhibit nuclear transfer of NF-κB induced by TNF-α without obviously affecting the degradation of IκBα (Fig. 2). The NF-κB p50 precursor, NF-κB p105, has been shown to be associated with NF-κB in the cytoplasm and efficiently inhibits its

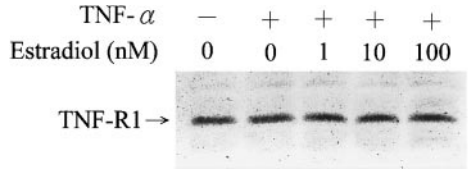

FIG. 3. Level of TNF-R1 receptor in 17β-estradiol-treated MCF-7 cells. Western blot analysis of TNF-R1 receptor level in MCF-7 cells treated with TNF-α (10 ng/ml) and 17β-estradiol for 3 h as indicated.

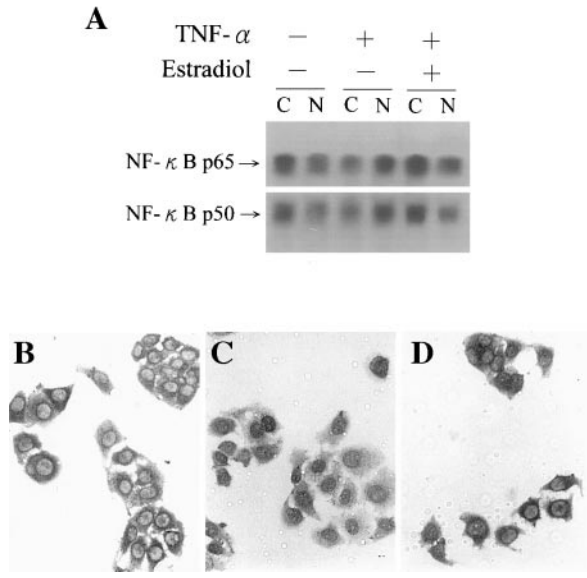

FIG. 4. 17β-Estradiol inhibits TNF-α-induced NF-κB nuclear localization in MCF-7 cells. (A) NF-κB p65 (upper panel) and NF-κB p50 (lower panel) levels of cytosol-containing (lanes 1, 3, and 5) and nuclei-containing (lanes 2, 4, and 6) cell extracts of MCF-7 cells treated with 17β-estradiol (1 nM) or TNF-α (10 ng/ml) for 3 h as indicated. (B–D) Immunostaining of NF-κB p65 in MCF-7 cells that left untreated (B), or treated with 10 ng/ml TNF-α (C), or 10 ng/ml TNF-α plus 1 nM 17β-estradiol (D) for 3 h.

nuclear translocation and activation. Therefore, we examined whether E_2 could affect the expression of NF-κB p105. Data in Fig. 5 show that E_2 treatment increased the level of NF-κB p105 protein in TNF-α-stimulated MCF-7 cells (Fig. 5A). E_2 itself was capable of increasing NF-κB p105 protein level in MCF-7 cells (Fig. 5B). 4OH-tamoxifen is an effective anti-estrogen agent, which can antagonize the effects of estrogen (20). Co-treatment with 4OH-tamoxifen inhibited the E_2-induced increase of NF-κB p105 protein level (Fig. 5C).

DISCUSSION

Nuclear translocation and activation of NF-κB classically involved the phosphorylation and dissociation of IκBα upon cell stimulation by various stimulators such as phorbol esters, cytokines, mitogenes, or some viruses (21). Thus, IκBα plays an important role in the regulation of NF-κB activation. Besides the IκB family, another mechanism of cytoplasm retention and inactivation of NF-κB is through the interaction of NF-κB with the NF-κB precursors, NF-κB p105 (13–17) or NF-κB p100 (22, 23). There are reports that demonstrate the inhibition of NF-κB through modulating the expression of the IκBα or NF-κB precursor molecules. In hepatocytes, dexamethasone inhibited TNF-α, IL-1β, and interferon-γ-induced NF-κB activation by upregulating the expression of IκBα (24). In HTLV-1 Tax-transfected monkey COS-7 cells, Tax protein was reported to be associated with NF-κB p105 and thus enhanced the nuclear localization of NF-κB without inducing degradation of IκBα (25, 26). Also, sequiterpene lactones, the extract of Mexican Indian medicinal plants, were reported to inhibit NF-κB by preventing the degradation of the IκB family (27).

Estrogen plays an important role in the initiation and progression of breast cancer cells, and the progression of breast cancer is often accompanied by altered function of the estrogen receptor (ER) (28, 29). Estrogen has been reported to inhibit NF-κB activated by various stimulators including phorbol ester, TNF-α, and lipopolysaccharide (30–32). It was reported that transfected, unliganded ER was able to repress NF-κB activity (33). In this circumstance, the ER may inhibit NF-κB activity through inhibiting the DNA binding activity of NF-κB by physically associating with NF-κB p65 and p50 (30, 33–35). But, data also showed that the presence of E_2 enhanced the NF-κB inhibition in ER-transfected cells (33). Thus, in addition to direct interaction of ER with NF-κB, other factors induced by E_2 may also be involved in the inhibition of NF-κB by E_2. We report here that TNF-α-induced NF-κB activation inhibited by E_2 is accompanied by increased expression of the NF-κB precursor p105 which possesses IκB function. In fact, E_2 itself was able to increase the level of NF-κB p105 (Fig. 5). In our data, E_2 treatment did not obviously affect IκBα levels (Fig. 2); a result the same

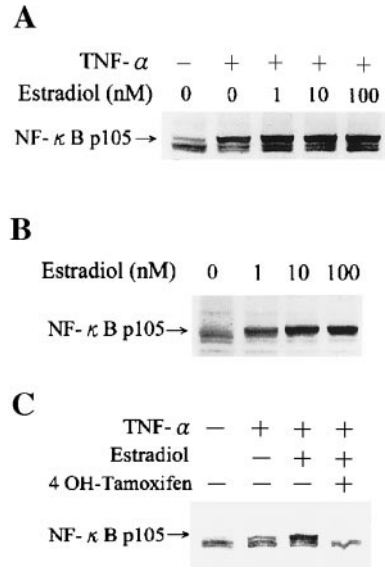

FIG. 5. Effects of 17β-estradiol and 4OH-tamoxifen on NF-κB p105 protein level in MCF-7 cells. (A) MCF-7 cells treated with 17β-estradiol and 10 ng/ml TNF-α for 3 h as indicated. (B) MCF-7 cells treated with 17β-estradiol for 3 h as indicated. (C) MCF-7 cells treated with 4OH-tamoxifen (100 nM), 17β-estradiol (1 nM), or TNF-α (10 ng/ml) for 3 h as indicated. Total cellular protein (50 μg) extracted from treated cells were subjected to SDS-polyacrylamide gel electrophoresis and Western blotting using anti-NF-κB p105 antibody.

with a previous report that E_2 did not significantly modulate IκBα levels in an ER- or IκBα-transfected U2-OS human osteoblast cell line (34). E_2 also did not decrease the expression of the TNF-R1 receptor which plays a primary role in TNF-α induced NF-κB activation (Fig. 3). Thus, neither TNF-R1 nor IκBα are involved in the modulation of E_2-induced NF-κB inhibition.

NF-κB p105 contains ankyrin repeats in the carboxyl terminal region and possesses IκB function (13). Characterization of the NF-κB p105 promoter showed some transcription factor regulatory sequences, including NF-κB, AP-1, and HIP-1, suggesting that the NF-κB p105 gene can be regulated by NF-κB or the AP-1 gene family (36). Since E_2 is an AP-1 activator (37), the increased NF-κB p105 expression induced by E_2 may result from the activation and interaction of the AP-1 transcriptional factor with the AP-1 element located on the promoter of NF-κB p105. Our data also show that TNF-α treatment induced some level of NF-κB p105 (Fig. 5A, lane 2). The increase of NF-κB p105 level by TNF-α treatment may result from the activation and interaction of the NF-κB transcriptional factor with the NF-κB element located on the p105 promoter. TNF-α has been reported to increase the expression of NF-κB p50 (36, 38). The increase of NF-κB p105 level by TNF-α treatment may probably serve as the pool of NF-κB p50. But, at the same time, TNF-α induced a greater IκBα decline in cells. As the result we show, TNF-α activated NF-κB in MCF-7 cells. However, in the presence of E_2, the induction of NF-κB p105 level by TNF-α was augmented. The increased NF-κB p105 could interact with NF-κB and block its nuclear translocation and activation, and thus E_2 attenuated TNF-α-induced NF-κB activity.

Recently, NF-κB has been shown to prevent cell apoptosis induced by various apoptosis stimuli (39). E_2 inhibits NF-κB activity and thus may enhance the TNF-α-induced cell apoptosis. But, in our studies, E_2 did not enhance the TNF-α-induced apoptosis in MCF-7 cells. On the contrary, E_2 treatment reduced the TNF-α-induced apoptosis in MCF-7 cells (data not shown). This is reasonable, since besides NF-κB, E_2 can also activate many genes and protein which may affect the survival of cells. In fact, estradiol can inhibit apoptosis of many cell types (40–42). Burow *et al*. have reported that estrogen prevent TNF-α-induced apoptosis in MCF-7 cells by increasing Bcl-2 expression (43).

NF-κB plays important roles in mediation of TNF-α-triggered activation of genes of many cytokines and factors of cells that make up the immune system's response to cancer (3). For example, the macrophages are also estrogen receptor positive and are involved in the immune system's response of TNF-α to cancer (44–46). Estrogen may also modulate NF-κB activity induced by TNF-α in macrophages. Thus, the fact that estrogen increases the NF-κB p105 level and inhibits TNF-α-induced NF-κB activation indicate that NF-κB p105 plays a role in modulating tumor progression.

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