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Role of NF-κB signaling pathway in increased tumor necrosis factor-α-induced apoptosis of lymphocytes in aged humans

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Abstract

In human aging, lymphocytes display increased sensitivity to tumor necrosis factor-α (TNF-α)-induced apoptosis. TNF-α induces both survival and apoptotic signals. The survival signal is mediated by the activation of NF-κB. Although a role of certain proapoptotic molecules in aging has been reported, a role of altered NF-κB signaling pathway has not been explored in detail. In this study, we have compared TNF-α-induced activation of NF-κB, phosphorylation of IκBα, and the expression of IKKβ between lymphocytes from young and aged humans. Furthermore, we have explored a role of IKKβ in increased susceptibility of lymphocytes from aged humans to TNF-α-induced apoptosis. Lymphocytes from aged humans displayed decreased activation of NF-κB, reduced phosphorylation of IκBα, and decreased expression of IKKβ. In addition, overexpression of IKKβ in lymphocytes from aged humans normalized TNF-α-induced apoptosis to the level of young subjects. These data suggest a deficiency of NF-κB signaling pathway and a role of IKKβ, at least in part, for increased sensitivity of lymphocytes from aged humans to TNF-α-induced apoptosis.

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Keywords: apoptosis; aging; TNF-α; NF-κB; T cells

Abbreviations: DD, death domain; EMSA, electrophoretic mobility shift assay; cIAPs, cellular inhibitor of apoptosis proteins; IKK, IκB kinase; MNC, mononuclear cells; TNF-α, tumor necrosis factor-α; TNFR, tumor necrosis factor receptor

Introduction

Tumor necrosis factor-α (TNF-α) exerts its biological activity by binding to type 1 and type 2 receptors (TNFR-1 and TNFR-2) and by activating several signaling pathways.[1–8] TNFRs belong to a large family of nerve growth factor receptors/TNFRs.[9–11] These are type I transmembrane receptors with one to five cysteine-rich repeats in their extracellular domains and a common death domain (DD) in their cytoplasmic tail. TNFR-1 contains DD whereas TNFR-2 lacks DD. Therefore, TNFR-1 signals both cell survival and cell death signals; whereas TNFR-2 primarily mediates a cell survival signal. However, recent data suggest that TNFR-2 might potentiate the death signal mediated by TNFR-1.[12–16] Both cell survival and cell death signals mediated by TNF-α require distinct sets of adapter and other downstream signaling molecules.[1–4]

NF-κB is a heterodimer of p50 and RelA (p65) subunits, which is sequestrated in the cytoplasm by its association with an inhibitory protein IκB. TNF-α induces phosphorylation of IκB at two serine residues, which triggers ubiquitination and subsequent degradation of IκB, resulting in the release and translocation of NF-κB to the nucleus where it binds to κB enhancer elements and induces gene expression, including antiapoptotic genes to provide a survival signal.[17–25] During human aging, both TNF-α production and TNF-α-induced apoptosis are increased.[26–29] In this study, we have examined whether decreased NF-κB survival signaling pathway plays a role in increased sensitivity of aged lymphocytes to TNF-α-induced apoptosis. Here, we provide evidence for a role of IKKβ (IκB kinase, IKK) in increased sensitivity of aged lymphocytes to TNF-α-induced apoptosis.

Results

TNF-α-induces less activation of NF-κB in T cells from aged humans

Since NF-κB plays an antiapoptotic role[22–25] and in aging TNF-α-induced apoptosis is increased,[27–29] we examined whether TNF-α-induced activation of NF-κB was different between lymphocytes from aged and young subjects. Lymphocytes from young and aged subjects were activated with 150 ng/ml of TNF-α for various time intervals and NF-κB.

DNA-binding activity was measured by electrophoretic mobility shift assay (EMSA) and ELISA assays. Figure 1 shows the results of EMSA analysis. The top panel shows a representative gel electrophoresis from one aged and one young subject and the bottom panel shows a quantitative mean of 10 young and 10 aged subjects. A decreased NF-κB activation was observed in aged subjects as compared to young subjects. Figure 2 shows data of ELISA assay for NF-κB activity from 10 young and 10 aged subjects. A significantly decreased ($P<0.05$) NF-κB activation was observed at each point of observation (5 min, 15 min, and 30 min). ELISA assay appears to be more sensitive than EMSA assay as the differences were observed at all points of observation with ELISA assay whereas significant differences between young and aged were observed only at 5 min of stimulation with TNF-α when analyzed by EMSA assay.

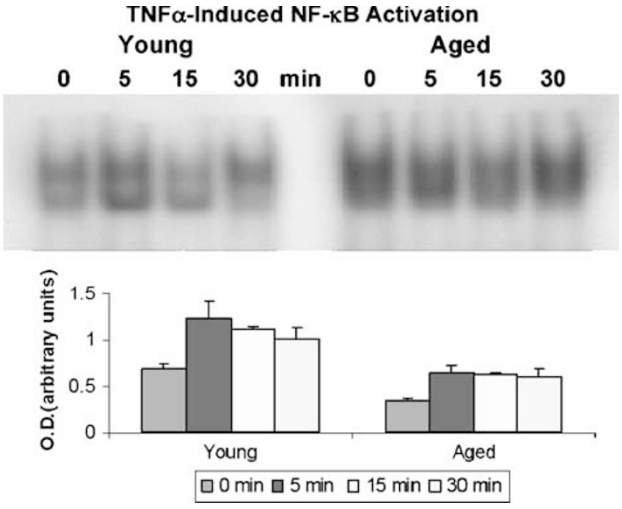

Figure 1 TNF-α-induced activation of NF-κB by EMSA assay. Lymphocytes from young and aged humans were activated with TNF-α for various time intervals and NF-κB activity was measured by EMSA. Top panel shows a representative gel from one young and one aged subject. Bottom panel shows quantitative analysis from 10 young and 10 aged subjects

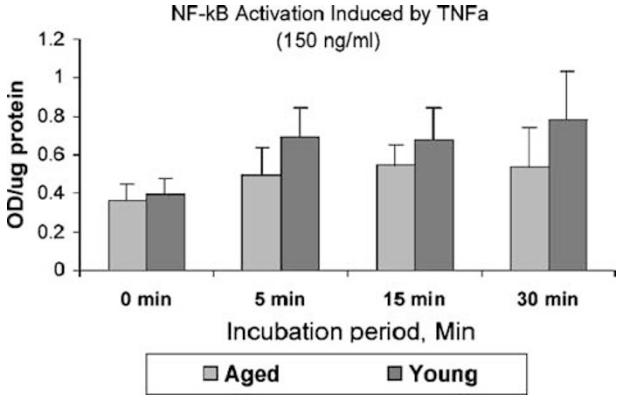

Figure 2 TNF-α-induced activation of NF-κB by ELISA assay. A time-dependent increase in NF-κB activity was observed; however, NF-κB activity in aged samples was significantly lower than in young subjects ($P<0.05$). Data are a mean ± S.D. of 10 subjects each

IκB phosphorylation is decreased in aged lymphocytes

NF-κB is held in the cytoplasm in an inactive state by IκB.[18–21] The ligation of TNFR with TNF-α results in the phosphorylation of IκB with subsequent ubiquitination and proteasomal degradation, leading to release of NF-κB, which then translocates to the nucleus and binds to its DNA binding sites to activate transcription of a number of genes.[20–22] Since we observed that NF-κB DNA-binding activity in aging is decreased, we examined if decreased NF-κB activity was due to decreased phosphorylation of IκB. Lymphocytes from young and aged subjects were activated with 150 ng/ml of TNF-α for 2 min, 5 min, and 10 min, protein was extracted and Western blotting was performed with anti-phosphorylated IκB monoclonal antibody (mAb). Actin was used as an internal loading control. The quantitative analysis was performed by

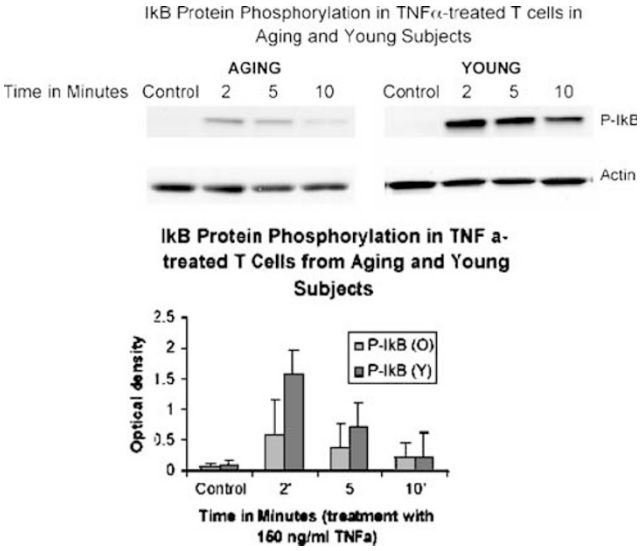

Figure 3 TNF-α-induced phosphorylation of IκB. Lymphocytes from young and aged subjects were activated with TNF-α for various time intervals and phosphor IκB was analyzed by Western blotting. Actin was used as internal loading control. The top panel shows a representative gel and the bottom panel shows quantitative analysis from 10 young and 10 aged subjects as determined by densitometry

densitometry. Figure 3 (top) shows a representative Western blot from one young and one aged subject. A marked decrease in phosphorylated IκB was observed in lymphocytes from aged subjects. Figure 3 (bottom) shows cumulative quantitative data (by densitometry) from 10 young and 10 aged subjects. A significant decreased phosphorylated IκB was observed at both 2 min ($P<0.01$) and 5 min ($P<0.05$), following activation of lymphocytes from aged subjects with TNF-α as compared to that in young subjects.

IKKβ expression is decreased in aging and increased expression of IKKβ *in vitro* corrects TNF-α-induced apoptosis in aged humans

IKKβ is essential for NF-κB activation and inhibition of TNF-α-induced apoptosis.[30–32] The phosphorylation of IκB is under the control of IKKβ,[33,34] and because IκB phosphorylation is decreased in aging, we examined the expression of IKKβ in lymphocytes from aged and young subjects. Lymphocytes from young and aged subjects were activated with anti-CD3, proteins were extracted and expression was determined by Western blotting, using specific mAbs. Figure 4 (top panel) shows a representative experiment; a decreased expression of IKKβ was observed in lymphocytes from aged subjects. The bottom panel (Figure 4) shows quantitative data from three young and three aged subjects with significantly decreased ($P<0.05$) expression of IKKβ in aging. To determine whether decreased expression of IKKβ was responsible for decreased phosphorylation of IκB and reduced NF-κB activity and therefore increased TNF-α-induced apoptosis, lymphocytes from both young and aged subjects were transfected with vector alone or IKKβ expression plasmid and then incubated with TNF-α and IKKβ expression and activity (phosphorylation

of IκB) was confirmed by Western blotting. IKKβ transfection resulted in increased IKKβ expression (Figure 8) and increased IKKβ activity as demonstrated by increased phosphoIκB (Figure 5). In addition, IKKβ overexpression resulted in increased NF-κB activity (Figure 6). IKKβ-tranfected lymphocytes in aged subjects showed increased NF-κB activity comparable to the basal NF-κB activity in young subjects. Figure 7 shows that overexpression of IKKβ resulted in decreased TNF-α-induced apoptosis in both young and aged subjects, resulting in comparable apoptosis between them.

Overexpression of IKKβ inhibits TNF-α-induced apoptosis by upregulating Bcl-2 and cIAP1

To determine the mechanism of IKKβ-induced inhibition of apoptosis, cells from aged and young subjects were treated with control plasmid and expression plasmid and examined for Bcl-2, Bcl-x_L, Bax, and cIAP1 (Cellular inhibitor of apoptosis protein, CIAP) expression by Western blotting. An over-expression of IKKβ resulted in the upregulation of both Bcl-2 and cIAP1 (Figure 8).

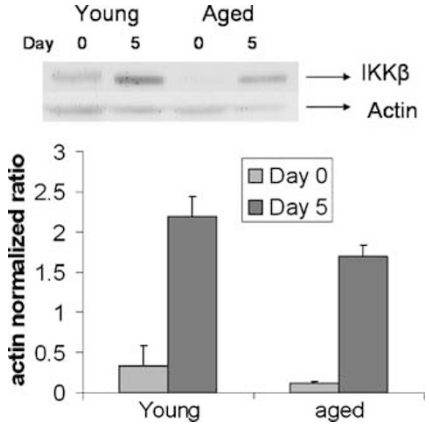

Figure 4 Expression of IKKβ in lymphocytes from aged humans. Lymphocytes from aged and young subjects were activated with anti-CD3 and IKKβ expression was measured at the baseline and on day 5 following activation by Western blotting. The top panel shows a representative gel from one young and one aged individual. The bottom panel shows a quantitative analysis (by densitometry) from three subjects from each group

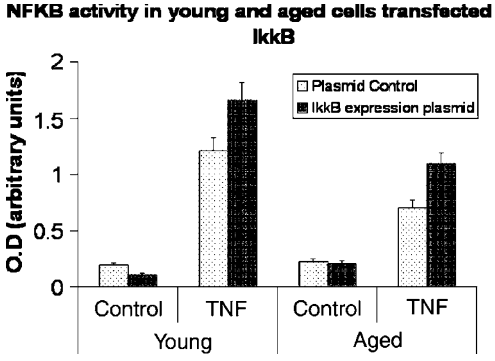

Figure 6 Effect of IKKβ transfection on NF-κB activity. To determine if the transfection of IKKβ was associated with increased NF-κB activity, lymphocytes transfected with control and expression plasmid and activated with TNF-α were analyzed for NF-κB DNA binding activity by ELISA assay. Data are mean of two such experiments

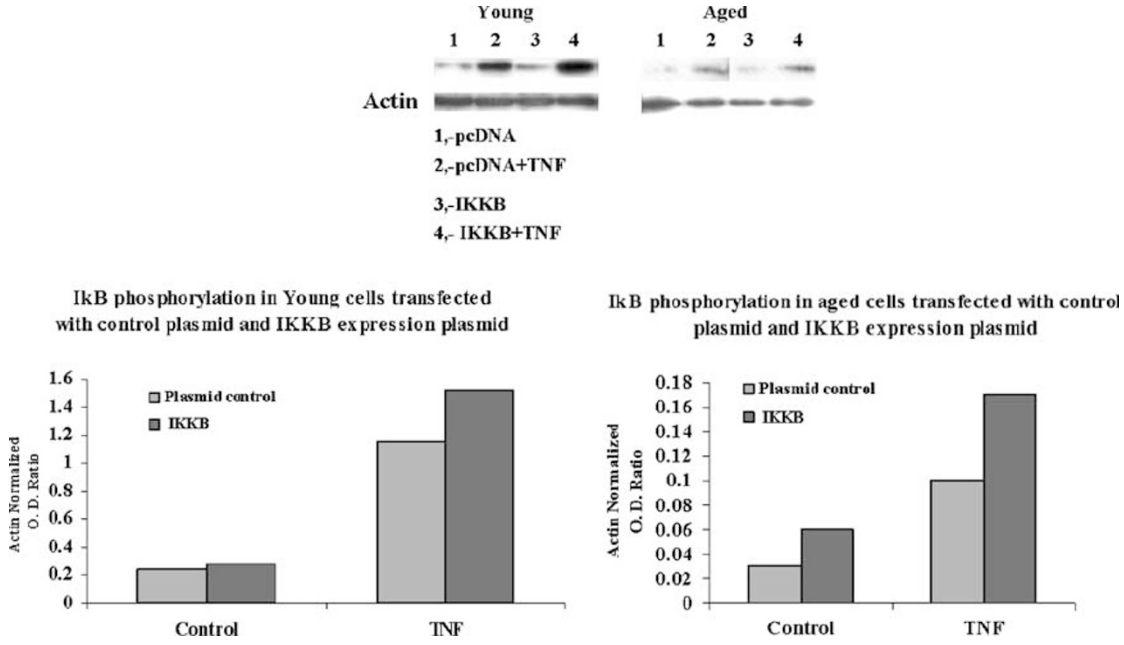

Figure 5 Effect of IKKβ transfection on IκB phosphorylation. To determine whether IKKβ transfection was associated with increased IKKβ activity, lymphocytes from young and aged subjects activated with TNF-α were transfected with control plasmid or expression plasmid and IκB phosphorylation was measured by Western blotting using antiphospho IκB antibody. Data show a mean from two such experiments. Please note the difference in the scale between the young and aged subjects. The top panel shows a Western blot from one aged and one young subject. Quantitative data from densitometry are shown in the bottom panel

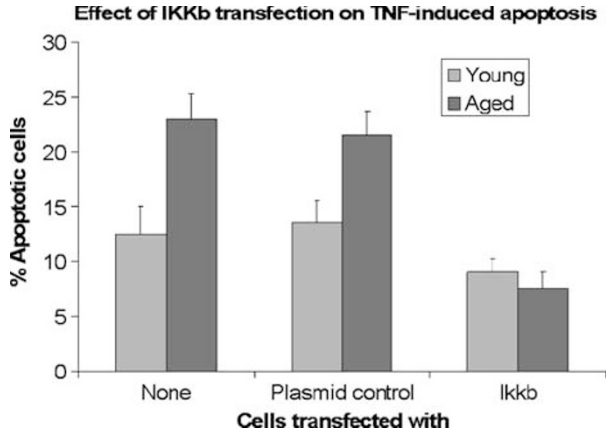

Figure 7 Effect of transfection with IKKβ on TNF-α-induced apoptosis. Lymphocytes from young and aged subjects were transfected with IKKβ expression plasmid or control vector and its effect on TNF-α-induced apoptosis was analyzed by TUNEL assay

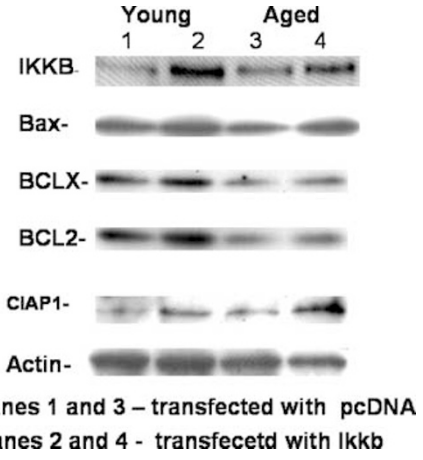

Figure 8 Effect of transfection with IKKβ on Bcl-2 family proteins and cIAP1. To determine the mechanism by which transfection with IKKβ resulted in decreased TNF-induced apoptosis, lysates from cells transfected with control and expression plasmid containing 25 μg of total protein were resolved by electrophoresis on 10% SDS-polyacrylamide gels and transferred onto PVDF membranes by electroblotting. The membranes were sequentially probed with the following antibodies: anti-Bcl-2, anti-Bcl-xL, anti-Bax, anti-cIAP, and anti-IκB. Blots were developed with ECL Plus detection system. To verify normalized protein loading and transfer efficiency, the blots were probed with antiactin antibody

Discussion

Aging is associated with progressive decline in immune functions,[35–38] which is assigned in part to increased apoptosis.[27–29,39–47] During aging there is an increased production of proinflammatory cytokines, including TNF-α.[26] Furthermore, lymphocytes from aged humans are associated with increased susceptibility to TNF-α-induced apoptosis in aged humans, which is associated with increased expression of TNFR-I and decreased expression of TNFR-2.[27] In addition, there appears to be an increased expression and activity of proapoptotic molecules.[27,41,48] However, a role of antiapoptotic pathway of apoptosis in TNF-α-induced apoptosis has not been studied in detail.

NF-κB is one of the transcription factors that play an important role in the regulation of immune response genes.[17–20] NF-kB exists as either heterodimers or homodimers of the subfamily of Rel family of proteins. The predominant form of NF-kB is a heterodimer comprising p50 (NF-κB1) and p65 (RelA). Other forms contain p52 (NF-κB2), RelB, and c-Rel subunits.[18] A number of genes, including cytokines, chemokines, cell surface receptors, and adhesion molecules, are targets of NF-κB.[49] In unstimulated cells, NF-κB is kept in the cytoplasm through interaction with the inhibitory proteins termed as IκB (inhibitor κB). When cells are exposed to inducers of NF-κB, such as TNF, IκB is phosphorylated, which is a signal for ubiquitination and degradation of IκB by the 26S proteasome. Free NF-κB dimmers are released and translocated to the nucleus, where they activate transcription of target genes. In this study, we observed decreased TNF-α-induced NF-κB activation in lymphocytes from aged subjects as determined by ELISA and EMSA assays. Trebilcock and Ponnappan[50] also reported decreased NF-κB activity in aged lymphocytes using EMSA assay. However, Aggarwal *et al*.[51] observed that diminished response of senescent human fibroblasts to TNF-dependent proliferation and cytokine production was not due to its effect on NF-κB activation.

IκB in response to proinflammatory signals (e.g. TNF-α) is phosphorylated by IKKβ.[33,34] Furthermore, it has been demonstrated that IKKβ is essential for the activating of NF-κB and protecting cells from apoptosis, including T cells from TNF-α-induced apoptosis.[30–32] In the present study, lymphocytes from aged humans had decreased expression of IKKβ and reduced phosphorylation of IκB. An overexpression of IKKβ in aged lymphocytes resulted in normalization of TNF-α-induced apoptosis, thus establishing a role of IKKβ in increased apoptosis in lymphocytes from aged humans.

NF-κB has been shown to inhibit apoptosis, especially that triggered by TNF-α.[21–23] The suppression of apoptosis by NF-κB depends on induction of a number of genes whose products regulate apoptosis, including Bcl-2 family[24,52–54] and IAP proteins.[25] cIAPs inhibit apoptosis by direct binding and inhibiting the activation of effector caspase-3 and caspase-7 and preventing activation of procaspase-9.[55] Therefore, decreased NF-κB activity may be associated with decreased Bcl-2/Bcl-X_L and/or cIAPs. Previously we have shown decreased Bcl-2[41] and cIAP expression in aged lymphocytes.[56] In the present study, we have demonstrated that overexpression of IKKβ resulted in increased IκB phosphorylation, increased NF-κB activity, upregulation of Bcl-2 and cIAP1, and inhibition of TNF-α-induced apoptosis, supporting a role of decreased NF-κB activity in increased sensitivity to TNF-induced apoptosis in lymphocytes from aged humans.

In summary, defects in NF-κB signaling pathway appear to be responsible, at least in part, for increased TNF-α-induced apoptosis in lymphocytes from aged humans.

Materials and Methods

Subjects

A total of 10 healthy young (18 to 30 y) and 10 healthy aged (66 to 88 y) persons, who are living independently and are of upper-middle social class, were the subjects for this study. There were seven female and three

male subjects in each group. The protocol was approved by the Institution Review Board of the University of California, Irvine. None of them had taken any antioxidants for at least 2 weeks prior to study.

Apoptosis

Apoptosis was measured by TUNEL assay. Cells (1×10^6/ml) were incubated for 48 h with or without 1 μg/ml of TNF-α. Cells were washed with PBS containing 1% BSA and 0.1% sodium azide and fixed in 2% paraformaldehyde for 30 min at room temperature. Cells were washed with PBS and permeabilized with sodium citrate buffer containing Triton X-100 for 2 min on ice. After washing, cells were incubated with FITC-dUTP in the presence of TdT enzyme solution containing 1 μM potassium cacodylate and 125 mM Tris-Hcl, pH 6.6 (In Situ Death Detection Kit, Boehringer Mannheim, Indianapolis, IN, USA) for 1 h at 37°C. Following incubation, cells were washed with PBS and 5000 cells were acquired and analyzed by FACScan.

Transfection of Mononuclear cells with IKKβ expression plasmid

Mononuclear cells (MNCs) were activated for 48 h with anti-CD3 mAb and subsequently cultured for 24 h in the medium supplemented with 10 ng/ml of IL-2 and transfected with Ikkβ expression plasmid using the Lipofectin reagent (GIBCO BRL, Gaithersberg, MD, USA). The activated MNCs were resuspended at 3×10^6 cells/ml in OPTI-MEM I medium, and 0.8 ml of the cell suspension was placed in each well of six-well plate. For liposome formation, 16 μl of Lipofectin was diluted in 84 μl of OPTI-MEM I medium and 2 μg of plasmid DNA was diluted in 98 μl of OPTI-MEM I medium. The vector plasmid without cloned gene was used as a negative control. After 45 min incubation at room temperature, the DNA and Lipofectin diluents were combined and incubated for 15 min at room temperature. Then, 200 μl of the DNA/Lipofectin mixture was added to each well and cells were incubated for 20 h at 37°C. IL-2 supplemented culture medium (3 ml) was added to each well and cells were allowed to express Ikkβ for 2 days. Expression of Ikkβ was confirmed by real-time RT-PCR.

NF-κB DNA-binding activity

Preparation of nuclear extract

Five million cells were washed with ice-cold PBS and ice-cold buffer A (10 nM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), and 0.2 mM phenylmethylsulfonyl fluoride (PMSF)). Cells were resuspended in 400 μl of buffer A, incubated on ice for 15 min, and homogenized by 15 passages through a 25-gauge needle. The nuclei were collected by centrifugation at $600 \times g$ for 6 min and resuspended in 30 μl of buffer C (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF). After 15 min incubation on ice, the nuclear extracts were microcentrifuged at 4°C for 2 min and the supernatants were collected and stored at −70°C until used for the gel mobility shift assay or NF-κB binding ELISA.

Gel mobility shift assay for NF-κB

The assay was performed using 10 μg of protein from each nuclear extract in 20 μl of binding mixture (10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, and 5% glycerol) containing 1 μg of poly(dI-dC) and 3×10^5 cpm of ^{32}P-labeled oligoprobe of NF-κB binding sequence (AGTTGAGGGGACTTTCCCAGG). The specificity of the binding was examined by competitive binding reaction with cold NF-κB oligoprobe before addition of ^{32}P-labeled oligoprobe. The binding mixture was incubated for 15 min at room temperature and 1 μl of 0.5 M EDTA was added to each reaction to terminate the binding reaction. The probe–protein complex was separated from the probe on a 6% Tris-glycine gel containing 10% glycerol and visualized by autoradiography.

ELISA for NF-κB activity

DNA-binding activity of NF-κB was assessed using an ELISA kit for NF-κB p65 according to the manufacturer's protocol (ActivMotif, San Diego, CA, USA). The 96-well plate was coated with the oligonucleotide specific for NF-κB binding and the bound NF-κB was measured using anti-NF-κB p65 antibody. Briefly, 30 μl of binding buffer was added to each well. NF-κB binding oligonucleotide or mutated oligonucleotide was added to the binding buffer for negative or positive controls, respectively. Then 20 μl of lysis buffer containing 10 μg of protein from each nuclear extract was added to each well and incubated for 1 h at room temperature with mild shaking. The plate was washed 3 times, treated with anti-NF-κB p65 antibody (1 : 1000 dilution) and incubated for 1 h at room temperature. The plate was washed, treated with HRP-conjugated secondary antibody (1 : 1000 dilution) for 1 h, and washed 4 times before addition of provided developing solution. The reaction was terminated in 5 min by addition of provided stop solution and optical density in each well of the plate was measured at 450 nm using the microtiter plate reader. DNA binding of NF-κB in each nuclear extract was presented as OD_{450} per milligram nuclear protein. This method provides advantages over EMSA: (1) a sensitive method without using radioactivity, (2) a large number of samples can be analyzed simultaneously.

Western blotting

Cells treated with or without TNF-α (150 ng/ml) for various time intervals were centrifuged and whole-cell extracts were prepared by lysing the cell pellet in 50 μl cold TGNT buffer with protease and phosphatase inhibitors (100 mM Tris-Cl pH 7.4, 20% glycerol, 100 mM NaCl, 2% Triton X-100, 20 mM EGTA, 100 mM NaF, 4 mM phenylmethylsulfonyl fluoride, 4 mM sodium orthovanadate, and 2 mM *p*-nitrophenol phosphate) and clarified by centrifugation at 4°C for 20 min. Protein concentration of the lysates was determined by Bradford assay (Bio-Rad, Richmond, CA, USA). Aliquots of cell lysates containing 25 μg of total protein are resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes by electroblotting. The membranes were blocked for 2 h at room temperature in TBS-T buffer with 5% nonfat dried milk, and sequentially probed by overnight incubation at 4°C with antiphospho IκB or IKKβ primary antibodies, anti-Bax, anti-Bcl-2, anti-Bcl-xL, anti-cIAP1 diluted in TBS-T buffer with 5% nonfat dried milk (1 : 2000 dilution; Transduction Laboratory, San Diego, CA, USA). The blots were washed 3 times for 15 min with TBS-T buffer and then incubated with HRP-conjugated anti-mouse secondary antibody (1 : 2000 dilution; Cell Signaling Technology, Beverly, MA, USA) for 1 h at room temperature. After washing 3 times for 20 min in TBS-T buffer, blots were developed with ECL Plus detection system (Amersham Pharmacia Biotech Inc, Piscataway, NJ, USA). Before each cycle of reprobing, blots were incubated at 50°C for 45 min in stripping buffer (62.5 mM Tris, pH 6.7, 2% SDS, and β-mercaptoethanol). To normalize protein loading and transfer efficiency, the blots were probed with anti-actin antibody (1 : 20 000 dilution).

Acknowledgements

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