THE ROLE OF NF-κB IN 1→3-β-GLUCAN (ZYMOSAN) INDUCED TNF-α PRODUCTION

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Abstract

The signal transduction pathway of inflammatory response to 1→3-β-glucans, a fungal cell wall component, is not well understood. Tumor necrosis factor (TNF)-α is an important cytokine in inflammation. The present study used zymosan A (a source of 1→3-β-glucans)-induced TNF-α production as a model to explore the signal transduction pathway of 1→3-β-glucans. The results show that zymosan A induced TNF-α production by mouse macrophage RAW264.7 cells in a time- and concentration-dependent pattern. The optimal concentration of zymosan A was 100 μg/ml for TNF-α stimulation. The TNF-α level reached a plateau around 12.5 hrs in the cell culture medium. The mechanism of zymosan A-induced TNF-α production was investigated at the gene transcription level. The gel shift assay was used to examine the DNA-binding activity of NF-κB in the zymosan A-treated cells. The results showed that NF-κB activity was increased by zymosan A, and this activation was associated with TNF-α production. A luciferase reporter controlled by the TNF-α promoter was utilized to examine promoter activity of the TNF-α gene in transiently transfected RAW264.7 cells. The results demonstrated that zymosan A could activate the gene promoter and the induced-promoter activity peaked at 24 hours after zymosan A stimulation. The promoter response depended on the NF-κB binding site as verified by using a NF-κB inhibitor (caffeic acid phenethyl ester) and by mutation of the NF-κB binding site in the promoter. Combined together, these results suggest that activation of NF-κB is a very important pathway by which zymosan A induces TNF-α expression.

Introduction

The role of fungi or yeast in organic dust toxic syndrome (ODTS) has attracted much attention recently. Zymosan A is a cell wall component in yeast Saccharomyces cerevisiae, and is used as a crude preparation for 1→3-β-glucans. Inhalation of zymosan A has been shown to induce an inflammatory response in animal experiments (Robinson et al., 1996). 1→3-β-Glucans are polymers of D-glucose which comprise the major structural components of fungal cell walls (Manners et al., 1973). 1→3-β-Glucans has been identified as a major reticuloendothelical stimulating component in zymosan (Riggi and Di Luzio, 1961). A broad range of cell types can be activated by zymosan A, such as macrophages (Tennent and Donald, 1976; Tapper and Sundler, 1995; Sorenson et al., 1998), polymorphonuclear (PMN) leukocytes (Morikawa et al., 1985; Adachi et al., 1997), and natural killer cells (Duan et al., 1994). The interaction of zymosan A with macrophages is generally considered as the first step in the initiation of immune response. Glucan-receptors play an important role in mediating binding of zymosan to macrophages (Tapper and Sundler, 1995). The zymosan-induced immune responses include the production of cytokines (Noble et al., 1993; Sakurai et al., 1997), hydrogen peroxide (Chiba et al., 1996), and arachidonic acid (Daum and Rohrbach, 1992). An intensive exposure to zymosan A leads to the infiltration of PMN and results in inflammation or toxicity.

TNF-α is a pro-inflammatory cytokine released mostly from macrophages or activated T cells in response to exposure to microbes and other agents. TNF-α plays a key role in the initiation of inflammation in the lung and other tissues. TNF-α acts as a chemotactic agent leading to accumulation of macrophages and PMN at the inflammatory site. Although expression of TNF-α is controlled at multiple levels, the transcriptional level is the first and most important step. Nuclear factor κB (NF-κB) is a critical transcription factor in the regulation of TNF-α transcription (Beg and Baltimore, 1996; Bohuslav et al., 1998; Carpentier et al., 1998). This transcription factor is a heterodimer protein composed of p65 and p50 in most cases. In addition to TNF-α, NF-κB is also involved in the regulation of gene transcription of many other cytokines (Blackwell and Christman, 1997). Nonactivated NF-κB is associated with an inhibitory protein, I-κB (Baeuerle and Henkel, 1994). I-κB is phosphorylated and degraded in response to inflammatory stimuli, leading to the release of NF-κB. The activated NF-κB translocates from the cytoplasm into the nucleus where it binds to promoter regions of target genes and regulates their transcription. When target genes are turned on by NF-κB, mRNA synthesis and protein expression will follow. Although it has been reported that 1→3-β-glucan is able to activate NF-κB (Battle et al., 1998) and induce TNF-α production (Ohno et al., 1995; Suzuki et al., 1996), the relationship between these two effects of 1→3-β-glucan remains to be established. This study investigates the mechanism of TNF-α expression induced by zymosan A at the cell and molecular level.

Materials and Methods

Cells and Reagents

The mouse macrophage cell line, RAW264.7, was purchased from the American Type Culture Collection (Rockville, MD). The cells were cultured in RPMI 1640 supplemented with 5 % fetal bovine serum (FBS), 2 mM glutamine, 25 mM HEPE

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buffer (pH=7.4), 100 U/ml of penicillin and 100 μg/ml of streptomycin. The cells were maintained in 75-cm^2 tissue culture flasks at 37°C in a humidified atmosphere of 5 % CO_2. Specific antibodies against the NF-κB p50 subunit (Cat # SC-114x, Santa Cruz Biotechnology) and p65 subunit (Cat # PC137, Oncogene) were used in the supershift assay. The NF-κB inhibitor, caffeic acid phenethyl ester (CAPE), was purchased from BIOMOL Research Lab (Plymouth Meeting, PA). CPRG (chlorophenolred-b-D-galactopyranoside monoso) was purchased from Bohringer Mannheim (Indianapolis, IN). Zymosan A and polymyxin B were obtained from Sigma (St. Louis, MO). Lipopolysaccharide (LPS) was supplied from DIFCO Laboratory (Detroit, Michigan).

Polymyxin B Pre-Treatment of Zymosan A

Endotoxin is a potent stimulator of TNF-α. In order to avoid any endotoxin contamination which may contribute to the stimulation of TNF-α by zymosan A, ploymyxin B (4.42 mg/ml, 1 ml) was used to pre-treat zymosan A (50 mg) for 10 min at room temperature. The treated zymosan A was centrifuged, washed with PBS once and kept in sterile PBS supplemented with 100 U/ml of penicillin, and 100 μg/ml of streptomycin to prevent bacterial growth.

Zymosan A Stimulation

For zymosan A stimulation, the normal cell culture medium was changed to 0.5 % FBS RPMI 1640 medium without phenol red. The stimulation time and zymosan A concentration were indicated in the text.

TNF-α ELISA Assay

An ELISA kit from Endogen (Woburn, MA) was used to determine the TNF-α level in the cell culture supernatant according to the manufacturer's instruction.

Statistical Analysis

Results are given as mean ± SEM. A paired one-tailed t-test (two-sample assuming equal variances) was performed and the differences were considered statistically significant for $p < 0.050$. All statistical analyses were performed by Microsoft® Excel 2000.

Nuclear Extraction

RAW264.7 cells were plated onto a 100-mm culture plate at a density of 8 $x10^6$ cells/plate 24 hrs before the experiment. Then, the cells were starved in RPMI 1640, with 0.5 % FBS for additional 24 hrs. The cells were treated with zymosan A or LPS for 4 hrs. At the end of treatment, the cells were harvested and treated with 500 μl lysis buffer (50 mM KCl, 0.5 % NP-40, 25 mM HEPES (pH 7.8), 1 mM PMSF, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 100 μM DTT) on ice for 4 min. After one minute centrifugation at 14,000 rpm, the supernatant was saved as a cytoplasmic extract. The nuclear pellet was washed once with the same volume of buffer without NP-40, then were treated with 300 μl of extraction buffer (500 mM KCl, 10 % glycerol with the same concentrations of HEPES, PMSF, leupeptin, aprotinin and DTT as the lysis buffer) and pipetted several times. After centrifugation at 14,000 rpm for 5 min, the supernatant was harvested as the nuclear protein extract and stored at -70°C. The protein concentration was determined using BCA protein assay reagent (Pierce, Rockford, IL).

Electrophoretic Mobility Shift Assay (EMSA)

EMSA assay was carried out as reported before (Ye et al., 1994): Briefly, the DNA-protein binding reaction was conducted in a 24 μl reaction mixture including 1 μg Poly dI.dC, 3 μg nuclear protein extract, 3 μg BSA, $4x10^4$ cpm of ^{32}P-labeled oligonucleotide probe and 12 μl of reaction buffer (24% glycerol, 24 mM HEPES (pH 7.9), 8 mM Tris-HCl (pH 7.9), 2 mM EDTA, 2 mM DTT). In some cases, the indicated amount of double stranded oligomer was added as a cold competitor. The reaction mixture was incubated on ice for 10 or 20 min (with antibody) in the absence of radiolabeled probe. The double-stranded IL-6 NF-κB probe was labeled with ^{32}P-ATP (Amersham, Arlington Heights, IL) using the T4 kinase (BRL, Gaithersburg, MD). It may be noted that although the IL-6 NF-κB probe was utilized for the measurement of NF-κB activation, it is expected that the same results would be obtained using TNF-α NF-κB probe. After addition of the radiolabeled probe, the mixture was incubated for 20 min at room temperature, then resolved on a 5 % acrylamide gel that had been pre-run at 200 V for 30 min with 0.5 x Tri-boric acid EDTA (TBE) buffer. The loaded gel was run at 200 V for 90 min, then dried and placed on Kodak X-OMAT film (Eastman Kodak, Rochester, NY) for autoradiography. The film was developed after an overnight exposure at -70°C.

Transfection and Luciferase Assay

Two TNF-α reporter vectors, the wild and the mutant, used in this study were gifts from Dr. S. T. Fan at the Scripps Research Institute (La Jolla, CA) (Yao et al., 1997). In the wild type vector, a TNF-α gene promoter fragment (-615/+15) controls the luciferase reporter gene. The mutant vector was derived from the wild type vector by point-mutation of the NF-κB (κB3) site in the promoter fragment. The murine macrophage cells ($0.5 x 10^6$ /well) were plated in a 24 well plate for 16 h, then were transfected with 0.5 μg reporter DNA per well using lipofectamine (Life Technologies, Grand Island, NY). After transfection, the cells were washed once in PBS solution and cultured in 1 ml of the RPMI medium with 0.5 % FBS at 37°C. Zymosan A was added 16 h later, and the cells were harvested at different times (as indicated) for the reporter assay. The luciferase activity was determined using an assay kit from Promega (Madison, WC) in combination with a luminometer (Monolight 3010, Analytical Luminescence Laboratory, Sparks, MD 21152).

β-Galactosidase Assay

β-Galactosidase is a useful internal control for normalizing variability in luciferase reporter activity due to transfection efficiency (Alam and Cook, 1990). One hundred microliter β-gal reaction buffer (80 mM Na_2HPO_4, 0.5M $MgCl_2$ and 104 mM 2-mercaptoethanol) and 20 µl CPRG (chlorophenolred-β-D-galactopyranoside monosodium salt) (80 mM) were added to 80 µl of cell lysate solution. The absorbance was measured after 20 min of incubation at 574 nm.

Results

Cells and Reagents

The mouse macrophage cell line, RAW264.7, was purchased from the American Type Culture Collection (Rockville, MD). The cells were cultured in RPMI 1640 supplemented with 5 % fetal bovine serum (FBS), 2 mM glutamine, 25 mM HEPE buffer (pH=7.4), 100 U/ml of penicillin and 100 µg/ml of streptomycin. The cells were maintained in 75-cm^2 tissue culture flasks at 37°C in a humidified atmosphere of 5 % CO_2. Specific antibodies against the NF-κB p50 subunit (Cat # SC-114x, Santa Cruz Biotechnology) and p65 subunit (Cat # PC137, Oncogene) were used in the supershift assay. The NF-κB inhibitor, caffeic acid phenethyl ester (CAPE), was purchased from BIOMOL Research Lab (Plymouth Meeting, PA).CPRG (chlorophenolred-b-D-galactopyranoside monoso) was purchased from Bohringer Mannheim (Indianapolis, IN). Zymosan A and polymyxin B were obtained from Sigma (St. Louis, MO). Lipopolysaccharide (LPS) was supplied from DIFCO Laboratory (Detroit, Michigan).

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Discussion

Zymosan A is a particulate 1→3-β-glucan that induces immune response by activating macrophages. Macrophages play an essential role in orchestrating the inflammatory response by the selective production of cytokines. In vitro studies have demonstrated that 1→3-β-glucans induce secretion of both interleukin 1 (IL-1) and TNF-α in mouse peritoneal macrophages (Ohno et al., 1996) and in human monocytes (Doita et al., 1991; Abel and Czop, 1992). Northern blot analysis showed that TNF-α mRNA was increased within 30 min, peaked at 2 hrs, and remained elevated for at least 8 hrs after exposure to 1→3-β-glucans in human monocytes. (Abel and Czop, 1992). The stimulation was mediated by β-glucan receptors (Abel and Czop, 1992). In vivo study shows that 1→3-β-glucans are able to induce a transient increase of both IL-1 and interleukin 6 (IL-6) in murine blood (Kondo et al., 1992). In addition to IL-1, TNF-α and IL-6, 1→3-β-glucans also induce expression of interleukin 8 (IL-8) (Noble et al., 1993). These cytokines may share a common mechanism in the response to 1→3-β-glucans.

Transcriptional regulation is a major mechanism controlling cytokine expression. Initiation of transcription is determined by the promoter region in a cytokine gene. NF-κB is an activator protein for many cytokine gene promoters including IL-1, IL-6, IL-8, and TNF-α (Baeuerle and Henkel, 1994). We hypotheses that NF-κB might be one of the major mediators of zymosan A signals for induction of these cytokines. The present study used TNF-α gene as a model for analysis of the transcriptional regulation of glucan response cytokine. The results show that the murine macrophage cell line, RAW264.7, is a proper in vitro system for studying macrophage response to zymosan A. The cells exhibited a similar pattern of TNF-α secretion to that measured with mouse primary macrophage cells or the human monocyte cell line. The cells produced TNF-α in a dose- and time-dependent manner, with the optimal TNF-α production at a zymosan A concentration of 100 μg/ml at 12.5 hrs post exposure (Fig. 1).

It has been suggested that gene transcription was activated quickly following 1→3-β-glucans exposure (Abel and Czop, 1992). In the present study, TNF-α promoter activity was investigated using a luciferase reporter. TNF-α promoter activity increased rapidly in the presence of glucan and remained elevated through 28 hrs. By 33 hrs after zymosan A stimulation, promoter activity had declined to basal levels.

Induction of TNF-α promoter activity by glucan is dependent on NF-κB activation. The role of NF-κB in the zymosan signaling pathway of TNF-α production was supported by the following results. (1) An increase in the DNA binding activity of NF-κB was observed in zymosan A-treated macrophages; (2) The NF-κB inhibitor, CAPE, inhibited zymosan A-induced DNA binding activity of NF-κB and decreased TNF-α production; (3) Mutation of the major NF-κB binding site (κ3) in the TNF-α gene promoter led to a dramatic reduction in the promoter response to zymosan A stimulation. Taken together, these data demonstrate that the transcription factor NF-κB mediates the TNF-α expression in macrophages in the response to zymosan A. Since IL-1, IL-6 and IL-8 gene promoters all contain NF-κB binding sites (Baeuerle and Henkel, 1994), we speculate that NF-κB might play a similar role in the induction of these cytokines by zymosan A.

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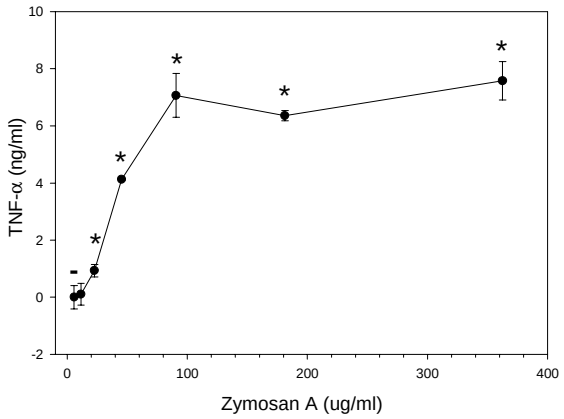

Figure 1A. Young et al. Dose-response curve for zymosan A-induced TNF-α production in RAW264.7 cells. The preparation of zymosan A was described in the section of Material and Methods. "*" indicates significantly higher than control "-".

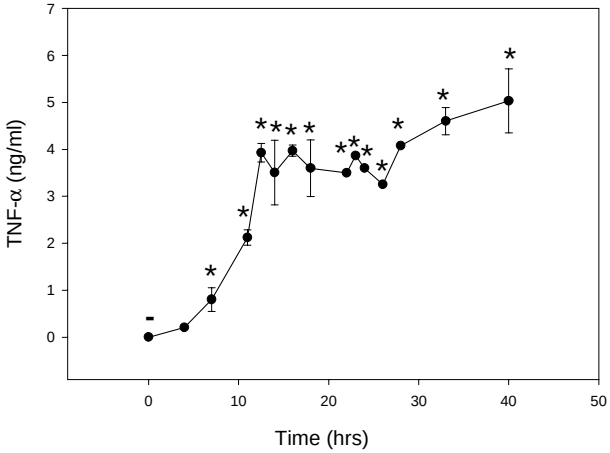

Figure 1B. Young et al. Time courses of zymosan A-stimulated TNF-α production in RAW 264.7 cells. The RAW264.7 cells were starved for 1 day before addition of zymosan A (100 μg/ml). The TNF-α level was measured in the supernatant by a TNF-α ELISA kit, as stated in the Materials and Methods. Values are means ± SEM of 3 experiments."*" indicates significantly higher than control "-".

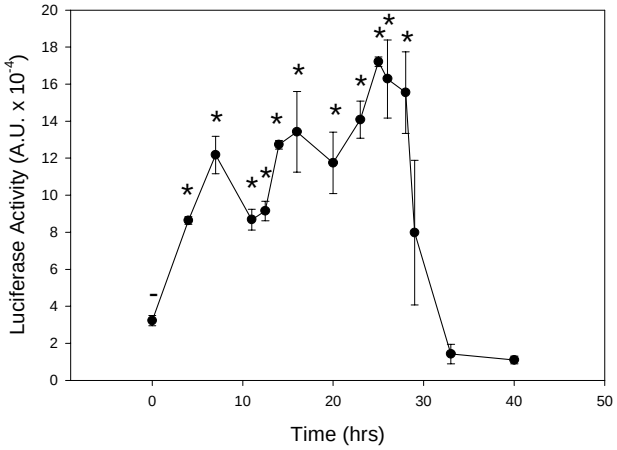

Figure 2. Young et al. The time course of zymosan A-stimulated TNF-α promoter activity in RAW 264.7 cells. RAW264.7 cells were transfected with a TNF-α wild type luciferase vector. Sixteen hour later, the cells were treated with zymosan A (100 μg/ml). The luciferase activity was measured in the cell lysate solution at various times after zymosan A treatment. Values are means ± SEM of 3 experiments.

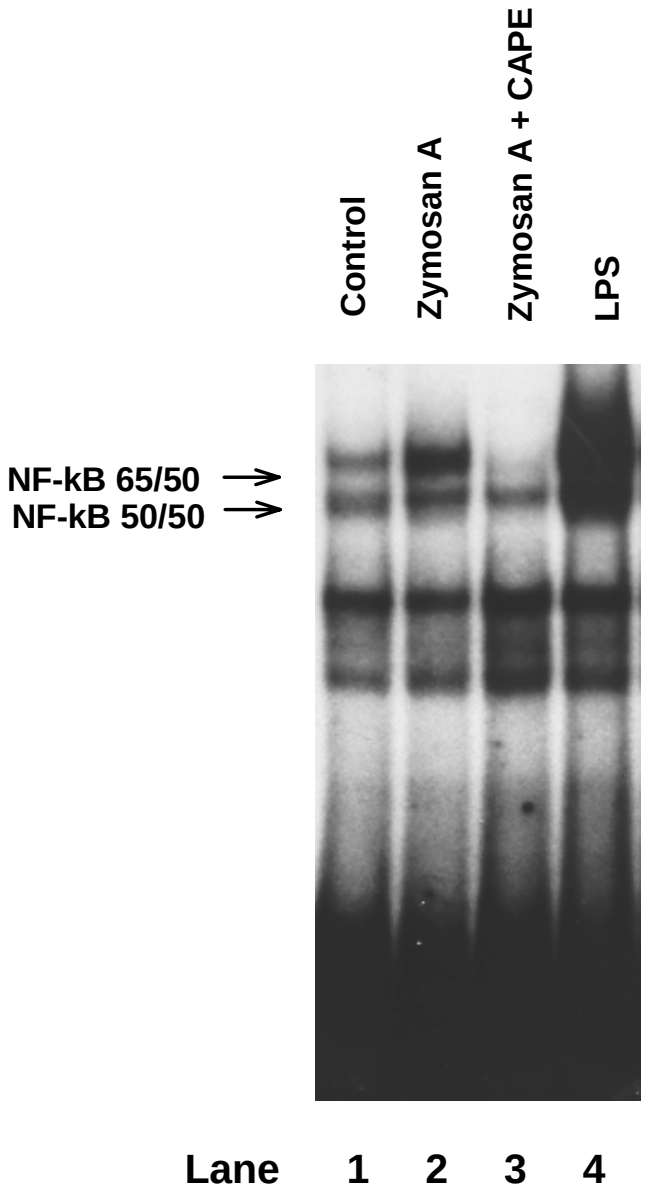

Figure 3A. Young et al. DNA binding activity of transcription factor NF-κB in the nuclear extract of RAW264.7 cells. The DNA binding activity of NF-κB in the nuclear extract was determined using the EMSA gel shift assay as stated in Materials and Methods. Lane 1, untreated cells. Lane 2, zymosan A-treated cells, which show enhanced NF-kB binding activity to the DNA. Lane 3, the NF-κB inhibitor, CAPE. Lane 4, the positive control LPS (10 μg/ml).

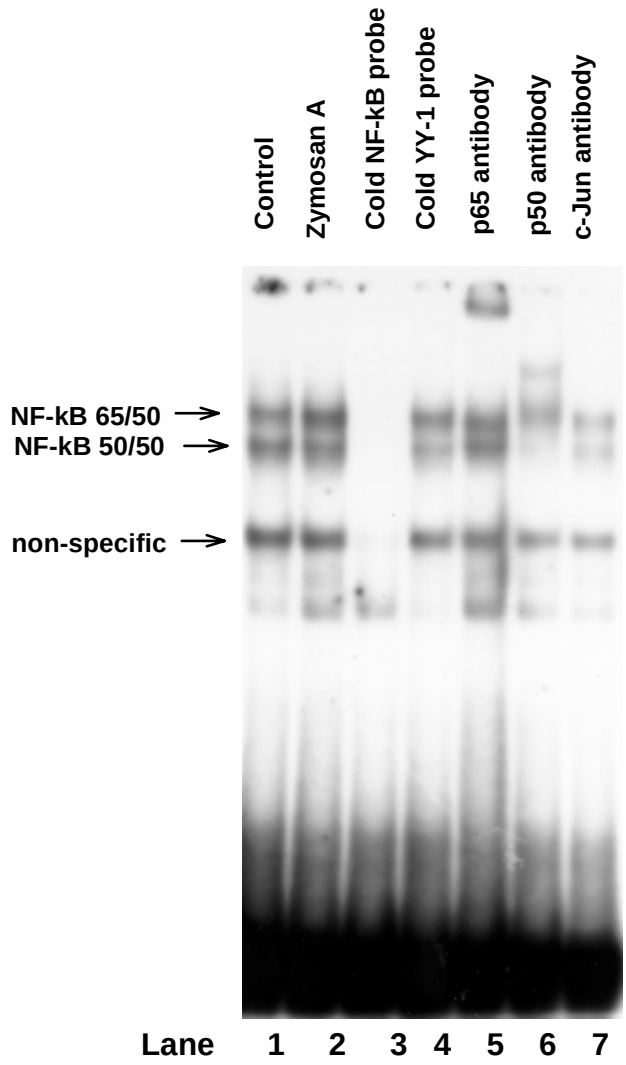

Figure 3B. Young et al. DNA binding activity of transcription factor NF-κB in the nuclear extract of RAW264.7 cells. Characterization of NF-κB complexes in the oligonucleotide competition assay and antibody supershift assay. Lane 1 served as a control for the oligonucleitide competition assay. Lanes 2-7 are nuclear proteins from the zymosan A-treated cells. The unlabeled NF-κB probe (0.2 μg) was used in Lane 3 as a specific competitor. The same amount of unlabeled YY-1 probe was used in the lane 4 for a nonspecific competition. The antibodys against the p65 or p50 subunit of NF-κB protein were added in Lane 5 or 6 to confirm the nature of DNA-protein complexes. Lane 7 contained 1 μg of c-Jun antibody to serve as a nonspecific antibody.

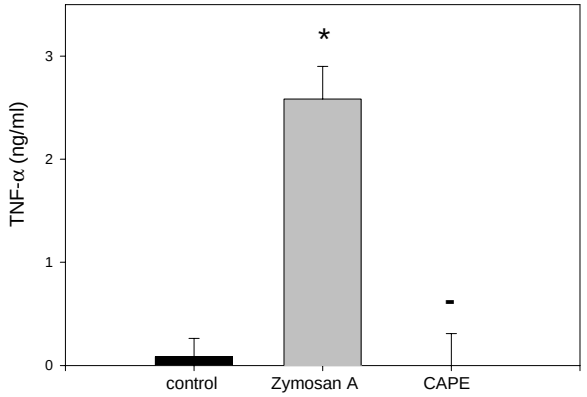

Figure 4. Young et al. Inhibition of TNF-α production by the NF-κB inhibitor, CAPE. RAW264.7 cells were pre-treated with CAPE (10 μg/ml) for 1 hr before addition of zymosan A into the culture medium. The TNF-α production was measured 24 hrs after zymosan A stimulation. Values are means ± SEM of 3 experiments. "*" indicates a significant increase from control. "-" indicates a significant decrease from zymosan A-induced level.

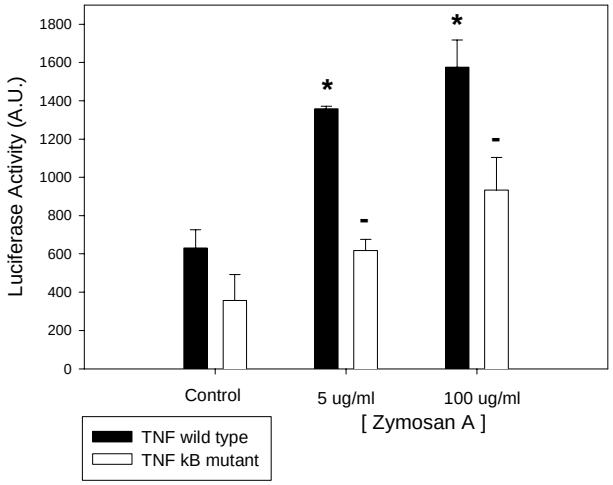

Figure 5. Young et al. The dependence of TNF-α gene expression on κB sites in the promoter. Zymosan A-induced promoter activity of TNF-α in the transient transfection assay. The wild type or κB mutated TNF-α luciferase reporters were transfected into the cells. The cells were treated with zymosan A 100 μg/ml for 24 hr. The reporter activity in the cell lysate was determined using the luminometer and the reading was normalized by β-galactosidase activity as stated in Materials and Methods. Values are means ± SEM of 3 experiments. "*" indicates a significant increase from the control. "-" indicates no significant change compared to control.