

Inhibition of Nuclear Transcription Factor- κ B by Specific I κ B Kinase Peptide Inhibitor

Navin Swaroop,¹ Fei Chen,² Liying Wang,² Sujatha Dokka,¹ David Toledo,³ and Yon Rojanasakul^{1,4}

Received June 11, 2001; accepted July 30, 2001

KEY WORDS: gene regulation; transcription factor; NF- κ B; I κ B; IKK; TNF- α .

INTRODUCTION

Aberrant activation and expression of genes is the major cause of many human diseases. Most genes are quiescent or have minimal activity in affecting physiologic processes. However, in certain pathologic conditions, these genes are abruptly turned on by a preexisting genetic switch, causing them to overexpress. The nuclear transcription factor- κ B (NF- κ B) is one such important factor that controls the genetic switch for many important genes that encode cytokines, growth factors, adhesion molecules, and some acute phase proteins (1). Abnormal activation of NF- κ B is associated with a number of diseases, including immune and inflammatory diseases (see Ref. 2 for review). Because of its pathophysiologic importance, the NF- κ B has been identified as a key target for pharmacologic manipulations. The purpose of this study is to investigate a gene inhibition approach using synthetic peptides that inhibit signal-induced degradation of the NF- κ B inhibitory subunit I κ B by I κ B kinases (IKK), thereby preventing NF- κ B activation.

NF- κ B belongs to a superfamily of protein dimers frequently composed of two DNA-binding subunits: NF- κ B₁ (p50) and RelA (p65) (3). It is normally kept in an inactive form in the cytoplasm by attachment of the inhibitory subunit I κ B. The activation of NF- κ B is accomplished by phosphorylation of the I κ B by specific I κ B kinases (IKK), which triggers the complete degradation of the inhibitor (4). The activated NF- κ B is then translocated into the nucleus where it binds to the promoter region of target genes and activates their transcription. The phosphorylation of I κ B by IKK occurs at two specific serine residues (ser32 and ser36), and mutation of these sites results in an inhibition of signal-induced phosphorylation and I κ B degradation (5). Because the interaction between the IKK and I κ B is sequence-specific, we hypothesize that specific peptides carrying the same amino

acid sequences as those of the I κ B recognition sites may be used to selectively inhibit I κ B phosphorylation and degradation, and thus NF- κ B activation. To test this hypothesis, we used hybrid peptides containing the normal or mutated sequence of the I κ B recognition site, covalently linked to a cell-permeable signal peptide to aid their cellular delivery. These peptides were then tested for their NF- κ B-inhibiting activity in signal-induced macrophage RAW 264.7 cell model.

MATERIALS AND METHODS

Cell Lines and Peptides

The macrophage cell line RAW 264.7 was obtained from the American Type Cell Culture Collection (Rockville, MD). Stably transfected RAW 264.7 cell line containing NF- κ B-luciferase reporter plasmid was prepared according to the method previously described (6). Peptides were synthesized by Alpha Diagnostic (San Antonio, TX). They were specifically modified to include C-terminal amidation and N-terminal acetylation to confer increased resistance to proteases. The peptides were purified by C₁₈ reverse-phase high-performance liquid chromatography and verified by mass spectrometry. Peptide 1 (see below) contains the I κ B recognition sequence for IKK (in italics) and a cell-permeable peptide sequence (in roman), derived from the hydrophobic region of the signal peptide fibroblast growth factor (7). This carrier peptide was previously shown to facilitate the cellular entry of other peptides into intact cells via a nonendocytic process (8). Peptide 2 is a control peptide containing a mutated I κ B sequence, linked to the same carrier peptide. Note that the essential serine residues that get phosphorylated by the IKK (ser32 and ser36) are replaced by alanine (underlined). The peptides were administered to the cells at the time of lipopolysaccharide (LPS) treatment.

Peptide 1: AAVALLPAVLLALLAPDDRHDSGLDSMKDE
Peptide 2: AAVALLPAVLLALLAPDDRHDAGLDAMKDE

Assays of Luciferase Activity and NF- κ B-Dependent Tumor Necrosis Factor (TNF- α) Expression

Luciferase activity was determined by using a luciferase assay kit (Promega, Madison, WI). Cells were washed and incubated for 10 min in 250 μ L of lysis buffer. Ten-microliter samples were loaded into an automated luminometer (Bio-Rad, Hercules, CA), and luciferase substrate (100 μ L) was injected into each sample. Luminescence output was quantitated as relative light units (RLU) per μ g protein of the sample. For analysis of TNF- α protein, cell-free supernatants were used. TNF- α levels were determined by using an ELISA kit (R&D System, Minneapolis, MN) according to the manufacturer's instructions.

Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared according to the method previously described (8). Nuclear protein-DNA binding reactions were conducted in a reaction mixture containing 3 μ g

¹ Department of Basic Pharmaceutical Sciences, West Virginia University, Morgantown, West Virginia 26506.

² Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, West Virginia 26505.

³ Merck & Co., Pharmaceutical Research Department, West Point, Pennsylvania 19486.

⁴ To whom correspondence should be addressed. (e-mail: yrojanasakul@hsc.wvu.edu)

nuclear protein extract, 1 μg poly dI.dC, 3 μg BSA, and 4×10^4 cpm of ^{32}P -labeled NF- κB oligonucleotide probe (5'-TGGGATTTTCCCATGAGTC T-3'). The probe was denatured at 80°C for 5 min and annealed with its complementary sequence at room temperature. The double-stranded probe was labeled with ^{32}P -ATP using T4 kinase. The mixture was incubated on ice for 10 min, with or without NF- κB /p50 antibody (200 μg ; Santa Cruz Biotechnology, Santa Cruz, CA), in the absence of radiolabeled probe and then 20 min at room temperature in the presence of probe. The mixture was resolved on a 5% polyacrylamide gel at 200 V for 90 min and then dried and developed on Kodak X-OMAT film after an overnight exposure at -70°C .

Western Blots

For I κB degradation studies, whole-cell proteins were extracted. The extracts were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The resolved proteins were transferred to a nitrocellulose membrane and incubated with rabbit polygonal anti-I $\kappa\text{B}\alpha$ serum raised against a peptide corresponding to amino acids 297–317 (Santa Cruz Biotechnology). After the membranes were washed, they were incubated with peroxidase-conjugated anti-rabbit IgG, and the antigen-antibody complexes were detected by using ECL Western blotting detection reagents.

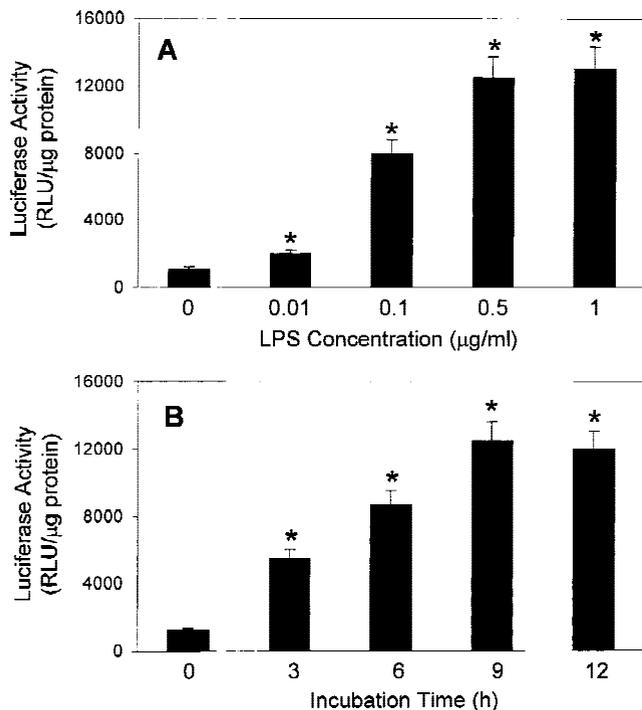


Fig. 1. Effect of LPS on NF- κB -dependent luciferase activity. Stably transfected RAW 264.7 cells containing NF- κB -luciferase reporter gene were treated with LPS in a 96-well plate (10^5 /well) at 37°C . (A) Dose effect of LPS (0–1 $\mu\text{g}/\text{mL}$) on luciferase activity determined 12 h after the treatment. (B) Effect of incubation time on luciferase activity determined at the LPS dose of 0.5 $\mu\text{g}/\text{mL}$. Data are shown as the mean \pm SEM, $n = 4$. *Indicates a significant increase over nontreated control ($P < 0.05$).

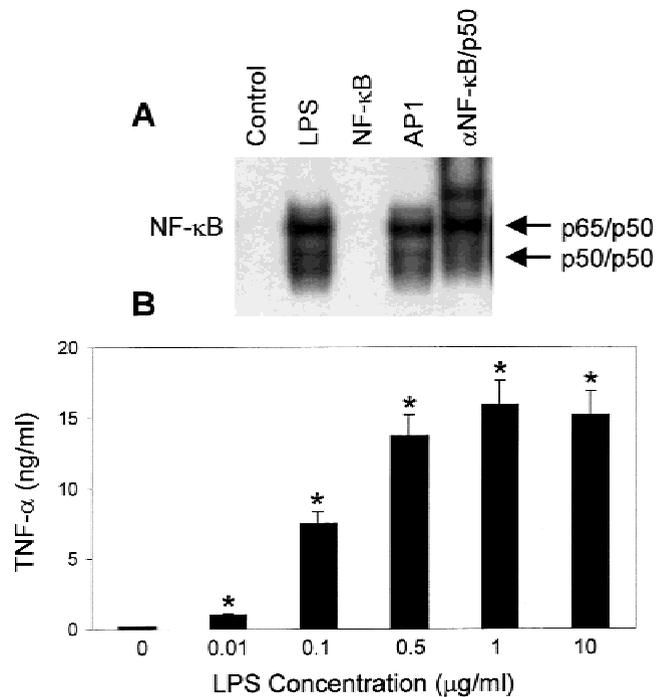


Fig. 2. Effects of LPS on NF- κB activity and TNF- α expression. (A) RAW 264.7 cells (10^5 /well) were treated with LPS (0.5 $\mu\text{g}/\text{mL}$) for 1 h and the NF- κB -DNA binding activity was determined by EMSA. (B) Cells were treated with LPS (0–10 $\mu\text{g}/\text{mL}$) for 12 h after which the TNF- α protein levels were determined by ELISA. Maximal NF- κB activity and TNF- α expression induced by LPS were found to be approximately 1 h and 12 h, respectively. Data are shown as the mean \pm SEM, $n = 4$. *Indicates a significant increase over nontreated control ($P < 0.05$).

RESULTS AND DISCUSSION

Luciferase Activity of Stably-Transfected Reporter Cells

A stably transfected RAW 264.7 cell line containing the NF- κB -luciferase reporter gene was used to assess the transcriptional activity of NF- κB . The cells were treated with LPS (0.01–1 $\mu\text{g}/\text{mL}$), and their luciferase activities were determined. Figure 1A shows that LPS was able to induce NF- κB activation in a dose-dependent manner. The optimal dose range of LPS stimulation was 0.5–1 $\mu\text{g}/\text{mL}$. Higher doses of LPS, i.e., 10 $\mu\text{g}/\text{mL}$, did not result in further activation of NF- κB . At the optimal dose range, LPS did not cause any cytotoxic effects as assessed by lactate dehydrogenase assay (result not shown). Time course studies (Fig. 1B) show that a saturation level of NF- κB activity was reached in about 9 h at the LPS concentration of 0.5 $\mu\text{g}/\text{mL}$.

NF- κB -DNA Binding Activity and TNF- α Expression

To test whether the enhanced reporter gene expression by LPS is causally related to the increased NF- κB -DNA interaction, EMSA studies were carried out. Figure 2A shows that LPS was able to induce the DNA binding activity of NF- κB as indicated by the strong NF- κB bands on the gel (lane 2). The binding specificity of NF- κB was determined by oligonucleotide competition and antibody supershift assays. The results show that the NF- κB binding activity could be competed by a nonlabeled NF- κB oligonucleotide probe

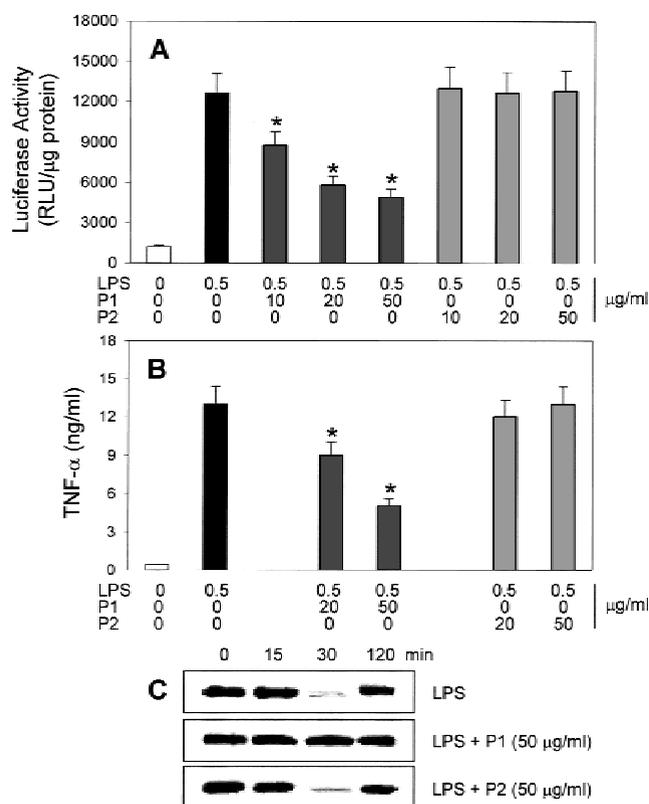


Fig. 3. Effects of P1 and P2 on LPS-induced luciferase activation, TNF- α production, and I κ B degradation. Stably transfected RAW 264.7 cells (10^5 /well) were treated with LPS ($0.5 \mu\text{g/ml}$) in the presence or absence of P1 or P2 (0 – $50 \mu\text{g/ml}$). (A) Luciferase activity determined 12 h after the treatment. (B) TNF- α levels determined 12 h after the treatment. (C) I κ B degradation determined at different time points after the treatment. Data are shown as the mean \pm SEM, $n = 4$. *Indicates a significant decrease over LPS-treated control ($P < 0.05$).

(lane 3) but not by a nonspecific AP-1 probe (lane 4) and that the antibody specific to NF- κ B p50 subunit caused a band shift of the NF- κ B complexes (lane 5). These results indicate the DNA binding specificity of NF- κ B under the experimental conditions.

The activation of NF- κ B should lead to an increased expression of its affected genes. Because TNF- α has been reported to be under the transcriptional control of NF- κ B (9), we tested whether LPS-induced NF- κ B activation would lead to an increased expression of TNF- α . Our ELISA results show that TNF- α expression did increase upon LPS stimulation (Fig. 2B). This effect was dose dependent and could be inhibited by specific NF- κ B inhibitors (see below).

Effects of Peptides on Luciferase Activity, TNF- α , and I κ B Degradation

To test whether the peptides could inhibit NF- κ B activation, we treated the cells with LPS ($0.5 \mu\text{g/ml}$) in the presence or absence of varying concentrations of P1 or P2 (0 – 50

$\mu\text{g/ml}$), and the luciferase activity and TNF- α protein expression were determined. The results show that P1, which contains the active recognition sequence of I κ B, was able to inhibit both the luciferase activity (Fig. 3A) and TNF- α production (Fig. 3B), whereas the mutated control P2 had no effects. Parallel LDH studies showed that these peptides were not toxic to the cells under the experimental conditions (results not shown).

To test whether the observed inhibitory effects were a result of blockage of I κ B degradation caused by LPS, Western blot analysis of I κ B was carried out. Figure 3C shows that LPS was able to induce a time-dependent degradation of I κ B, which was maximal at 30 min post-treatment, and that P1 effectively inhibited this degradation. In contrast, P2 had only minimal effect on I κ B degradation (Fig. 3C). These results strongly indicated that the inhibitory effects of P1 on LPS-induced NF- κ B activation were due to blockage of I κ B degradation.

In summary, we have demonstrated that transcriptional inactivation of NF- κ B can be achieved by using functional peptides that inhibit I κ B degradation. This inhibition requires the presence of active I κ B recognition sequence, and mutation of this sequence negates the inhibitory effect, indicating sequence specificity of the effect. This inhibitory peptide may be used as a research tool or as a novel therapeutic agent for diseases whose etiology depends on NF- κ B activation.

ACKNOWLEDGMENT

This work was supported by the National Institutes of Health Grant HL62959.

REFERENCES

1. T. S. Finco and A. S. Baldwin. Mechanistic aspects of NF- κ B regulation: emerging role of phosphorylation and proteolysis. *Immunity* **3**:263–272 (1995).
2. F. Chen, V. Castranova, X. Shi, and L. M. Demers. New insights into the role of nuclear factor- κ B, a ubiquitous transcription factor in the initiation of diseases. *Clin. Chem.* **45**:7–17 (1999).
3. P. A. Baeuerle and T. Henkel. Function and activation of NF- κ B in the immune system. *Annu. Rev. Immunol.* **12**:141–179 (1994).
4. B. M. Traenckner, S. Wilk, and P. A. Baeuerle. A proteasome inhibitor prevents activation of NF- κ B and stabilizes a newly phosphorylated form of I κ B- α that is still bound to NF- κ B. *EMBO J.* **13**:5433–5441 (1994).
5. E. B. Traenckner, H. L. Pahl, T. Henkel, K. N. Schmidt, S. Wilk, and P. A. Baeuerle. Phosphorylation of human I κ B α on serine 32 and 36 controls I κ B α proteolysis and NF- κ B activation in response to diverse stimuli. *EMBO J.* **14**:2876–2883 (1995).
6. X. Zhang, J. Ye, L. Wang, J. Manosroi, X. Shi, and Y. Rojanasakul. Rapid and sensitive assay of tumor necrosis factor- α gene transcription. *Pharm. Res.* **18**:408–411 (2001).
7. G. von Heijne. The signal peptide. *J. Memb. Biol.* **115**:195–201 (1990).
8. Y. Rojanasakul, L. Luo, J. Ye, J. Antonini, and D. Toledo. Cellular delivery of functional peptides to inhibit cytokine gene expression. *J. Control. Release* **65**:13–17 (2000).
9. M. A. Collart, P. A. Baeuerle, and P. Vassal. Regulation of tumor necrosis factor alpha transcription in macrophages: involvement of four κ B-like motifs and of constitutive and inducible forms of NF- κ B. *Mol. Cell Biol.* **10**:1498–1506 (1990).