BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **231,** 99–102 (1997)
ARTICLE NO. RC976057

Tetrandrine Inhibits Signal-Induced NF-κB Activation in Rat Alveolar Macrophages

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Received December 13, 1996

Tetrandrine is a bisbenzylisoquinoline alkaloid isolated from a natural Chinese herbal medicine. While this alkaloid has been shown to exhibit antifibrotic and anti-inflammatory activities, its mechanism of action is unknown. The present study was designed to investigate the inhibitory effect of tetrandrine on NF-κB activation in the alveolar macrophage. Three different provocative stimuli were used to activate NF-κB in these cells. The results indicate that tetrandrine can inhibit the activation of NF-κB and NF-κB-dependent reporter gene expression by LPS, PMA, and silica in a dose-dependent manner. In contrast, at the doses used, tetrandrine did not interfere with Sp-1 DNA binding activity or Sp-1-dependent reporter gene expression in these cells. Western blot analysis suggests that the inhibitory effect of tetrandrine on NF-κB activation can be attributed to its ability to suppress signal-induced degradation of IκBα, a cytoplasmic inhibitor of the NF-κB transcription factor.

Inappropriate activation of NF-κB is considered to be a crucial step in the pathological processes of inflammation, injury, stress, radiation, cancer, atherosclerosis, and viral replication (i.e. HIV-1) (1-5). For that reason, control of NF-κB activation is a potential therapeutic strategy to reduce the untoward tissue damage. Recent studies have shown that a major part of anti-inflammatory effects of several widely prescribed drugs such as NSAID's (including aspirin) and glucocorticoids can be attributed to their ability to suppress NF-κB activation (6,7). The liberal use of these drugs however, can be limited due to their well known side effects. Tetrandrine, a bisbenzylisoquinoline alkaloid, isolated from a traditional Chinese herbal medicine, has been used in China for several decades for the treatment of silicosis and arthritis, two disease states associated with considerable inflammatory mediator release (8-10). Although there have been a number of studies describing the anti-inflammatory effects of tetrandrine, its mechanism of action is still unknown. Recent studies have shown that silica, a pro-inflammatory substance, can induce the activation of NF-κB transcription factor (11, 12). In the studies reported here, using the rat alveolar macrophage cell line NR8383, we investigated the effects of tetrandrine on the activation of NF-κB transcription factor induced by several extracellular inflammatory stimuli including bacterial endotoxin, a phorbol ester and silica.

MATERIALS AND METHODS

Reagents. Crystalline silica (particle diameter 2-7μm) was provided by U.S. Silica Corp., (Berkeley, Springs, WV). Lipopolysaccharide (LPS) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma Chemical Co. (St. Louis, MO). All molecular biology agents, non-radioactive cell proliferation assay kits and luciferase assay kits were from Promega (Madison, WI). [^{32}P]-dCTP was obtained from DuPont NEN (Boston, MA). All polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell line and culture. The rat alveolar macrophage cell line NR8383 was a gift provided by Dr. Ronald Helmke (University of Texas Health Science Center, San Antonio, TX) (13). The cells were maintained in F12 medium (Mediatech, Washington, DC) supplemented with 15% fetal bovine serum, 2 mM glutamine, and 1000 units/ml penicillin-streptomycin. For the stimulation assay, 2×10^6 cells were seeded into 6-well culture plates in 5 ml medium and stimulated with 5 μg/ml LPS, 40 μg/ml PMA or 100 μg/ml silica and indicated agents for indicated time periods. Cell viability was determined by a non-radioactive cell proliferation assay system according to the provided protocol. Cell viability was expressed as the absorbance at 562-630 nm.

Nuclear extracts. Nuclear extracts were prepared as previous described (11, 12). NR8383 cells were cultured in 5% FCS in 6-well plates at 2×10^6 cells/ml for 3 days, the medium was then replaced with fresh medium and cultured with LPS, PMA or silica with or without tetrandrine for various time periods as indicated in the Fig-

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0006-291X/97 $25.00

ure legends. At the end of culture period, the cells were harvested and resuspended in hypotonic buffer A (10 mM HEPES, pH 7.6, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF) for 10 min on ice, then vortexed for 10 sec. Nuclei were pelleted by centrifugation at 12000 × g for 20 sec and were resuspended in buffer C (20 mM HEPES, pH 7.6, 25% glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF) for 30 min on ice. The supernatants containing nuclear proteins were collected by centrifugation at 12000 × g for 2 min and stored at −70°C.

Electrophoretic mobility shift assay (EMSA). The preparation of ^{32}P-labeled double-stranded oligonucleotide containing NF-κB or Sp-1 consensus sequence was performed as described (12). Briefly, single-stranded DNA was synthesized using a Millipore Cyclone Plus automated synthesizer. To prepare double-stranded DNA, the first strand of DNA was annealed with a complementary decameric primer to its 3′-tail in 2 × anneal buffer. The second strand was extended with DNA polymerase Klenow fragment in a reaction mixture containing 250 μCi [^{32}P]dCTP and 5 mM dATP, dGTP and dTTP. For EMSA, 4 μg of nuclear extract was mixed with the labeled double-stranded probe and incubated at room temperature for 30 min. The reaction solution was electrophoresed on native 6% polyacrylamide gel in 0.25 × TBE buffer for 2 to 3 h.

Western blot analysis. Whole-cell proteins (10-15μg) were subjected to electrophoresis in a 12% SDS-polyacrylamide gel. Resolved proteins were transferred to a nitrocellulose membrane and incubated with affinity-purified rabbit polyclonal anti-IκBα serum raised against a peptide corresponding to amino acids 297 to 317 mapping within the carboxy terminal domain of the human IκBα molecule. After three 10 min washes with PBS-Tween-20, the membranes were incubated with peroxidase-conjugated anti-rabbit immunoglobulin and the antigen-antibody complexes were detected using the ECL Western blotting detection reagents (Amersham) according to the manufacturer's instructions.

Transient transfection and luciferase assay. Reconstructed PGL2 basic plasmid containing a luciferase gene transcribed from artificial promoters dependent on two NF-κB binding sites or five Sp-1 binding sites were introduced into NR8383 cells using the DEAE-Dextran method (14). Briefly, the cells were washed with PBS and incubated in 1 ml transfection buffer (DMEM medium containing 2 μg/ml of recombinant plasmid DNA, 0.5mg/ml of DEAE-Dextran and 50μg/ml of chloroquine) at 37 °C for 2 h. The transfection buffer was aspirated and the cells were challenged with 10% DMSO in PBS for 2 min. After two washes with PBS, the cells were cultured in 15% FCS for 40 h at 37 °C. Luciferase activity was measured after LPS stimulation with or without tetrandrine for another 6 h.

RESULTS

Activation of the nuclear transcription factor NF-κB by LPS, PMA and silica in inflammatory cells has been recently described in several laboratories (1-5, 12). To evaluate the inhibitory effects of tetrandrine on LPS, PMA and silica-induced NF-κB activation, alveolar macrophages were stimulated with these inducers in the presence of various concentrations of tetrandrine. As demonstrated by an electrophoretic mobility shift assay (EMSA), induction of NF-κB DNA binding activity by LPS, PMA and silica was inhibited by tetrandrine in a dose-dependent manner (Fig. 1a). This effect

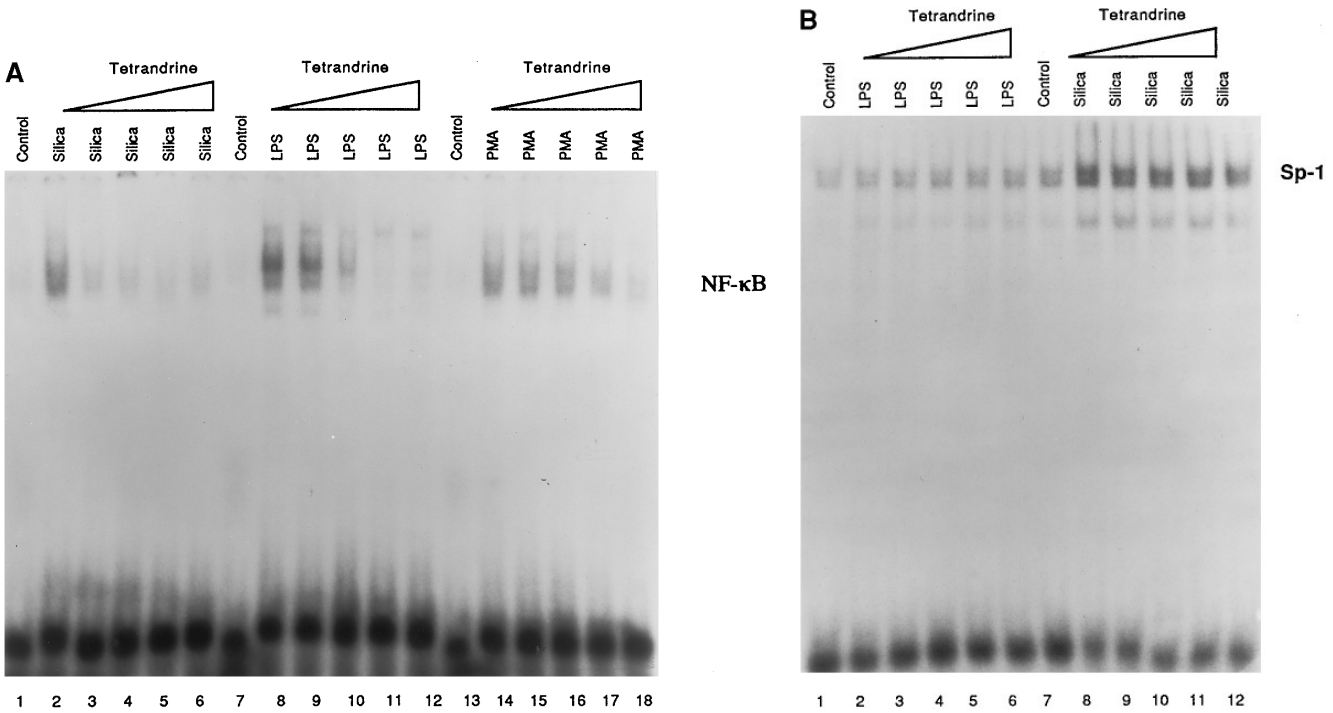

FIG. 1. (a) EMSA of inhibitory effect of tetrandrine on NF-κB activation induced by silica (100 μg/ml, lanes 2 to 6), LPS (5 μg/ml, lanes 8 to 12), and PMA (40 μg/ml, lanes 14 to 18) for 6 h. The concentrations of tetrandrine are 0, 12.5, 25, 50, and 100 μM, respectively. The results shown are a representative experiment from three separate experiments. (b) Effect of tetrandrine on Sp-1 DNA binding activity in the nuclear protein extracted from NR8383 cells stimulated by silica (lanes 2 to 6) or LPS (lanes 8 to 12) for 6 h. The concentration of tetrandrine is the same as (a). The results shown are a representative experiment from four repeated experiments.

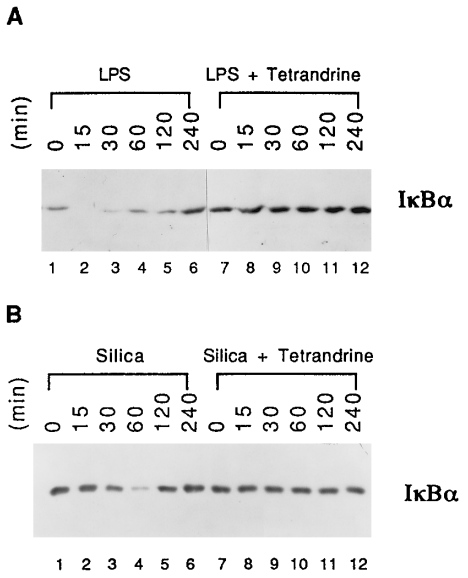

FIG. 2. Effect of tetrandrine (40 μM) on LPS (5 μg/ml, upper panel) and silica (100 μg/ml, lower panel) induced IκBα degradation. The results shown are a representative experiment from three separate experiments.

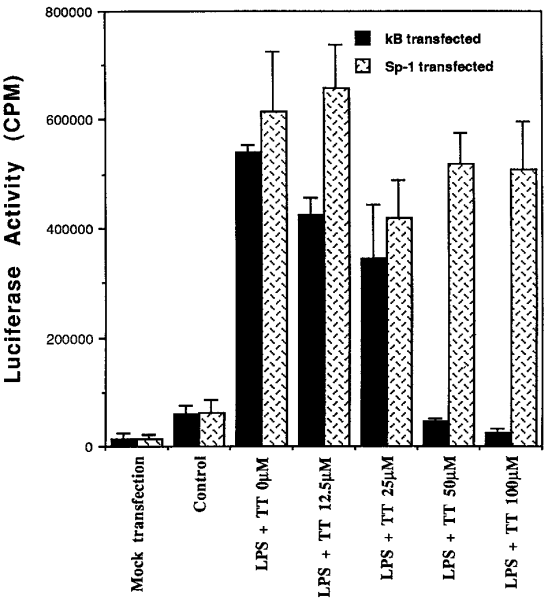

FIG. 3. Effect of tetrandrine on NF-κB- and Sp-1-dependent transcription (n=4).

appeared to be specific for NF-κB since tetrandrine over the same dose range was ineffective in inhibiting Sp-1 DNA binding activity (Fig. 1b).

IκBα degradation is a necessary step for signal-induced activation of NF-κB. To determine the potential effect of tetrandrine on signal-induced IκBα degradation, whole-cell extracts were prepared from cells treated with LPS (Fig. 2A) or silica (Fig. 2B) either in the absence or presence of tetrandrine. These extracts were then subjected to SDS-PAGE and western blotting to assess the IκBα protein. As depicted in Fig. 2, both LPS and silica were capable of inducing IκBα degradation in a time dependent manner. IκBα degradation appears to peak at 15 to 30 min following LPS or silica exposure. As shown in the lanes 7 to 12 in Figure 2A and 2B, the accelerated degradation of IκBα by LPS or silica was significantly inhibited in the presence of 40 μM tetrandrine.

To extend this observation, a luciferase reporter gene assay was performed using the NR8383 cells. The cells were transiently transfected with a PGL2 plasmid inserted with two κB sites or five Sp-1 sites upstream of luciferase reporter gene. Cells were challenged with LPS in the presence or absence of various concentration of tetrandrine for an additional 6 h after two days of the transfection experiment. As shown in Fig. 3, tetrandrine effectively inhibited LPS-induced NF-κB-dependent transcription. In contrast, tetrandrine did not influence Sp-1-dependent transcription at the same concentration range.

To explore the possibility that the inhibitory effect of tetrandrine on NF-κB activation and NF-κB-dependent transcription might be due to cytotoxicity of tetrandrine on these alveolar macrophages, cell viability was determined with different doses of tetrandrine using the Promega non-radioactive cell proliferation assay system. As shown in Figure 4, following 6 h of tetrandrine treatment, there was no apparent cytotoxicity at doses up to 100 μM. We did observe however, cytotoxicity of the cells when the dosage of tetrandrine exceeded 100 μM.

DISCUSSION

The results obtained in the present study support the potential use of tetrandrine, a natural Chinese herbal

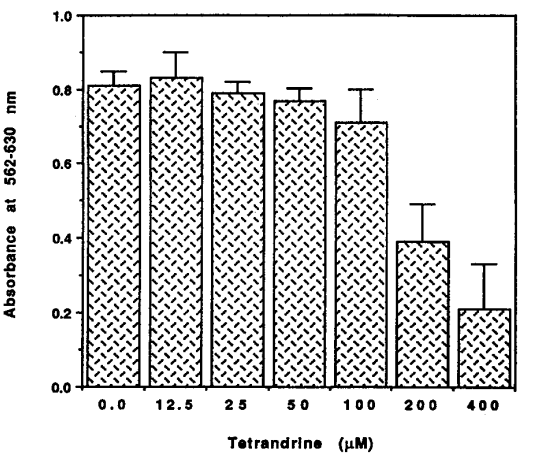

FIG. 4. Determination of cell viability treated with various concentrations of tetrandrine. The cell viability was expressed as the absorbance at 562-630 nm (n=4).

medicine, as an inhibitor of NF-κB activation when cells are exposed to pro-inflammatory agents such as bacterial endotoxin, PMA and silica. The signaling events that lead to the activation of NF-κB by different agonists is still poorly understood. It is well documented however, that IκBα phosphorylation and its subsequent degradation is a common key step in this process (1-5). Various agents had been reported to interfere with the degradation of IκBα and the activation of NF-κB by acting on proteasomes, proteases, redox states or even by directly enhancing IκBα synthesis. These agents include proteasome inhibitors (15, 16), antioxidants (17), protease inhibitors (18), nitric oxide (12, 19), and glucocorticoids (7, 20). Earlier studies have suggested that tetrandrine is a Ca^{2+} channel blocker (21) as well as an antioxidant (22). Both calcium signaling and redox changes are important in the activation of NF-κB. It is reasonable therefore, to consider that the inhibitory effects of tetrandrine on NF-κB activation might be by way of interfering with calcium signaling mechanisms and/or influencing the redox state of the cell. This potential mechanism is improbable however since calcium signaling and redox changes are relatively generalized signals for most of the transcription factors such as NF-κB, AP-1, Sp-1, etc. (23, 24). In this report, we observed that tetrandrine specifically inhibited NF-κB activation and NF-κB-dependent transcription without affecting Sp-1 or Sp-1-dependent transcription in these cells. Additional studies are needed to characterize the effects of tetrandrine on other transcription factors such as AP-1, Egr-1 or CREB.

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