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RESEARCH PAPER

Thymic Peptides Inhibit Nuclear Factor Kappa B Activation in Human T Lymphocytes

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ABSTRACT

Nuclear factor kappa B (NF-κB), which has been implicated in the regulation of gene transcription, is essential for the expression of genes controlled by the long terminal repeat of human immunodeficiency virus type 1. Studies have shown that reactive oxygen species are involved in signal transduction pathways leading to NF-κB activation. We have reported that calf thymic peptides (TP) protect various cell types from oxidant injury. In this study, we determined the effects of TP on NF-κB activation in human T lymphocytes (Jurkat cells) induced by two stimuli: tumor necrosis factor α and phorbol 12-myristate 13-acetate. Activated NF-κB in nuclear extracts was measured by an electrophoretic mobility shift assay (EMSA) using ^{32}P-labeled probe. TP consistently exhibited a dose-dependent inhibition of NF-κB activation induced by both stimuli. Supershift with specific antibodies to NF-κB subunits confirmed that the inducible retarded bands observed in the EMSA are p65-p50 heterodimer of the NF-κB/Rel protein. Our data suggest that TP may act via antioxidant mechanisms to block NF-κB activation in Jurkat cells.

Key Words: *Antioxidant; Jurkat cells; Phorbol myristate acetate; Reactive oxygen species; Thymic peptides; Tumor necrosis factor*

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INTRODUCTION

Nuclear factor kappa B (NF-κB), a transcriptional activator involved in human immunodeficiency virus type 1 expression (1), signals an immediate early gene activation during inflammatory processes (2). The DNA-binding subunits of NF-κB comprise six members in mammals: p50, p65 (RelA), v-Rel, c-Rel, p52, and RelB (3). Existing in the cytoplasm as an inactive form, NF-κB is stabilized by an inhibitory subunit IκB that inhibits its DNA binding activity (4,5). Stimulating the cells with a wide variety of pathogens and cytokines dissociates IκB from NF-κB, allowing free NF-κB to migrate to the nucleus. NF-κB then binds to the κB consensus motif, thereby transmitting various signals from the cytosol to the nucleus (6,7). Agents that induce release of IκB from the cytoplasmic form of NF-κB include viruses, tumor necrosis factor α (TNF-α), protein synthesis inhibitors, and T cell mitogens such as phorbol 12-myristate 13-acetate (PMA), and lectin (1).

Conditions that induce NF-κB in T cells share a common reactive oxygen species (ROS)-dependent pathway, in which a transient increase in ROS causes IκB to be released from the cytoplasmic NF-κB-IκB complex (4,5,8), Activation of NF-κB and κB-dependent genes under control of the human immunodeficiency virus type 1 long terminal repeat (HIV-1 LTR) by micromolar amounts of hydrogen peroxide (H_2O_2) provides additional evidence that ROS play a role in the activation of NF-κB.

Since ROS are involved in the signal transduction mechanisms for NF-κB activation, it is logical to investigate the possibility of using antioxidants to prevent NF-κB activation. Indeed, thiol reagents known to scavenge oxygen radicals have been shown to suppress induction of NF-κB by H_2O_2, cycloheximide, double-stranded RNA, interleukin-1, and the viral transactivators (9).

The thymus, which plays a key role in immunoregulation and in the neuroendocrine-immune network (10–12), produces hormonal-like peptides that control development of the thymic-dependent lymphoid system (13). These peptides have been shown to restore impaired immune reactivities in thymectomized animals (14). Other extracts such as thymostimulin, thymic factor X, and T activin have been used to treat immunodeficiencies associated with HIV (15), herpes (16), and hepatitis B viral infections (17). Our laboratory has previously shown that thymic peptides (TP) isolated from calf thymus protected vascular endothelial cells from oxidant injury induced by H_2O_2, indicating that TP has antioxidant activity (18,19).

Antioxidants such as vitamin E derivatives and α-lipoic acid have been shown to inhibit NF-κB activation in human T cells induced by TNF-α and PMA (20,21). Another antioxidant, N-acetyl-L-cysteine, was shown to suppress NF-κB activation in HeLa cells induced by PMA (22). In this study, we investigated the effects of TP on NF-κB activation in human T cells induced by two stimulants: PMA and TNF-α.

MATERIALS AND METHODS

Reagents

The composition of TP, obtained from Haerbin Songhe Pharmaceutical Factory, Heilongjiang, P. R. China, was previously described (18). PMA, dithiothreitol (DTT), Nonidet P-40, TNF-α, and phosphate buffered saline (PBS) were obtained from Sigma Chemical Co. (St. Louis, MO). RPMI 1640 medium was purchased from Mediatech Co. (Washington, DC). Fetal calf serum (FCS) was obtained from Gemini Bioproducts (Calabasas, CA). HeLa nuclear extract and NF-κB-specific oligonucleotide were purchased from Promega Co. (Madison, WI). Poly (dI-dC) was purchased from Pharmacia Biotech Inc. (Piscataway, NJ). Specific antibodies to NF-κB subunits p50 and p65 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). [γ-^{32}P]ATP was obtained from ICN Biomedicals, Inc. (Irvine, CA). Coomassie protein assay reagent was obtained form Pierce (Rockford, IL). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

Jurkat T (human lymphoma) cells were obtained from American Type Culture Collection (Rockville, MD). Cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, penicillin (500 U/ml), and gentamicin (0.1 mg/ml) at 37°C and 5% CO_2. Cell density was maintained between 5×10^5 and 1×10^6/ml. The viability of cells used in experiments was always greater than 95% as determined by tryphan blue exclusion.

Experimental Design

To determine the appropriate concentrations of NF-κB activators, different concentrations of PMA or TNF-α were added to Jurkat cells (10 ml, 1×10^6 cells/ml) in individual tissue culture dishes (Falcon 3003, Becton

Dickinson, Franklin Lakes, NJ), incubated at 37°C and 5% CO_2 for 2 hr, and followed by nuclear extraction. EMSA using HeLa nuclear extract as a control was then performed. To determine the effects of TP on NF-κB activation, various concentrations of TP were preincubated with Jurkat cell culture for 20 hr, incubated for another 2 hr after addition of TNF-α or PMA, and followed by nuclear extraction and EMSA. To ascertain that the DNA-binding protein activated by PMA or TNF-α was κ-specific, a binding competition analysis was performed in which a nuclear extract from 200 nM PMA or 20 ng/ml TNF-α-treated Jurkat cells was used. Five hundred-fold of unlabeled NF-κB-specific oligonucleotide was mixed with the nuclear extract 10 min before the ^{32}P-labeled NF-κB probe was added. Antibodies against p50 and p65 were used in the supershift assays to identify the composition of NF-κB complexes induced by PMA and TNF-α.

Nuclear Extracts

Nuclear extracts were prepared as described by Staal et al. (23). Cells were harvested by centrifugation for 10 min at 1200 rpm at 4°C, resuspended in 1 ml of ice-cold PBS, and centrifuged again for 15 sec at 14,000 rpm at 4°C. Lysing buffer (0.4 ml of: 10 mM Hepes, 2 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol [DTT], 0.1 mM phenylmethylsulfonyl fluoride [PMSF], 5 μg/ml antipain, and 5 μg/ml leupeptin) was added to the pellet, vortexed for 15 sec, and kept on ice for 10 min. Twenty microliters of 10% Nonidet P-40 solution was added, and cells were vigorously mixed for 15 sec and centrifuged for 30 sec at 14,000 rpm at 4°C. Pelleted nuclei were resuspended in 60 μl of extraction buffer (50 mM Hepes, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10% [v/v] glycerol), vortexed for 15 sec, kept on ice for 20 min, and then centrifuged for 10 min at 14,000 rpm at 4°C. The supernatant containing the nuclear proteins was stored at −70°C. Protein concentration was determined by using the Coomassie protein assay reagent.

EMSA

NF-κB-specific oligonucleotide was end-labeled with [γ-^{32}P]ATP (7,000 Ci/mM) and purified using a NAP-5 column (Pharmacia Biotech, Inc., Piscataway, NJ). EMSAs were performed according to the method of Staal et al. (23). Binding reaction mixtures (2 μg of nuclear protein, 1 μg poly(dI-dC), ^{32}P-labeled probe, 50 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 2% [v/v] glycerol, and 10 mM Tris-HCl) were incubated for 20 min at 25°C. In competition assays, 500-fold excess unlabeled oligonucleotide competitors were added 10 min before addition of radiolabeled probes. DNA–protein complexes were separated from the unbound DNA probe by electrophoresis through a native 6% polyacrylamide gel in 0.5 × TBE (pH 8.0). Gels were vacuum dried and examined by autoradiography. Films were scanned by the DeskScan II program (Hewlett-Packard Co., Boise, ID). Relative intensity of NF-κB bands was quantified by densitometry scanning of autoradiographs using the Bio Image Whole Band Analyzer, version 3.0 (Millipore Corp., Ann Arbor, MI). In supershift assays, nuclear extracts were incubated with 1 μg and 2 μg of anti-p65 and 2 μ of anti-p50 for 60 min at room temperature, followed by EMSA.

Statistical Analyses

Data were presented as means ± SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple range test for honestly significant difference (HSD). The level of significance was defined as $p < 0.05$. All statistical procedures were performed with Statgraphics software version 5.1 (STSC, Inc., Rockville, MD).

RESULTS

To determine the appropriate concentration of PMA for NF-κB activation, Jurkat T cells were incubated with PMA (50–250 nM) for 2 hr. Nuclear extracts were prepared and analyzed for NF-κB using EMSA. PMA exhibited a dose-dependent increase of NF-κB activation [Fig. 1a]. PMA-induced NF-κB complexes were competitively inhibited by an excess of the unlabeled κB oligonucleotide. To determine the effects of TP on NF-κB activation by PMA, Jurkat cells were pretreated with different concentrations of TP for 20 hr before stimulation with 200 μM PMA (the concentration of PMA that had no effect on cell viability) for 2 hr. Figs. 1(b) and 2(a) show a concentration-dependent inhibition of the NF-κB activity by TP. Nuclear extract of Jurkat cells treated with 500 μg/ml of TP alone had no effect on DNA-protein complex (Fig. 2(a), lane 7).

To determine the appropriate concentration of TNF-α for NF-κB activation, cells were treated with 2.5–20 ng/ml of TNF-α. A dose-dependent increase of NF-κB was obtained [Fig. 3a]. Cell viability was not decreased

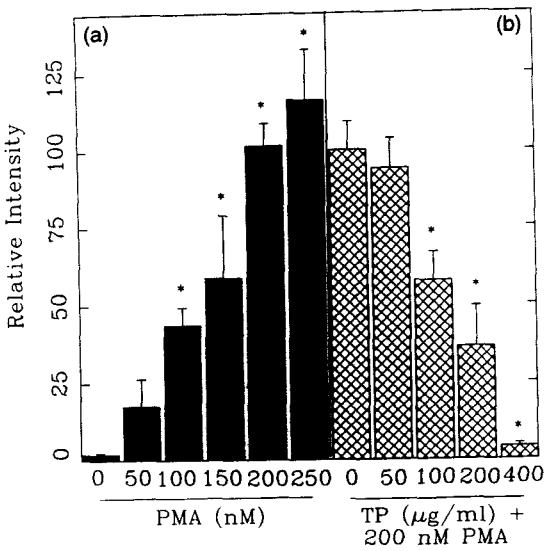

Figure 1. The effect of TP on the activation of NF-κB by PMA. (a) ^{32}P-labeled NF-κB oligonucleotide probe was incubated with nuclear extracts from Jurkat cells stimulated with PMA, and followed by EMSA. A dose-dependent increase of NF-κB activation was noted. (b) Jurkat T cells were pretreated with 0, 50, 100, 200, and 400 μg/ml of TP for 20 hr, followed by 200 nM PMA for 2 hr. Nuclear extracts were incubated with radiolabeled NF-κB oligonucleotide, and followed by EMSA. TP displayed a concentration-dependent inhibition of NF-κB activation by PMA. Values were expressed as relative intensity of radioactivity. Data represent means ± SD of three experiments. Asterisks denote significant difference compared with respective zero points ($p < 0.05$).

by concentrations of TNF-α less than 40 ng/ml. To test the effects of TP on NF-κB activation by TNF-α, cells were preincubated with different concentrations of TP followed by treatment with 20 ng/ml of TNF-α. As shown in Figs. 2(b) and 3b, the induction of NF-κB was decreased by increasing concentrations of TP.

To identify the NF-κB complexes induced by TNF-α, nuclear extract from 20 ng/ml TNF-α-treated Jurkat cells was authenticated by antibody supershift assays. Results are presented in Figure 2c. Lane 1 shows that nuclear extract from Jurkat cells has two bands. Nuclear extract from HeLa cells was used as a control in lane 2. Two micrograms of antibody specific for the p65 subunit of NF-κB supershifted the slower migrating upper band (lane 3) and 1 μg of anti-p65 caused about 50% less band shifting (lane 4). The antibody specific for p50 reduced most of the faster migrating lower band and removed some of the upper complex (lane 5). The supershift assay was also performed for nuclear protein from 200 nM PMA-treated Jurkat cells. The result was essentially the same as that for TNF-α-treated cells (data not shown).

To determine whether TP exerted any toxicity in cell cultures, Jurkat cells were incubated for 24 hr with various concentrations of TP. When the concentration of TP was less than 500 μg/ml, there was no decrease in cell viability. The results thus indicated that the inhibition of NF-κB activation by TP was not as a result of any toxic effects.

DISCUSSION

Jurkat cells are widely used in the study of T cell activation and provide a model system for studying induction of NF-κB (20,21,24). To investigate the effect of TP on NF-κB activation in this cell line, we used two inducers: PMA and TNF-α. These two agents act intracellularly by distinct signalling pathways with a final common pathway. PMA is thought to activate NF-κB by protein kinase C by enhancing the activity of an NADPH oxidase-like enzyme producing superoxide anions and hydrogen peroxide from oxygen and NADPH (25). TNF-α has been shown to activate NF-κB by stimulating production of superoxide anions and hydrogen peroxide, and causing a rapid depletion of glutathione levels in Jurkat cells (23,26,27).

The data presented in this paper show that PMA orTNF-α treatment of Jurkat cells results in the increased expression of the slower migrating part of NF-κB, which can be separated from uninducible part by the differences in their electrophoretic mobilities. The binding of the two complexes was specific, as both bands were competitively inhibited by an excess of unlabeled κB oligonucleotide. The most prominent form of NF-κB has been described as a heterodimer consisting of two proteins, p50 and p65. Results of the supershift assay confirm that the faster migrating complex is the p50. The inducible slower migrating band is the p65-p50 heterodimer. A large increase of the upper band from the nuclear extract of PMA and TNF-α treated Jurkat cells demonstrated that either PMA or TNF-α can induce a high level of the p50/p65 heterodimer.

Significant progress has been achieved over the last 30 years in the understanding of the thymus and its extracts (13,28). Thymus extracts have been shown to reconstitute primary and secondary immunological deficiencies in humans (29–32). The thymic extract used in this study is a commercial product isolated from calf thymus. Using electrophoresis and amino acid sequenc-

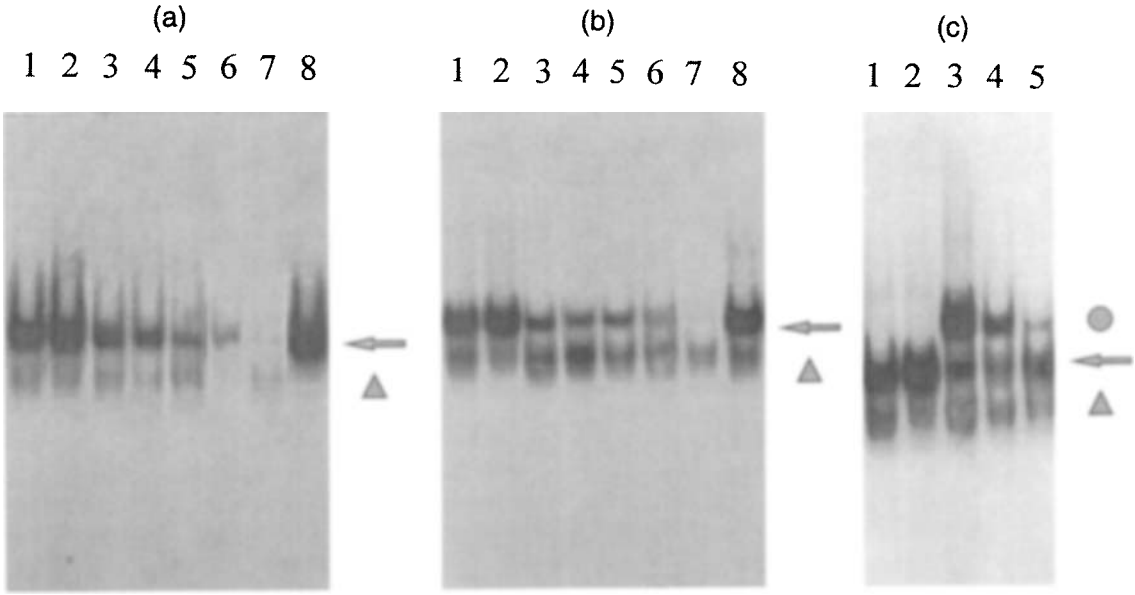

Figure 2. (a) TP displays a concentration-dependent inhibition of NF-κB binding activities by PMA. Cells were pretreated with TP for 20 hr and followed by PMA for 2 hr. Nuclear extracts were prepared and analyzed by EMSA. Lanes 1–5 = cells were treated with 0, 50, 100, 200, and 400 μg/ml of TP, respectively, followed by 200 nM PMA. Lane 6 = untreated cells. Lane 7 = cells treated with 500 μg/ml of TP only. Lane 8 = nuclear extract from HeLa cells as control. The arrow indicates the specific binding complex. The triangle indicates the position of constitutive NF-κB. (b) TP exhibits a dose-dependent inhibition of NF-κB activation by TNF-α. Jurkat cells preincubated with TP for 20 hr, followed by TNF-α for 2 hr. Nuclear extracts were prepared and analyzed by EMSA. Lanes 1–5 = cells pretreated with 0, 50, 100, 200, and 300 μg/ml of TP, respectively, followed by 20 ng/ml of TNF-α. Lane 6 = untreated cells. Lane 7 = cells were treated with 500 μg/ml of TP only. Lane 8 = nuclear extract from HeLa cells as control. TNF-α as inducible bands are indicated by arrow. Faster migrating bands indicated by triangle were not inducible by TNF-α. (c) TNF-α induced NF-κB complex comprises NF-κB p65 and p50. Lane 1 = nuclear extract from cells treated with 20 ng/ml TNF-α. Lane 2 = nuclear extract from HeLa cells. Lanes 3 and 4 = nuclear extracts from TNF-α (20 ng/ml) treated cells with 2 μg and 1 μg anti-p65, respectively. Lane 5 = nuclear extracts from 20 ng/ml TNF-α treated cells with 2 μg anti-p50. TNF-α inducible bands are indicated by arrow. Faster migrating bands indicated by triangle were not inducible by TNF-α. Circle indicates the shift bands by the antisera.

ing analysis, we have shown that this thymus extract consists of two subunits. The fast-moving subunit with a molecular weight of 3 kDa has nine amino acid residues at the NH_2 terminal and accounts for 92% of the total quantity, while the other subunit (6 kDa) has 18 amino acid residues at the NH_2 terminal and amounts to 8% (18). Both subunits are required to demonstrate the biological activity (our unpublished data). We have also shown that TP reversed decreased GSH level, increased lipid peroxidation, and elevated leakage of intracellular lactate dehydrogenase induced by t-butyl hydroperoxide in bovine pulmonary artery endothelial cells (PAEC) (18). This antioxidant effect has also been demonstrated by other studies in our laboratory: TP prevented decreased cell viability, increased intracellular lactate dehydrogenase release, and elevated malondialdehyde production in PAEC induced by hydrogen peroxide (19). Similar data were obtained from the study with murine macrophage cell line J774, in which TP exhibited a concentration-dependent suppression of the oxidative burst triggered by zymosan. Suppression of TP was shown to be mediated by increasing the glutathione level, and activities of both glutathione-peroxidase and glutathione-reductase (33). Results of these studies suggest that the antioxidant effect of TP may be due to its modulation of the glutathione redox cycle.

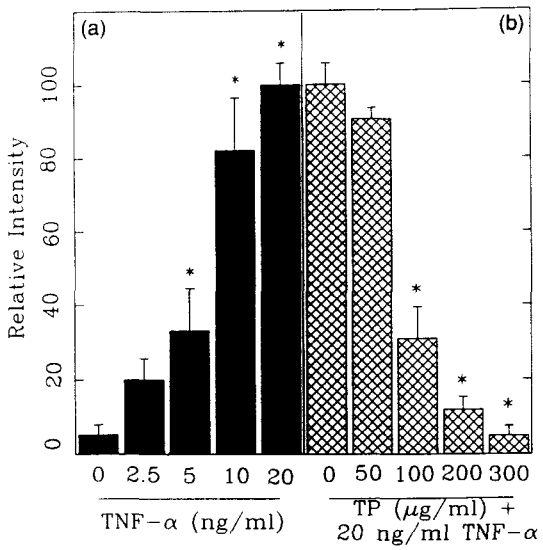

Figure 3. The effect of TP on the activation of NF-κB by TNF-α. (a) Nuclear extracts from cells treated with 0, 2.5, 5, 10, and 20 ng/ml of TNF-α were used. Samples were assayed for NF-κB binding activity with a radiolabeled probe by EMSA. TNF-α induced a dose-dependent activation of NF-κB. (b) Cells were preincubated with 0, 50, 100, 200, and 300 μg/ml of TP for 20 hr, followed by 20 ng/ml TNF-α for 2 hr. Nuclear extracts were prepared and analyzed by EMSA. TP showed a concentration-dependent inhibition of NF-κB activation by TNF-α. Values were expressed as relative intensity of radioactivity. Data represent means ± SD of three experiments. Asterisks denote significant difference compare with respective zero points ($p < 0.05$).

In the present study, the gel retardation analyses demonstrate that TP selectively inhibited the activation of NF-κB p50/p65 heterodimer induced by PMA and TNF-α. TP did not affect NF-κB activation in the absence of an inducer. Neither did it affect cell viability. Thus the decrease of NF-κB activation effected by TP was not due to cell death. Since TP can block the NF-κB activation by both stimuli, these data imply that as an antioxidant, TP can interfere with a common intermediate of PMA and TNF-α to decrease ROS. The exact mechanism may include an increase of glutathione by TP, as demonstrated in our previous studies, and/or an inhibition of protein kinase C by TP, which remains to be investigated.

Most NF-κB target genes in T cells and other cell types encode proteins involved in immune, inflammatory, and acute-phase responses (34). For instance, NF-κB is believed to be essential for the expression of genes controlled by HIV-1 LTR by HIV-infected cells (25,35). The HIV-1 LTR contains two binding sites for NF-κB (25,36). These binding sites can serve as response elements that activate genes following treatment with TNF-α, PMA, or H_2O_2 (9,37,38). This study demonstrates that TP can inhibit NF-κB activation induced by TNF-α and PMA. It will be of interest to determine whether or not TP may be effective in preventing not only immune deficiencies, but also oxidative damage and NF-κB-mediated disorders.

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