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Suppression of Phorbol Ester-Induced NF-κB Activation by Capsaicin in Cultured Human Promyelocytic Leukemia Cells

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Capsaicin, a major pungent constituent of red pepper (*Capsicum annuum* L.) possesses a vast variety of pharmacologic and physiologic activities. Despite its irritant properties, the compound exerts anti-inflammatory and anti-nociceptive effects. Previous studies from this laboratory revealed that capsaicin, when topically applied onto dorsal skin of female ICR mice, strongly attenuated activation of NF-κB and AP-1 induced by the typical tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), which may account for its anti-tumor promoting activity in mouse skin. In the present work, we have found that capsaicin suppresses TPA-stimulated activation of NF-κB through inhibition of IκBα degradation and blockade of subsequent nuclear translocation of p65 in human promyelocytic leukemia HL-60 cells. Methylation of the phenolic hydroxyl group of capsaicin abolished its inhibitory effect on NF-κB DNA binding. Likewise, TPA-induced activation of AP-1 was mitigated by capsaicin treatment.

Key words: Activator protein-1 (AP-1), Capsaicin, HL-60 cells, NF-κB, TPA

INTRODUCTION

Many components from dietary plants have been identified to possess potential chemopreventive properties (Surh, 1999 & 2002). Capsaicin (*trans*-8-methyl-*N*-vanillyl-6-nonenamide; structure shown in Fig. 1), a major pungent ingredient present in red pepper (*Capsicum annuum* L., Solanaceae) has a broad spectrum of pharmacologic and toxicologic properties (Surh, 1999 & 2002; Surh and Lee, 1995 & 1996; Surh *et al.*, 1997; Szallasi and Blumberg, 1999). Capsaicin exerts chemopreventive or chemoprotective effects through modulation of metabolism of certain carcinogens and mutagens and/or their covalent interaction with target cell DNA (reviewed by Surh and Lee, 1995 & 1996 also by Surh *et al.*, 1997 see references therein). Capsaicin has an antioxidant activity as revealed by amelioration of oxidative tissue damage in rats (De and Ghosh, 1992). The antioxidative or free radical scavenging activities of capsaicin have been confirmed in other studies (Brar *et al.*, 2001; Joe and Lokesh, 1994; Lee *et al.*, 2000; Savitha and Salimath, 1995). This pungent vanilloid also displays anti-inflammatory and antiplatelet properties (Janusz *et al.*, 1993; Reddy and Lokesh, 1994; Wang *et al.*, 1985). Interestingly, direct injection of capsaicin into B16 mouse melanoma transplanted in C57BL/6 mice significantly suppressed the growth of tumors (Morré *et al.*, 1996), and the administration of capsaicin to neonatal rat profoundly affected T cell differentiation (Santoni *et al.*, 2000). Topical application of capsaicin prior to 12-*O*-tetradecanoylphorbol-13-acetate (TPA) suppressed 7,12-dimethylbenz(*a*)anthracene-induced mouse skin carcinogenesis (Park *et al.*, 1998). According to a study by Morré *et al.* (1995), capsaicin preferentially induced growth inhibition and apoptosis of some transformed cells of human origin including HeLa, ovarian carcinoma, mammary adenocarcinoma, and HL-60 cells. Proliferation of M11619 melanoma

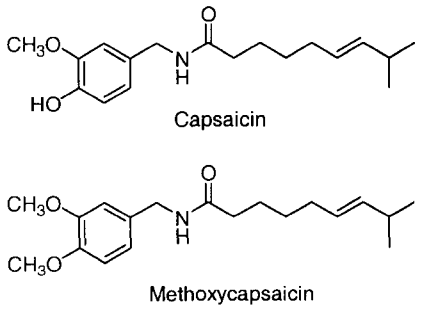

Fig. 1. Structures of capsaicin and methoxy-capsaicin

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cells was also suppressed by capsaicin (Brar *et al.*, 2001) at a concentration blocking the NAD(P)H:quinone oxidoreductase-activity. Capsaicin-induced apoptosis and antiproliferation were also observed with other cell lines (Jung *et al.*, 2001; Kim *et al.*, 1997; Lee *et al.*, 2000; Macho *et al.*, 1998 & 1999; Morré *et al.*, 1996; Wolvetang *et al.*, 1996).

One of the most ubiquitous transcription factors that regulate the expression of genes responsible for encoding proteins regulating cellular proliferation, inflammatory responses, cell adhesion, etc. is nuclear transcription factor kappa-B (NF-κB). The functionally active NF-κB exists mainly as a hetero-dimer consisting of subunits of Rel family, which is normally sequestered in the cytosol as an inactive complex by binding to IκB (Baeuerle, 1998). Phosphorylation and subsequent ubiquitination of IκB upon exposure of cells to various extracellular stimuli lead to rapid degradation of this inhibitory subunit by proteosomes. The resulting free NF-κB is translocated to the nucleus, where it binds to the κB binding sites in the promoter regions of target genes, thereby controlling their expression Baeuerle and Baltimore, 1996; Barnes., 1997, Thanos and Maniatis, 1995. Another transcription factor, activator protein (AP-1) also has a central role in controlling eukaryotic gene expression. AP-1 is composed of Jun and Fos proteins, which interact via a leucine-zipper domain. Like NF-κB, DNA binding of AP-1 is influenced by the intracellular redox state (Abate *et al.*, 1990; Sen and Packer, 1996). In consideration of the important roles of NF-κB and AP-1 in cellular proliferation and malignant transformation, we have examined the effects of capsaicin on TPA-induced activation of these transcription factors as part of our research program to understand molecular mechanisms underlying the anti-tumor promoting property of capsaicin (Park *et al.*, 1998).

MATERIALS AND METHODS

Materials

Capsaicin and gentamicin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Capsaicin was dissolved in dimethyl sulfoxide (DMSO). Methoxycapsaicin was generously provided by Professor Jeewoo Lee of College of Pharmacy, Seoul National University. TPA was supplied from Alexis Biochemicals (San Diego, CA, USA).

Cell culture and preparation of the nuclear extract

HL-60 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 5 μg/ml gentamicin at 37°C in a humidified 5% CO_2 incubator. HL-60 cells (1×10^7) treated with DMSO alone or 10 nM TPA for 1 h were lysed by incubation at 4°C for 10 min in 400 μl of buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride (PMSF)]. Unless specified, capsaicin was added to the media 30 min prior to TPA treatment. The cell lysate was centrifuged at 14,800 *g* for 6 min, and the supernatant was stored at -70°C as a cytosolic extract after measurement of protein content. The pellet was resuspended in 100 μl of ice-cold buffer C [20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 20% (v/v) glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF], followed by incubation at 4°C for 20 min. The resulting extract was centrifuged, and the supernatant was collected, aliquoted, and stored at -70°C.

Electrophoretic mobility gel shift assay (EMSA)

EMSA was performed using a DNA-protein binding detection kit (Gibco-BRL, Rockville, MD, USA) for NF-κB binding according to the manufacturer's protocol with minor modifications. Briefly, the NF-κB oligonucleotide was labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase and purified on a Nick column (Pharmacia Biotech., Uppsala, Sweden). The binding reaction was carried out in 25 μl of mixture containing 5 μl of incubation buffer [10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 4% (v/v) glycerol, and 0.1 mg/ml sonicated salmon sperm DNA], 10 μg of nuclear extracts, and 100,000 cpm of the labeled probe. After 20 min incubation at room temperature, 2 μl of 0.1% bromophenol blue was added, and samples were electrophoresed through a 6% nondenaturing polyacrylamide gel at 150V at room temperature. Finally, the gel was dried and exposed to an x-ray film. EMSA for AP-1 was carried out in the same manner as that for NF-κB except that the AP-1 oligonucleotide (Promega, Madison, WI, USA) was used as a probe. Both nuclear and cytosolic extracts prepared from HL-60 cells were subjected to 12% SDS-polyacrylamide gel electrophoresis for measuring p65 and IκBα levels. After 3-h transfer of the gel to PVDF membrane (Amersham Life Sciences, Arlington Heights, IL, USA), the blots were blocked with 5% fat-free dry milk in phosphate-buffered saline containing 0.1% Tween-20 for 2 h at room temperature and then washed in the same buffer.

Immunoblot analysis of p65 and IκBα

p65 protein was detected with a rabbit p65 polyclonal antibody (Santa Cruz Biotech., Santa Cruz, CA, USA) diluted 1:2000. IκBα protein was detected with a rabbit IκBα polyclonal antibody (Santa Cruz Biotech., Santa Cruz, CA, USA) diluted 1:1000. Goat anti-rabbit immunoglobulin G-conjugated horseradish peroxidase (diluted 1:5000) was used as a secondary antibody. The transferred proteins were visualized with an enhanced chemiluminescence (ECL)

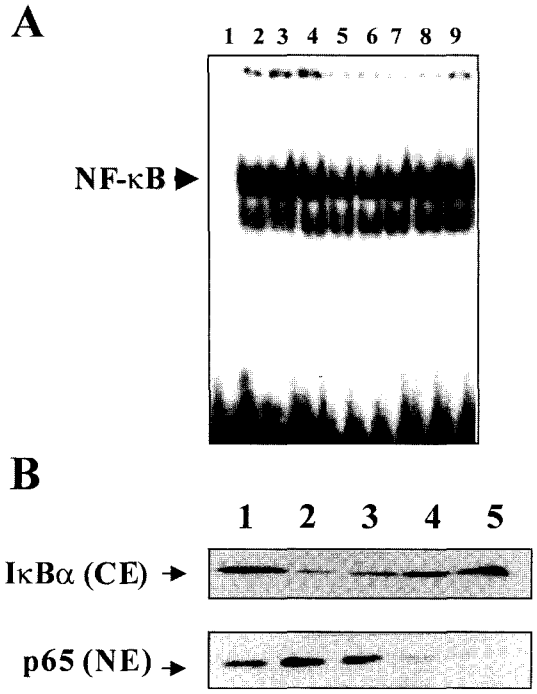

Fig 2. A. Effect of capsaicin and its methoxy-capsaicin on TPA-induced NF-κB activation in HL-60 cells. HL-60 cells (1×10^6/ml) were treated with various concentrations of capsaicin and methoxy-capsaicin for 30 min prior to TPA (10 nM) treatment. Lane 1, probe only; lane 2, DMSO control; lane 3, TPA alone; lane 4, 1 μM capsaicin ± TPA; lane 5, 5 μM capsaicin ± TPA; lane 6, 10 μM capsaicin ± TPA; lane 7, 1 μM methoxy-capsaicin ± TPA; lane 8, 5 μM methoxy-capsaicin ± TPA; lane 9, 10 μM methoxy-capsaicin ± TPA. Nuclear extracts (10 μg) were subjected to EMSA as in the text. The specificity of NF-κB activated by TPA in HL-60 cells was verified by competition of its DNA binding with excess amounts of unlabeled oligonucleotide (Han *et al.*, 2002). The relative intensity of bands has been analyzed by densitometric analysis is: 1.0 (lane 2), 1.63 (lane 3), 1.02 (lane 4), 0.71 (lane 5), 0.73 (lane 6), 1.32 (lane 7), 1.55 (lane 8), 1.61 (lane 9). **B.** Effects of capsaicin on levels of IκBα in cytosol and p65 in nucleus. Nuclear and cytosolic extracts from HL-60 cells (1×10^6/ml) pretreated with various concentrations of capsaicin were assayed for IκBα and p65 by Western blot. Lane 1, DMSO control; lane 2, TPA alone; lane 3, 1 μM capsaicin ± TPA; lane 4, 5 μM capsaicin ± TPA; lane 5, 10 μM capsaicin ± TPA. Abbreviations: NE, nuclear extract; CE, cytosolic extract.

detection kit (Amersham Life Sciences, Arlington Heights, IL, USA) as described elsewhere (Han *et al.*, 2002).

RESULTS AND DISCUSSION

When HL-60 cells were cultured with various concentrations of capsaicin and its methoxy derivative for 30 min in prior to 10 nM TPA treatment, capsaicin inhibited the NF-κB activation concentration dependently, while methoxy-capsaicin exerted no substantial inhibitory effect (Fig. 2A). To determine whether capsaicin could inactivate NF-κB through suppression of degradation of IκBα and

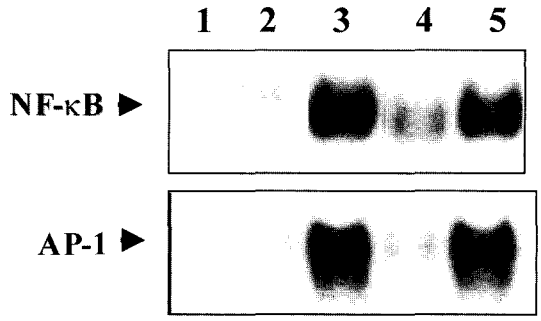

Fig. 3. Effect of capsaicin on DNA-binding activity of NF-κB and AP-1 in HL-60 cells. HL-60 cells were treated with DMSO (lane 2) or 10 nM TPA in the absence (lane 3) or presence (lane 4) of 10 μM capsaicin. For lane 5, nuclear extracts from TPA-stimulated HL-60 cells were treated with 10 μM capsaicin for 20 min at room temperature before EMSA with oligonucleotide probe for NF-κB or AP-1.

the subsequent nuclear translocation of NF-κB, the levels of p65 in nucleus and those of IκBα in cytosol were measured. As illustrated in Fig. 2B, capsaicin pretreatment strongly inhibited the degradation of IκBα induced by TPA, thereby attenuating the nuclear translocation of p65 subunit. Besides blocking the translocation of p65 subunit as well as the IκBα degradation, capsaicin might interrupt the DNA binding of translocated p65. To test this possibility, nuclear extracts from TPA treated HL-60 cells were mixed with 10 μM capsaicin *in vitro* for 20 min at room temperature, and EMSA was performed. While capsaicin inhibited NF-κB DNA binding when treated to HL-60 cells before TPA, the compound did not much attenuate the DNA binding activity of preactivated NF-κB (Fig. 3). Likewise, the DNA binding activity of AP-1 in TPA-stimulated nuclear extracts was not suppressed by direct addition of capsaicin to the incubation mixture for EMSA, while it suppressed AP-1 DNA binding when added to the media before TPA stimulation (Fig. 3).

Recent advances in our understanding of biochemical and molecular basis of inflammatory processes reveal that the transcription factors NF-κB and AP-1 are implicated in the inducible expression of a variety of genes in responses to cytokines, oxidative stress and a variety of chemical agents (Angel and Karin, 1991; Barnes and Karin, 1997; Chabot-Fletcher, 1996; Sen and Packer, 1996). There is substantial body of data supporting that activation of NF-κB regulates the expression of many proteins involved in tumor-promotion, which is suppressed by certain chemopreventive agents (Surh *et al.*, 2001 and see references therein). Capsaicin caused concentration-dependent abrogation of NF-κB activation induced by TPA through suppression of IκBα degradation, thereby attenuating nuclear translocation of p65. Similarly, capsaicin abolished the degradation of cytosolic IκBα and the nuclear translocation of the p65 subunit of NF-κB complex

stimulated with TNF-α (Singh *et al.*, 1996). Consistent with the results of our present *in vitro* work, previous studies from this laboratory revealed that topical application of capsaicin onto dorsal skin of female ICR mice strongly suppressed TPA-stimulated activation of NF-κB by preventing IκBα degradation in the cytosol with subsequent inhibition of nuclear translocation p65 (Han *et al.*, 2001). It has been well recognized that a receptor for TPA is protein kinase C (PKC), and one of the plausible mechanisms underlying the inhibitory effect of capsaicin on TPA-stimulated NF-κB may involve blockade of the receptor binding of TPA (Harvey *et al.*, 1995). Therefore, capsaicin may down-regulate NF-κB activation by inhibiting the upstream signaling kinases (e.g., PKC) responsible for phosphorylation of IκBα or through a mechanism independent of PKC activation (Kong *et al.*, 2000). In consideration of the antioxidant capacity cap-saicin possesses, it is conceivable that the compound protects against IκBα phosphorylation and its subsequent degradation. Interestingly, the NF-κB inhibitory activity of capsaicin was abolished when its phenolic hydroxyl functional group was blocked by methylation, suggesting that the 4-hydroxyl functional group of capsaicin is essential for its inhibitory effect on TPA-induced NF-κB activation. It would be worthwhile determining whether or not the suppression of NF-κB DNA binding by capsaicin is related to the antioxidant capacity of this phenolic phytochemical.

AP-1 is another well defined transcription factor that plays a crucial role in intracellular signal transduction mediating cellular growth, proliferation and malignant transformation (Angel and Karin, 1991). The binding site of AP-1 on DNA is recognized as the TPA response element (TRE), which is present in the promoter region of several genes including metallothioneine IIA gene, collagenase, interleukin-2, etc. (Abate *et al.*, 1990; Sen and Packer, 1996). Beside NF-κB activation, activation of AP-1 induced by TPA was also inhibited by capsaicin. Capsaicin treatment also reduced the AP-1 DNA binding in cultured Jurkat cells treated with TPA and ionomycin (Macho *et al.*, 1998). Recently, Han *et al.* (2001) have reported that TPA-stimulated activation of AP-1 is abolished by capsaicin pretreatment in the dorsal skin of female ICR mice. It seems likely that the suppression of AP-1 activation as well as that of NF-κB by capsaicin is attributable, in part, to its anti-oxidant activity and may account for the anti-tumor promoting effect of this chemopreventive agent.

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