Modulatory Properties of Various Natural Chemopreventive Agents on the Activation of NF-κB Signaling Pathway

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Purpose. To study and compare effects of selected natural chemopreventive agents on the transcription activation of nuclear factor-kappa B (NF-κB) in human HT-29 colon cancer cells.

Methods. The natural chemopreventive compounds isothiocyanates (ITCs) found in cruciferous vegetables, flavonoids found in green tea, resveratrol (RES) and procyanidin dimers found in red wine, and curcumin (CUR) found in turmeric curry food were examined in this study. HT-29 cells were stably transfected with NF-κB luciferase construct, and stable clones were selected. One of the clones, HT-29 N9 cells, was selected and treated with various concentrations of the natural chemopreventive agents and subsequently challenged with NF-κB stimulator lipopolysaccharide (LPS), and the luciferase activities were measured. Western blot analysis of phosphorylated IκBα was performed after treatments with the natural chemopreventive agents. The effects of these agents on cell viability and apoptosis were also evaluated by a nonradioactive cell proliferation MTS assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-arboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt], Trypan blue staining, and caspase assay.

Results. Treatments with the natural chemopreventive compounds resulted in different responses in the NF-κB–luciferase assay. ITCs such as phenethyl isothiocyanate (PEITC), sulforaphane (SUL), allyl isothiocyanate (AITC), and curcumin (CUR) strongly inhibited LPS-induced NF-κB–luciferase activations, whereas RES increased activation at lower dose, but inhibited activation at higher dose, and tea flavonoids and procyanidin dimers had little or no effects. ITCs, CUR, (–)-epigallocatechin-3-gallate (EGCG), and RES reduced LPS-induced IκBα phosphorylation. Furthermore, in the MTS assay, PEITC, SUL, and CUR also potently inhibited cell growth. Caspase-3 activity was induced by chemopreventive compounds, however, the kinetics of caspase-3 activation varied between these compounds within the 48-h time period.

Conclusions. These results suggest that natural chemopreventive agents have differential biological functions on the signal transduction pathways in the colon and/or colon cancer.

KEY WORDS: natural chemopreventive agents; nuclear factor kappa B (NF-κB); transcription regulation.

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ABBREVIATIONS: AITC, allyl isothiocyanate; CUR, curcumin; EC, (–)-epicatechin; ECG, (–)-epicatechin-3-gallate; EGC, (–)-epigallocatechin; EGCG, (–)-epigallocatechin-3-gallate; ITCs, isothiocyanates; LPS, lipopolysaccharide; NF-κB, nuclear factor-kappa B; PEITC, phenethyl isothiocyanate; RES, resveratrol; SUL, sulforaphane.

INTRODUCTION

Colon cancer is one of the leading causes of cancer morbidity and mortality worldwide and the second leading cause of cancer death in the United States, although it is considered as one of the most preventable forms of visceral cancer (1,2). Epidemiological studies have shown that certain components of fruits, vegetables, grains (3), and nuts may prevent or reduce the risk of cancer as well as some chronic diseases (4,5). During the last several decades, numerous chemopreventive and/or chemotherapeutic compounds have been identified from natural products including food and plant sources. Of the natural compounds, isothiocyanates (ITCs) in cruciferous vegetables, polyphenols in green tea and red wine, and curcuminoids in turmeric have gained much attention as potential chemopreventive agents. However, the chemopreventive mechanisms of these compounds, in particular their roles in the modulation of cellular signal transduction pathways, are not fully understood.

Nuclear factor-kappa B (NF-κB) is a transcription factor that consists of homo- and heterodimeric complexes formed from the Rel family of proteins (3,6). In vertebral cells, there are five members of the Rel/ NF-κB proteins including p65 (Rel A), p50/p105, p52/100, c-Rel, and Rel B (3,6,7). The most common NF-κB is a heterodimer composed of p65 and p50. NF-κB is activated by a wide variety of stimuli such as tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), T- and B-cell mitogens, bacterial lipopolysaccharide (LPS), viruses, UV light, gamma rays, and oxidative stress (8,9). In most cells, NF-κB is sequestered in the cytosol, associated with inhibitor proteins, IκBs. A variety of extracellular stimuli lead to the activation of the upstream IκB kinases (IKKs), resulting in rapid phosphorylation and proteolytic degradation of IκB, which makes NF-κB translocate to the nucleus where it regulates gene transcription (9–11).

NF-κB has extensively been studied over the past two decades. It has been reported to play a critical role in several signal transduction pathways involved in various cancers as well as in chronic inflammatory diseases (12,13). Inappropriate regulation of NF-κB is also involved in neurodegenerative diseases, ataxiatelangiectasia, arthritis, and asthma (9). Activation of NF-κB has been linked to apoptotic cell death; either promoting or inhibiting apoptosis, depending on the cell type and conditions (14). In most cells, activation of NF-κB protects the cells from apoptotic stimuli, presumably through the induction of survival genes (9). To date, the cellular signal transduction events that are elicited by many chemopreventive agents are not well characterized and these may be dependent on the types and the concentrations of the agents as well as the cell/tissue types (15). In the current study, we investigated and compared the modulatory effects of several natural chemopreventive agents on the transcription activation of NF-κB and IκBα phosphorylation as well as their roles in cell viability and apoptosis in HT-29 human colon cancer cell line

MATERIALS AND METHODS

Materials

Phenethyl isothiocyanate (PEITC), allyl isothiocyanate (AITC), sulforaphane (SUL), curcumin (CUR), resveratrol

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(RES), (−)-epicatechin (EC), (−)-epicatechin-3-gallate (ECG), (−)-epigallocatechin (EGC), (−)-epigallocatechin-3-gallate (EGCG), Bay 11-7085, and lipopolysaccharide (LPS) derived from *Esherichia coli* serotype 026:B6 were purchased from Sigma Chemicals Co. (St Louis, MO, USA).

Procyanidins B1 and B2 were obtained from Extrasynthese (Genay, France). Fluorogenic peptide substrate of caspase-3 (Ac-DEVD-MCA) was purchased from Peptides International, Inc. (Louisville, KY, USA). All other chemicals were of analytical grade and were purchased from commercial sources. HT-29 human colon cancer cell line was obtained from American Type Culture Collections (Manassas, VA, USA). Cells were cultured in Minimal Essential Medium supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg streptomycin, in a humidified atmosphere of 95%, 5% CO_2 at 37°C. NF-κB–luciferase reporter plasmid constructs in which two copies of the κB promoter containing the NF-κB-binding site were fused to Luc were kindly provided by Drs. Anning Lin (University of Chicago, Chicago, IL, USA) and Michael Karin (University of California, San Diego, CA, USA), and previously described (16,17).

Stable Transfection

NF-κB–luciferase construct and pCDNA3.1-neomycin plasmid were stably transfected into HT-29 cells by Lipofectamine 2000 (LF2000, Invitrogen Life Technology, Carlsbad, CA, USA), following the manufacturer's instruction. Briefly, the HT-29 cells were seeded in 100-mm-diameter culture plates at a density 5×10^6 cells/well and allowed to grow for 24 h to 90% confluency. For each plate, 10 μg of DNA (5 μg of each plasmids) was diluted in 500 μl OPTI-MEM I Reduced Serum Medium, and 30 μl of LF2000 reagent was diluted separately in 500 μl OPTI-MEM I Reduced Serum Medium and incubated for 5 min at room temperature. The diluted DNA and the LF2000 reagent were combined and incubated for 20 min at room temperature to allow DNA–LF2000 reagent complexes to form. The growth media were removed from the cell culture plates and replaced with 10 ml of serum-free media containing NEAA. Then, 1 ml of the DNA–LF2000 reagent mixture was added directly to each plate, mixed gently by rocking the plate, and incubated in a humidified atmosphere of 95%, 5% CO_2 at 37°C. After 4–5 h incubation, the media were replaced with 10 ml of growth media containing 10% FBS. Stable clones were selected with 0.8 mg/ml of G418 sulfate (Invitrogen Life Technology), and the luciferase activities of the selected clones were confirmed by a SIRIUS luminometer (Berthold Detection System GmbH, Pforzheim, Germany) as described below. One of the stable clones (HT-29 N9) was subcultured and used for subsequent study.

Reporter Gene Activity Assay

The HT-29 N9 cells were pretreated for 1 h with various chemopreventive agents. The cells were then treated with LPS (1 μg/ml), followed by 6 h of incubation. Luciferase activity was determined with a luciferase kit from Promega (Madison, WI, USA) according to the manufacturer's instructions and described briefly as follows. After treatments, the cells were washed twice with ice-cold phosphate-buffered saline and harvested in 1 × Reporter lysis buffer. After brief centrifugation, a 10-μl aliquot of supernatant was assayed for luciferase activity with a SIRIUS luminometer (Berthold Detection System GmbH, Pforzheim, Germany). The luciferase activity was normalized against protein amount (Bio-Rad, Hercules, CA, USA) and expressed as fold of induction over the luciferase activitiy of control untreated cells.

Western Blot Analysis of Phosphorylated IκBα

Cells were treated with DMSO (0.1%) or natural chemopreventive compounds for 1 h and challenged with LPS (1 μg/ml) for 0.5 h Then, cells were washed with ice-cold PBS (pH 7.4) and harvested with 200 μl of a lysis buffer (pH 7.4) containing 10 mM Tris-HCl, 50 mM sodium chloride, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 μM sodium orthovandate, 2 mM iodoacetic acid, 5 mM $ZnCl_2$, 1 mM phenylmethylsulfonyl fluoride, and 0.5% Triton-X 100. Cell lysates were homogenized by passing through a 23-G needle three times and left on ice for 30 min. The homogenates were centrifuged at 13,000 rpm for 15 min at 4°C. The supernatants were collected and 20 μg of total protein of each sample, as determined by Bio-Rad protein assay, were mixed with 4 × loading buffer, and heated at 95°C for 3 min. The samples were then separated by 10% mini SDS-polyacrylamide gel eletrophoresis at 200 V and transferred onto polyvinylidene difluoride (PVD) membrane for 1.5 h using a semi-dry transfer system (Fisher, Pittsburgh, PA, USA). The membrane was blocked with 5% bovine serum albumin (BSA) in TBST buffer (20 mM Tris-HCl, pH 7.4, 8 g/l NaCl, 0.2 g/l KCl, and 0.1% Tween 20) for 1 h at room temperature, washed with TBST buffer three times, and was incubated with anti-phospho-IκBα polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) in 3% BSA (1:1,000 dilution) overnight at 4°C. After hybridization with primary antibody, membrane was washed three times with TBST, then incubated with anti-rabbit antibody with horseradish peroxidase (Santa Cruz Biotechnology Inc.) for 45 min at room temperature and washed with TBST three times. Final detection was performed with enhanced chemiluminescence (ECL) Western blotting reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Bands were visualized with BioRad Chemi-Doc XRS system (Richmond, CA, USA), and densitometric analyses were performed using the BioRad Quantity One software. The intensity of the bands of each treatment was compared with the intensity of the control. Data were statistically analyzed by ANOVA, unpaired Student's *t* test.

MTS Assay

The MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay was performed with CellTiter 96 Aqueous nonradioactive cell proliferation assay kit (Promega Corp., Madision, WI, USA) by the manufacturer's instructions. Briefly, the cells were plated on 24-well plates at a density of 10^5 cells/well. After 24 h of incubation, cells were treated with different doses of each chemopreventive compound for 48 h. Then, media were removed, and culture media containing MTS and phenazine methosulfate solution were added. After 1–2 h, the absorbance was measured at 490 nm with μQuant ELISA reader (BIO-TEK Instruments, Inc., Winooski, VT, USA).

Caspase-3 Activity Assay

After treatments with chemopreventive comopounds for various times, the HT-29 N9 cells were washed twice with ice-cold PBS (pH 7.4) and harvested in a lysis buffer containing 50 mM Tris-HCl (pH 7.4), 50 mM β-glycerophosphate, 15 mM $MgCl_2$, 15 mM EDTA, 100 μM phenylmethylsulfonyl fluoride, 1 mM dithiothrietol (DTT), and 150 μg/ml digitonin. The cell lysates were homogenized by passing through a 23-G needle three times and kept on ice for 30 min. The homogenates were then centrifuged at 12,000 × *g* for 15 min at 4°C. The supernatants were collected and analyzed for their protein concentrations (Bio-Rad). The caspase-3 activity of each sample was determined in a reaction mixture containing 10 μg protein and 200 μM Ac-DEVD-MCA as a substrate in assay buffer (100 mM HEPES, 10% sucrose, 10 mM DTT, and 0.1% CHAPS). After 1–2 h incubation of the reaction mixture, the fluorescence was measured using FLx 800 microplate fluorescence reader (BIO-TEK Instruments, Inc.)

Trypan Blue Staining

After treatments, the floating and attached cells were collected and centrifuged for 10 min at 1000 × *g*. Cell pellets were resuspended in PBS and incubated with 0.4% Trypan blue solution for 10 min. The number of both stained and unstained cells was counted using a hemocytometer. The percentage of viable unstained cells was calculated as the ratio between the numbers of unstained over the total cell counts.

RESULTS

Effects of Chemopreventive Agents on the Transcription Activation of NF-κB in HT-29 Human Colon Cancer Cell Line

To investigate and compare the modulatory effects of various natural chemopreventive agents on transcription activation of NF-κB, we performed stable transfection of HT-29 human colon cancer cells with NF-κB–luciferase construct reporter gene, and the modulation of the luciferase activity by these compounds were examined at different concentrations. When NF-κB transfectants (HT-29 N9) were treated for 6 h with LPS (1 μg/ml), the NF-κB–luciferase activity was highest (data not shown), and therefore, we measured the luciferase activity after 6 h treatments with LPS thereafter. The effects of three different categories of natural chemopreventive compounds on the LPS-induced NF-κB–luciferase activity in HT-29 N9 are listed in Figs. 1, 2, and 3. These three categories of compounds were selected based on their structural similarity and include ITCs (Fig. 1), CUR and RES (Fig. 2), and tea catechins and procyanidins (Fig. 3). The luciferase activities were expressed as the fold of induction over control treatment. As shown in Figs. 1, 2, and 3, different categories of compounds display different responses with respect to the NF-κB luciferase activity. Compared to control, which was treated with DMSO (final concentration of 0.1%), LPS treatment (1 μg/ml) resulted in a 4- to 5-fold induction of NF-κB-luciferase activity (Fig. 1). As shown in Fig. 1, all of the ITCs

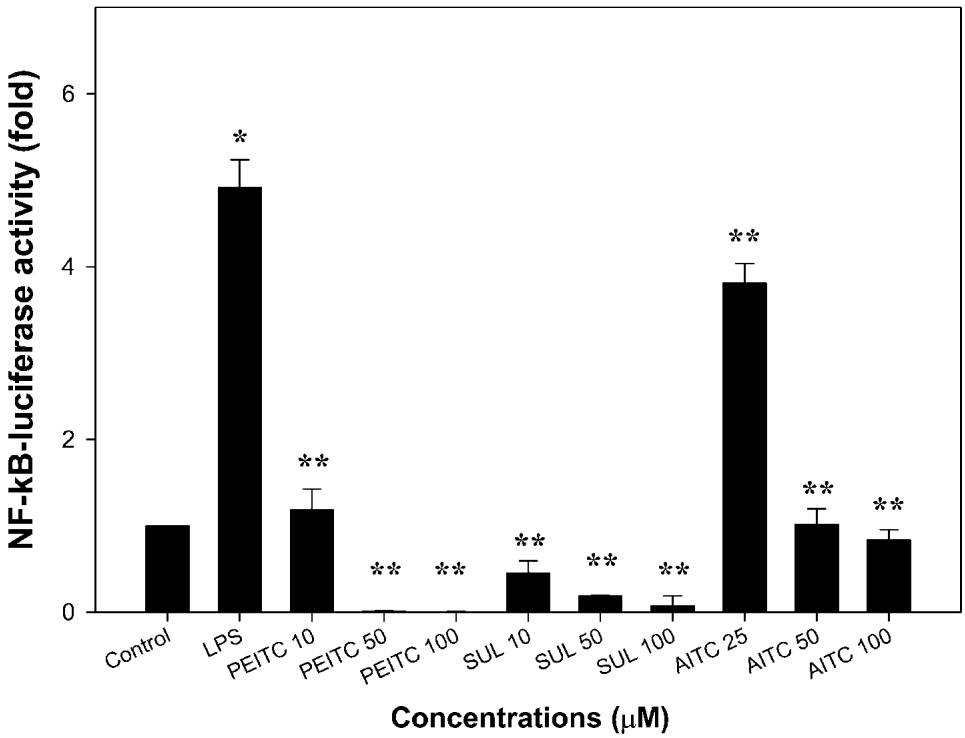

Fig. 1. Effects of isothiocyanates on LPS-induced NF-κB reporter gene activity in HT-29 cells. Cells were treated with DMSO (0.1%) or isothiocyanates for 1 h with the indicated concentrations, challenged with LPS (1 μg/ml), and incubated for additional 6 h. Results are expressed as fold induction over control and values are means ± SD (n = 3). *$p < 0.0005$ compared to control and **$p < 0.01$ compared to the cells treated by LPS, using an analysis of variance (ANOVA) followed by unpaired Student's *t* test.

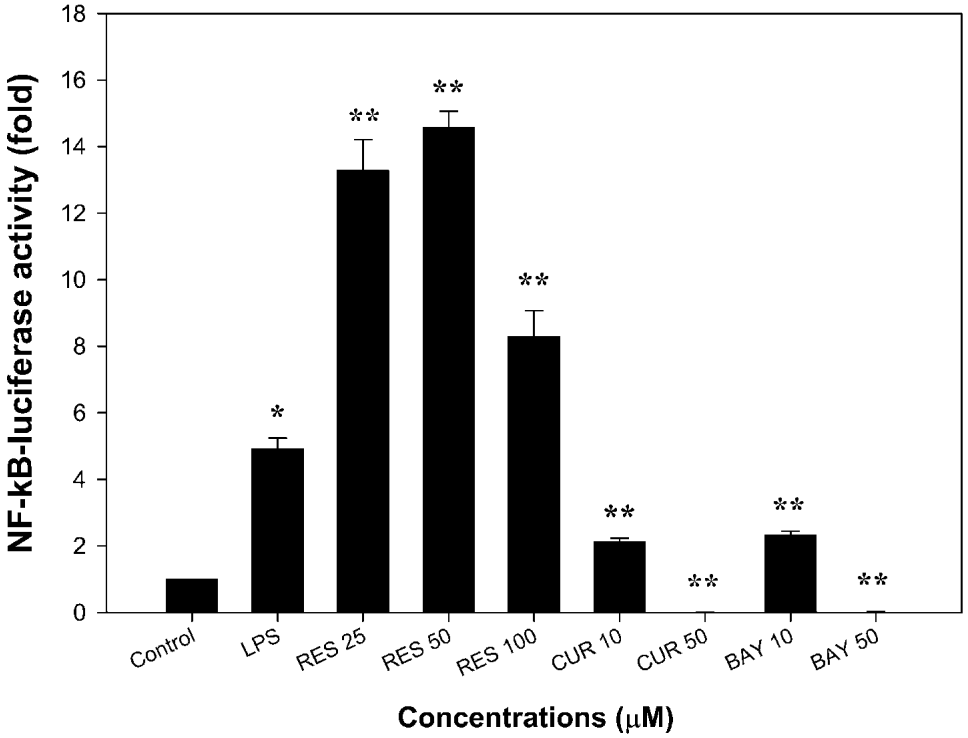

Fig. 2. Effects of RES, CUR, and BAY on LPS-induced NF-κB reporter gene activity in HT-29 cells. Cells were treated with DMSO (0.1%) or compounds for 1 h at the indicated concentrations and challenged with LPS (1 μg/ml) for 6 h. Results are expressed as fold induction over control and values are means ± SD (n = 3). *$p < 0.0005$ compared to control and **$p < 0.01$ compared to the cells treated by LPS, using an analysis of variance (ANOVA) followed by unpaired Student's *t* test.

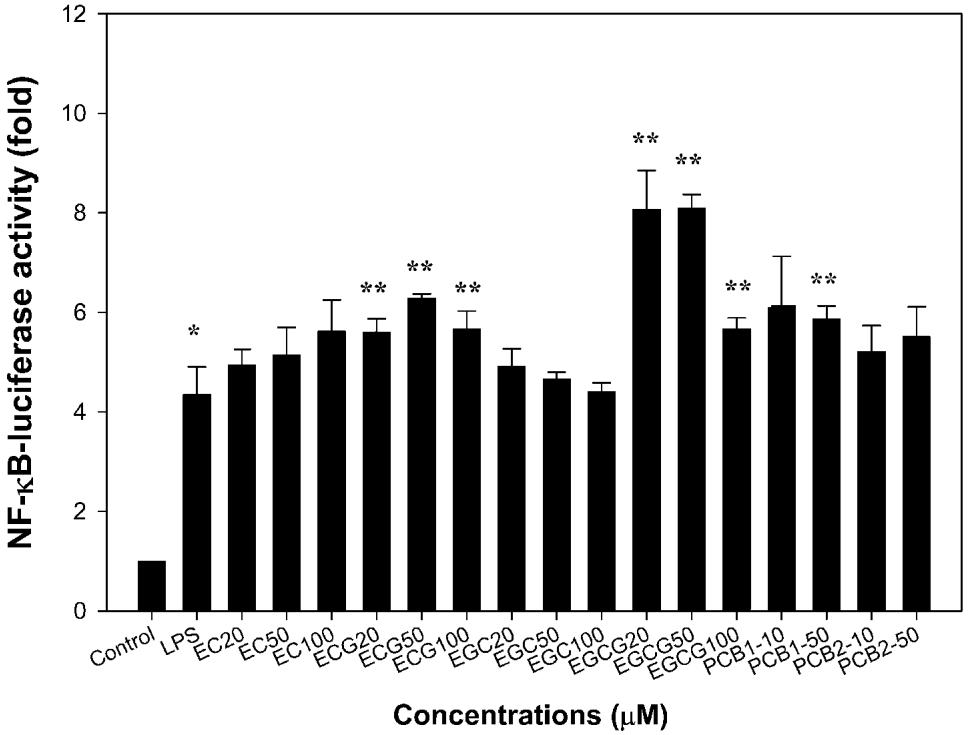

Fig. 3. Effects of tea catechins and procyanidin dimers on LPS-induced NF-κB reporter gene activity in HT-29 cells. Cells were treated with DMSO (0.1%) or compounds for 1 h at the indicated concentrations and challenged with LPS (1 μg/ml) for 6 h. Results are expressed as fold induction over control and values are means ± SD (n = 3). *$p < 0.0005$ compared to control and **$p < 0.05$ compared to the cells treated by LPS, using an analysis of variance (ANOVA) followed by unpaired Student's *t* test.

(PEITC, SUL, AITC) significantly affected the LPS-induced NF-κB-luciferase activity at the concentrations used. PEITC and SUL at ≥50 μM almost completely abolished the LPS-induced NF-κB–luciferase activity, which might be due to cytotoxicity (Table I). At lower doses, SUL (10 μM) decreased the activity to a level below the basal and PEITC to nearly basal level, whereas AITC at 25 μM attenuated the activity by 23%, the doses where little cytotoxicity were observed (Table I). Figure 2 shows the effects of CUR and RES as well as Bay 11-7085 (a known NF-κB inhibitor) (18) on the LPS-induced NF-κB–luciferase activity. CUR and RES, which have two side phenolic rings in the structures, demonstrated opposite effets. CUR at 10 μM decreased the LPS-induced NF-κB–luciferase activity by about 60% (about 2-fold compared to control), whereas 10 μM CUR completely abolished LPS-induced NF-κB–luciferase activity; which may be contributed in part to cytotoxicity (Table I). Treatments with RES at 25 and 50 μM dramatically increased the activity to 13- and 15-fold, respectively, whereas its pretreatment at 100 μM decreased the induction to 8-fold, despite cytotoxicity might have occurred at 100 μM (Table I).

None of the polyphenolic tea catechins and procyanidin dimmers tested in the experiment showed any inhibition on the LPS-induced NF-κB–luciferase activity in HT-29 cells (Fig. 3). Rather, they showed some increase in the activations of LPS-induced NF-κB–luciferase activity. Of the tea catechins, EGCG increased the luciferase activity about 8-fold at 20 and 50 μM, despite some cytotoxicity might have occurred at 50 μM (Table I). Procyanidin dimmers B1 and B2 caused a slight increase in the luciferase activity at both 10 and 50 μM.

Western Blot Analysis of IκBα

One of the major mechanisms involved in the transcription activation of NF-κB is through the phosphorylation of IκBα by IKK, allowing the release of NF-κB and its translocation to the nucleus. In order to further investigate effects of the natural chemopreventive agents on the transcription activation of NF-κB, we therefore examined the modulatory abilities of the natural chemopreventive agents on the phosphorylation of IκBα. The HT-29 N9 cells were treated with various natural chemopreventive agents at the concentration of 50 or 100 μM for 1 h, and LPS was applied to the cells to induce the phosphorylation of IκBα. As shown in Fig. 4, the phosphorylation of IκBα increased after treatment with LPS (1 μg/ml) for 30 min. Treatments with several natural chemopreventive agents prior to LPS treatment decreased the LPS-induced phosphorylation of IκBα. Consistent with the inhibitions of LPS-induced NF-κB–luciferase activation, there were significant inhibition on the LPS-induced IκBα phosphorylation by CUR (49%) and ITCs (27–40%). However, RES and EGCG decreased the LPS-induced IκBα phosphorylation by 35 and 24%, respectively, whereas these agents showed increased activations of NF-κB–luciferase activity. The dichotomy between NF-κB–luciferase activity and IκBα phosphorylation induced by these agents suggests that there might be other mechanism(s) involved in the NF-κB transcription activation by some of these chemopreventive agents.

Effects of Chemopreventive Agents on the Viability of HT-29 N9

The effects of various natural chemopreventive agents on the viability of HT-29 N9 cells were determined by a colorimetric MTS assay after 48-h treatments. The data were expressed as percent cell viability compared to that of control (DMSO, 0.1%) (Table I). The concentrations of the treatments with chemopreventive agents varied from 1 μM to 100 μM. Several compounds significantly affected the cell viability. Of the chemopreventive agents, PEITC, SUL, and CUR exhibited the most potent inhibitory effect against cell viabil-

Table I. Effects of Various Chemopreventive Compounds on Cell Viability of HT-29 N9 Cells in the MTS Assay

	Cell viability inhibition (%) Concentration (μM)				
	1	10	25	50	100
PEITC	104.4 ± 4.3*	88.6 ± 2.9†	2.7 ± 0.2†	2.2 ± 0.2†	—§
SUL	99.6 ± 7.6	104.8 ± 5.0	53.7 ± 5.9†	7.4 ± 1.1†	—
AITC	94.8 ± 3.1	—	102.0 ± 6.3	79.7 ± 7.6†	39.7 ± 1.8†
CUR	73.1 ± 5.5†	59.5 ± 9.0†	35.5 ± 5.3†	11.9 ± 0.6†	—
RES	100.5 ± 1.4	—	97.3 ± 6.0	93.6 ± 7.0	39.7 ± 6.4†
EC	99.6 ± 3.9	—	98.4 ± 7.8	94.1 ± 6.2	92.0 ± 5.5
ECG	98.5 ± 6.1	—	92.8 ± 6.5	65.9 ± 2.0†	54.7 ± 3.1†
EGC	94.9 ± 1.1†	—	103.4 ± 2.2	96.5 ± 0.8†	93.6 ± 4.0
EGCG	94.8 ± 2.2‡	—	92.0 ± 2.1†	66.5 ± 2.1†	34.5 ± 2.2†
PCB1	92.5 ± 2.0†	85.3 ± 2.5†	—	74.3 ± 1.11‡	78.7 ± 9.4‡
PCB2	88.5 ± 4.8‡	86.4 ± 2.3†	—	91.2 ± 2.9†	89.1 ± 5.9‡

PEITC, phenethyl isothiocyanate; SUL, sulforaphane; AITC, allyl isothiocyanate, CUR, curcumin; RES, resveratrol; EC, (−)-epicatechin; ECG, (−)-epicatechin-3-gallate; EGC, (−)-epigallocatechin; EGCB, (−)-epigallocatechin-3-gallate; PCBI, procyanidin B1; PCB2, procyanidin B2.

* The values are expressed as means ± SD (n = 4).

† $p < 0.01$, Student's *t* test.

‡ $p < 0.05$, Student's *t* test.

§ Not determined.

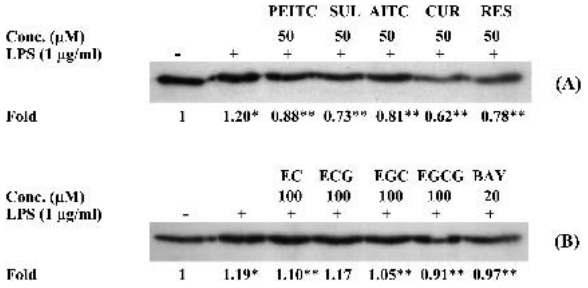

Fig. 4. Western blot analysis of phosphorylated IκBα prepared from the HT-29 N9 cells. Cells were treated with DMSO (0.1%) or natural chemopreventive compounds for 1 h at the indicated concentrations and challenged with LPS (1 μg/ml) for 0.5 h. The results are representative of two different experiments with similar results. *$p < 0.0001$ compared to control and **$p < 0.05$ compared to the cells treated by LPS, using ANOVA followed by unpaired Student's *t* test.

ity. Treatments of the cells with PEITC at 25 μM or greater for 48 h caused almost complete inhibition in the cell viability. SUL reduced the cell viability of these NF-κB transfectants to nearly 50% at 25 μM. However, AITC demonstrated little or weak effects on the cell viability even up to 50 μM concentration, although it almost completely inhibited LPS-induced NF-κB–luciferase activity back to the basal level. At higher concentration of 100 μM, AITC inhibited the cell viability by about 60%. CUR also strongly reduced the cell viability in a dose-dependent manner. The effects of RES on cell viability were similar to that of AITC. RES had little inhibition on cell viability up to 50 μM, but almost 60% inhibition of cell growth was seen at 100 μM. Among the tea catechin compounds, ECG and EGCG showed inhibitory activities against cell viability in a dose-dependent manner, whereas EC and EGC had little effect even up to 100 μM. At 100 μM, EGCG reduced more cell viability (34%) than EGC (54%). Procyanidin dimmers B1 and B2 were not effective in the inhibition of cell viability at the given conditions. According to our results, the inhibitory activity of the tested chemopreventive compounds at 50 μM could be ranked as follows: PEITC > SUL > CUR >> ECG > EGCG > PC B1 > AITC > PC B2 > RES > EC > EGC.

To examine further the effects of these compounds on the cell viability in the absence and presence of LPS, the Trypan blue staining assay was carried out after 48-h treatments of the cells. For this experiment, approximate or near IC_{50} values of the compounds based on the MTS assay were used. As shown in Fig 5, treatment with LPS (1 μg/ml) for 48 h resulted in an increase in the cell viability of the HT-29 N9 cells when compared to control ($p < 0.05$). In most cases, the LPS treatments displayed certain inhibitory effects on the cytotoxicity of the tested compounds. In this assay, some compounds did not show cell death at or near the 50%, even though we had used approximate IC50 values from the MTS assay. The reason(s) for this difference is not clear, but it could be due to the possible interactions between the compounds and the assay reagents. Even though the MTS and trypan blue assays are widely used to examine the cell viability, these assays determine only the metabolic activity and membrane integrity of the cells, and therefore more precise measurements would be needed to examine the viability of the cells by the natural chemopreventive agents.

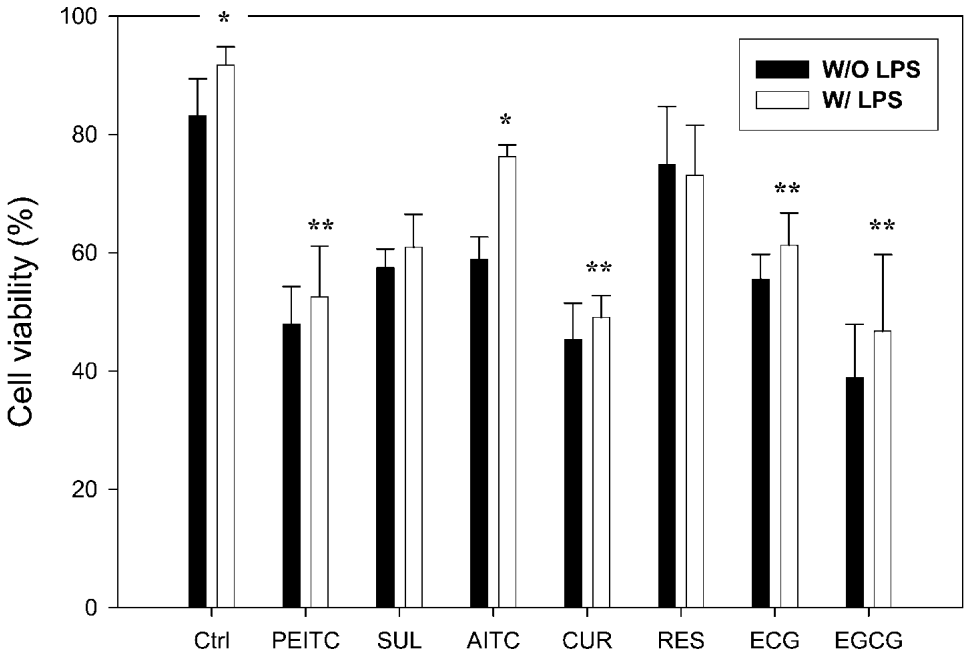

Fig. 5. Effects of various chemopreventive compounds on the cell viability of HT-29 N9 cells in the Trypan blue assay. Cells were pretreated with DMSO (0.1%) or compounds for 1 h, followed by 48-h treatment with and without LPS (1 μg/ml). The doses of compounds are as follows: PEITC, 17.5 μM; SUL, 25 μM; AITC, 60 μM; CUR, 17.5 μM; RES, 75 μM; EGCG, 75 μM; ECG, 100 μM. Results are expressed as percent cell viability calculated from the ratio between the number of viable cells and the total cells. Values are means ± SD ($n = 4$). *$p < 0.05$ and **$p < 0.5$ compared to the cells without LPS treatment, using an unpaired Student's *t* test.

Effects of Chemopreventive Agents on the Caspase-3 Activity

Caspases, a family of cysteine proteases, have been implicated to be central components of apoptotic machinery in cells (19). To investigate the apoptotic properties of the chemopreventive compounds in HT-29 N9 cells, we analyzed the caspase-3 activity in the cells after treatments with the selected compounds at different time intervals. The treatment doses were approximated or at the IC_{50} value of each compound based on the results of the MTS assay. As shown in Fig. 6, most chemopreventive compounds in this assay induced the caspase-3 activity, which potentially explains that one of the chemopreventive actions of these natural compounds could be through apoptosis of cancerous cells. However, the kinetics of caspase-3 activation by these compounds was quite different. The activation of caspase-3 peaked differently; 12 h for PEITC, 24 h for CUR and RES, 36 h for EGCG, and 48 h for SUL and ECG. The greatest induction of caspase-3 activity was observed in the EGCG-treated group with 17-fold induction over the control cells. Treatments with PEITC, CUR, and ECG resulted in almost 7- to 8-fold induction. Effects of SUL and RES on the caspase-3 activation were relatively lower than others under these conditions.

DISCUSSION

During the last several decades, a large number of chemopreventive compounds have been discovered from various natural sources. Of these natural products, the chemopreventive compounds identified from foods and edible plants have gained much attention because of their relatively low toxicity, low cost and availability, as well as because they are found in abundance in our everyday diets (20). However, the potential chemopreventive mechanisms of many of these natural compounds have not been fully understood. In addition, studies on the relative bioactivity of a diverse group of these compounds in colon cancer are lacking. Colon cell model may be one of the better ways to investigate the efficacy of dietary chemopreventive compounds due to their high possibility of direct exposure to this organ during our daily dietary intake. The chemopreventive compounds used in this study included three ITCs found in cruciferous vegetables, four catechins in tea, two procyanidin dimers present in various fruits including grapes, nuts, and cocoa, RES in grape seeds and red wine, and CUR found in turmeric curry food.

ITCs are produced from glucosinolates in cruciferous vegetables by myrosinase of intact plant tissue during processing or intestinal bacteria during digestion (21), which probably makes the ITCs directly accessible to intestinal epithelial cells. Despite a number of studies on the chemopreventive properties of ITCs, only a limited number of studies involved signal transduction pathways, in particular on the transcription activation of NF-κB in colon cancer model. SUL has been reported to reduce selectively DNA binding of NF-κB in macrophages without interfering with LPS-induced degradation of the IκBα or with nuclear translocation of NF-κB (22). These authors suggested that the thiol group of SUL was responsible for the modification of NF-κB subunits. Patten and DeLong (23), however, reported that treatments with benzyl isothiocyanate at 25 μM increased NF-κB binding to NF-κB transcription factor binding site in unstimulated HT-29 cells, reaching a maximum at around 6 h. In our current study, PEITC and SUL at 10 μM and AITC at 50 μM substantially reduced the LPS-induced NF-κB–luciferase ac-

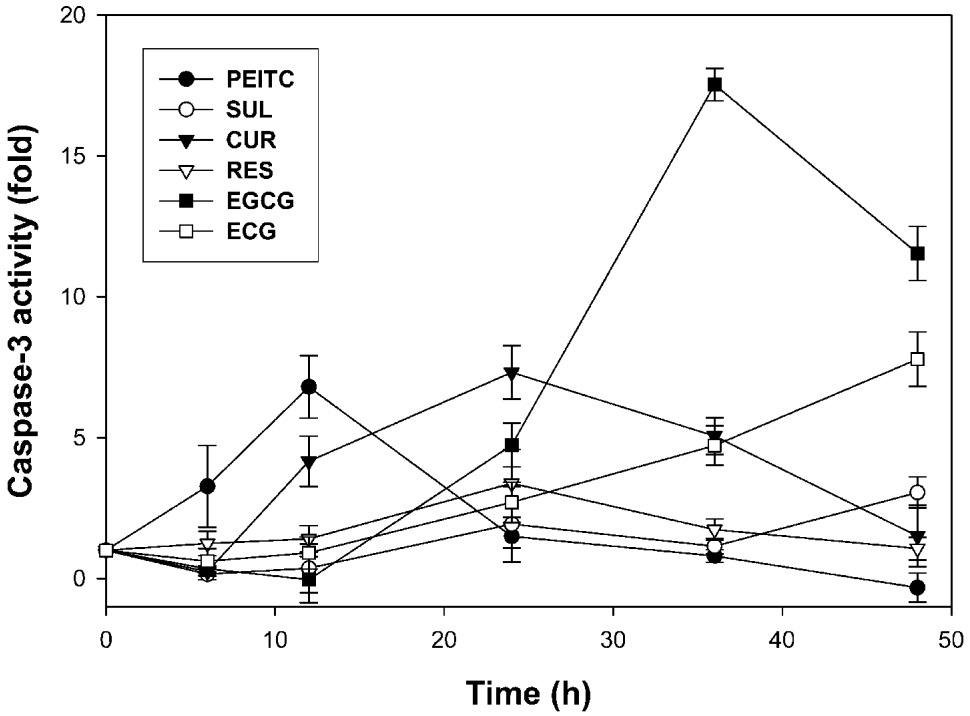

Fig. 6. Time-dependent activation of caspase-3 by natural chemopreventive compounds in HT-29 N9 cells. Cells were treated with DMSO (0.1%) or compounds with doses of their approximate IC_{50} at the indicated times (PEITC, 20 μM; SUL, 25 μM; CUR, 20 μM; RES, 75 μM; EGCG, 75 μM; ECG, 100 μM). Results are expressed as fold induction over control and values are means ± SD (n = 3).

tivity in HT-29 cells (Fig. 1), as well as phosphorylation of IκBα (Fig. 4).

CUR has been known as a potent inhibitor of prostaglandin synthesis (24). It also has been reported to be a potent inhibitor of NF-κB activation in various human cell lines including human umbilical vein endothelial, myeloid ML-1a, promyelocytic leukemia, and colon cancer cells (25–29). A recent study revealed that CUR suppressed osteopontin-induced IκBα phosphorylation and degradation by inhibiting the IKK activity (30). Our results agree with these previous studies in that 10 μM CUR has potent inhibitory activity against NF-κB–luciferase activity in HT-29 N9 cells, as well as inhibition of LPS-induced phosphorylation of IκBα.

Studies on RES have also shown an inhibitory role of RES on the NF-κB activation in various cell lines, although there has been no study with colon cell model (31–35). Results from our HT-29 cell model, however, demonstrated dramatic inductions of the LPS-induced NF-κB–luciferase activity at concentrations between 25 and 100 μM, while it showed an inhibition on the LPS-induced phosphorylation of IκBα at 50 μM (1.5 h incubation), which might decrease the nuclear localization of NF-κB. The exact mechanism for the difference and the contribution of these opposite process to the net effect is not clear, but may suggest an involvement of additional mechanism(s) in NF-κB pathway elicited by RES, which may be independent of IκBα phosphorylation. In fact, the cell viability was not significantly reduced with the treatment of RES at 50 μM for 48 h, suggesting an involvement of protective mechanism due to activation of NF-κB by RES at the given concentration.

Tea catechins are considered to be main chemopreventive ingredients in tea, and their effects on NF-κB have widely been studied in many cell lines but little in colon cells (36). Tea catechins including EC, ECG, EGC, and EGCG, as shown in the current study, appear to have little or slightly stimulatory effects on the LPS-induced NF-κB–luciferase activity under the current conditions. In Western blotting analysis, however, EGCG diminished the LPS-induced IκBα phosphorylation. Park *et al.* (37) have reported the effects of polymerization degree of flavonoids on nitric oxide (NO) production, TNF-α secretion, and NF-κB–dependent gene expression in an interferon-γ–stimulated RAW 264.7 macrophage cell line. Procyanidin dimmers B1 and B2 had a weak inhibitory activity on NO production, TNF-α secretion, and NF-κB–dependent gene expression, as compared to the monomeric flavonoids such as catechin, epicatechin, and taxifolin. The trimeric procyanidin C2 and pine bark extract (PBE), which is rich in oligomeric procyanidins, instead have been shown to activate these parameters in the same cells under the same conditions. In contrast, PBE has been reported to inhibit ultraviolet (UV) radiation-induced NF-κB–dependent gene expression in a concentration-dependent manner in the human keratinocyte cell line HaCaT, whereas NF-κB–DNA-binding activity was not prevented, suggesting that PBE may affect different transactivation pathways of NF-κB (38).

Flavonoids such as EGCG have been reported to induce formation of H_2O_2 in cancer cells (39). H_2O_2 is known to activate NF-κB activity through the activation of IκB kinase (IKK) (40). Recently, it has been suggested that EGCG mediates Fenton reaction by reducing ferric ion to ferrous ion, which causes hydroxy radical production, resulting in the activation of caspase and finally apoptotic cell death (41). Li *et al.* (42) have also implicated that early activation of NF-κB may be one of the mechanisms of apoptosis in intestinal epithelial cells by reactive oxygen species. However, most recently, we found that catalase, which abolished hydrogen peroxide-induced signaling, had no effect on EGCG-induced signaling such as JNK and ERK pathways (43). Therefore, the exact mechanism(s) leading to the induced activities of NF-κB–luciferase in HT-29 cells by various phenolic compounds such as tea catechins, procyanidins, and RES is unclear, but potentially might involve some other reactive intermediates.

Our cell viability study with various natural chemopreventive agents using MTS assay appears to provide certain structure–activity relationships. For the ITCs, allyl group is less effective than other functional group in the cell viability inhibition. When comparing CUR and RES, which have two side phenolic rings, the presence of the diketone in CUR appears to be responsible for its more potent inhibitory activity against cell viability than RES (44,45). Among the tea catechins, the gallate group at C-3 position at C-ring may account for the inhibitory activities of ECG and ECGC (46). Our results from Trypan blue staining assay imply a possible cell proliferative effect of the NF-κB activation that is induced by LPS. In case of ITCs and CUR, inhibition of NF-κB activation decreased cell survival induced by LPS, suggesting a NF-κB–mediated survival mechanism. However, for the polyphenolic compounds such as RES, ECG, and EGCG at the given concentrations, the additive activation of NF-κB did not protect the cells from dying, implying that the activation of NF-κB *per se* is not sufficient to protect cell death. Therefore, unknown mechanisms were operational.

To investigate further whether the cytotoxicity elicited by these compounds is related to apoptotic cell death, we tested their effects on the activity of caspase-3, which is a converging point of the diverse caspase-dependent apoptosis pathway. A number of chemopreventive and/or chemotherapeutic agents have been reported to induce apoptosis in various cell types (39,47–51). In the current study, we compared the effect of natural chemopreventive compounds on caspase-3 activity in these HT-29 N9 cells during the 48-h period after treatments with the compounds. Of the compounds tested in this assay, the induction of caspase-3 activity was the fastest by PEITC treatment (peak at 12 h) (Fig. 6). A delayed peak induction of caspase-3 activity was observed by EGCG (36 h), whereas CUR and RES displayed an intermediate peak induction (24 h). Caspase-3 activation induced by ECG and SUL increased gradually during the 48-h period. We have recently reported an early activation of caspase-3 activity by PEITC (12 h) and a gradually increased response by EGCG during 24-h period in HT-29 untransfected cells (43,52), analogous to the results we obtained here using the HT-29 N9 clone. The reason why different chemopreventive compounds differentially activated the caspase-3 pathway in this cell is not clear, but could be due to different reactive intermediates formed, mitochondrial damage, and/or gene expression. However, understanding the different kinetics of apoptotic enzyme activation by different chemopreventive agents could provide insights for future *in vivo* applications or possible combination of these agents. The constitutive activation of NF-κB has been implicated to cause cell proliferation and resistance to apoptosis in certain cancer cells (12), which may, in part, explain the chemopreventive inhibitory roles of

some of these compounds used in this study. Further studies would be needed to elucidate the modulatory mechanism between NF-κB signaling and apoptosis as well as the expression of NF-κB–mediated endogenous genes by natural chemopreventive compounds.

In conclusion, this report demonstrates that ITCs and CUR have more potent modulatory effects on the NF-κB signaling pathway as well as inhibition of the cell viability in HT-29 colon cancer cells than other phenolic-based compounds tested in this study, such as RES, tea catechins, and procyanidin dimers. Most chemopreventive compounds in the current study induce the caspase-3 activity but with different extent and kinetics. Due to the highly complicated mechanisms of signal transduction pathways in cancer, however, blocking or inducing a single signaling pathway may or may not account for the complete chemoprevention mechanism demonstrated by natural compounds. Moreover, different chemopreventive agents seem to play one or more roles at different targets in the highly complicated cellular signal transduction events that may be dependent on the types and concentrations of the chemopreventive agents as well as the cell/tissue types. Therefore, future studies on other signaling pathways as well as their biological consequences elicited by these natural agents are needed to further elucidate the chemopreventive mechanisms involved.

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