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Inhibition of Activator Protein-1, NF-κB, and MAPKs and Induction of Phase 2 Detoxifying Enzyme Activity by Chlorogenic Acid*

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Chlorogenic acid, the ester of caffeic acid with quinic acid, is one of the most abundant polyphenols in the human diet. The antioxidant and anticarcinogenic properties of chlorogenic acid have been established in animal studies. However, little is known about the molecular mechanisms through which chlorogenic acid inhibits carcinogenesis. In this study, we found that chlorogenic acid inhibited the proliferation of A549 human cancer cells *in vitro*. The results of the soft agar assay indicated that chlorogenic acid suppressed 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced neoplastic transformation of JB6 P^+ cells in a dose-dependent manner. Pretreatment of JB6 cells with chlorogenic acid blocked UVB- or TPA-induced transactivation of AP-1 and NF-κB over the same dose range. At low concentrations, chlorogenic acid decreased the phosphorylation of c-Jun NH_2-terminal kinases, p38 kinase, and MAPK kinase 4 induced by UVB/12-*O*-tetradecanoylphorbol-13-acetate, yet higher doses were required to inhibit extracellular signal-regulated kinases. Chlorogenic acid also increased the enzymatic activities of glutathione *S*-transferases (GST) and NAD(P)H: quinone oxidoreductase. Further studies indicated that chlorogenic acid could stimulate the nuclear translocation of Nrf2 (NF-E2-related factor) as well as subsequent induction of GSTA1 antioxidant response element (ARE)-mediated GST activity. The phosphatidylinositol 3-kinase pathway might be involved in the activation of Nrf2 translocation. These results provide the first evidence that chlorogenic acid could protect against environmental carcinogen-induced carcinogenesis and suggest that the chemopreventive effects of chlorogenic acid may be through its up-regulation of cellular antioxidant enzymes and suppression of ROS-mediated NF-κB, AP-1, and MAPK activation.

Epidemiological evidence demonstrates that consumption of healthy foods containing phytochemical compounds might reduce the incidence of cancer and chronic degenerative diseases (1). Chlorogenic acid, the ester of caffeic acid with quinic acid, is one of the most abundant polyphenols in human diet and has been reported to decrease the incidence of chemical carcinogenesis in several animal models of cancer (2, 3). However, the molecular mechanisms for its anti-carcinogenic properties are poorly understood. Thus, elucidation of such mechanisms of action is essential before the possibility for chlorogenic acid application in chemoprevention can be considered.

AP-1[1] and/or NF-κB signal transduction pathways are known to be important molecular targets of chemopreventive strategies (4–6). Activation of AP-1 or NF-κB induces the expression of target genes that are involved in many disease processes, such as inflammation, neoplastic transformation, tumor progression, metastasis, and angiogenesis (5–7). AP-1 activity has been shown to be involved in the tumor promotion and progression of various types of cancers (4–6). Active NF-κB is found in the nucleus of many different cancer cells (8). The potential proapoptotic effect of NF-κB on normal cells and its antiapoptotic and cytoprotective effect on tumor cells have also been reviewed (8, 9). The components of AP-1 are activated by three distinct but parallel MAPKs: ERKs, JNKs, and p38 kinase. Each consists of a module of three kinases: MAPK, a MAPK kinase (MAPKK) that is responsible for the phosphorylation of MAPK, and a MAPKK kinase that phosphorylates and activates MAPKK (10, 11). NF-κB can also be activated at the MAPKK kinase level, by MAPK/ERK kinase 1 or 3 (5, 12). AP-1, NF-κB, and associated MAPK signal transduction pathways are believed to be crucial in cell transformation and tumor promotion (13–16). Because of the critical roles of NF-κB, AP-1, and MAPK signaling in carcinogenesis, they have been proposed as targets for chemopreventive agents (5).

It has been reported that the induced AP-1 activity and neoplastic transformation can be blocked by chemopreventive agents, such as pyrrolidine dithiocarbamate (4), tea polyphenols (6), resveratrol (17), and blackberry extract (18). Many of these inhibitory agents have been shown to be active not only in the JB6 transformation model but also in mouse skin tumor promotion *in vivo* (5). Thus, the mouse epidermal cell line, JB6, provides a validated model for screening cancer chemopreventive agents and elucidating their mechanisms at the molecular level (4, 5).

The transcription factor Nrf2 is a member of the basic leucine zipper NF-E2 family and plays an essential role in the antioxidant response element (ARE)-mediated expression of phase 2 detoxifying enzymes and stress-inducible genes (19–21). Inducers of phase 2 and antioxidative enzymes are known to enhance the detoxication of environmental carcinogens in

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[1] The abbreviations used are: AP-1, activator protein-1; MAPK, mitogen-activated protein kinases; MAPKK, MAPK kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal kinase; MKK4, MAP kinase kinase 4 (alternatively designated SEK1 or MEK4); NQO1, NAD(P)H:quinone oxidoreductase; GST, glutathione *S*-transferase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; ECIS, electric cell-substrate impedance sensing; ROS, reactive oxygen species; ARE, antioxidant response element; FBS, fetal bovine serum; PI, phosphatidylinositol.

animals, often leading to protection against neoplasia (22–25). Regulation of both basal and inducible expression of cytoprotective genes is mediated in part by the ARE, a *cis*-acting sequence found in the 5′-flanking region of genes encoding many phase 2 enzymes, including heme oxygenase-1, glutathione *S*-transferase (GST) A1, NAD(P)H:quinone oxidoreductase (NQO1), and Nrf2 itself (23–27). Among these defensive systems, GST may block JNK-induced Jun activation and subsequently inhibit mitogenic signaling induced by oncogenic Ras-p21 (28). As the endogenous inhibitor of apoptosis signal-regulating kinase 1, GST may also inhibit apoptosis signal-regulating kinase 1-activated JNK and p38 signaling pathways (29). Thus, GSTs display broad substrate specificity and are associated with cancer chemopreventive and cytoprotective effects (26, 30). Studies with *nrf2*-disrupted mice indicated that Nrf2 was essential for the induction of GST and NQO1 activities *in vivo* by different classes of chemopreventive agents, including dithiole-thiones, isothiocyanates, and phenolic antioxidants (31, 32). The likely importance of these protective enzymes is highlighted by recent observations that *nrf2*-null mice were considerably more sensitive to the tumorigenicity of benzo[a]pyrene (33) and form higher levels of DNA adducts following exposure to carcinogens (34). Thus, the induction of phase 2 gene expression is an effective strategy for achieving protection against carcinogenesis (22).

Given the animal data suggesting cancer prevention properties of chlorogenic acid, we tested the effects of chlorogenic acid on proliferation of cancer cells and neoplastic transformation induced by tumor promoter in JB6 cells. To elucidate the mechanism of the anti-tumorigenic effect of chlorogenic acid, we also investigated the effects of chlorogenic acid on NF-κB, AP-1, and MAPK activation induced by tumor promotors as well as on the induction of Nrf2 transactivation and phase 2 enzyme activities. Our results demonstrate possible chemoprevention activity of chlorogenic acid and characterize mechanisms of the inhibitory actions on tumor promotion and the inductive effects on Nrf2 transactivation and phase 2 enzyme activity.

EXPERIMENTAL PROCEDURES

Materials—Chlorogenic acid, 1-chloro-2,4-dinitrobenzene, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, NADP, FAD, menadione, glucose 6-phosphate, yeast glucose-6-phosphate dehydrogenase, and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma. LY294002 was obtained from Calbiochem. Eagle's minimal essential medium and Dulbecco's modified Eagle's medium were obtained from Whittaker Biosciences (Walkersville, MD). Opti-MEM I medium was from Invitrogen. Fetal bovine serum (FBS), gentamicin, and L-glutamine were purchased from Invitrogen. Fugene 6 Transfection reagent was from Roche Applied Science. Antibody against MKK4 or Nrf2 was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Phospho-SEK1/MKK4 and PhosphoPlus MAPK antibody kits were purchased from Cell Signaling Technology (Beverley, MA). The luciferase assay kit was obtained from Promega (Madison, WI).

Plasmids and Cell Culture—AP-1 or NF-κB luciferase reporter plasmid is the construct containing the collagenase promoter bearing AP-1- or NF-κB-binding sites that drives a luciferase reporter gene. A DNA sequence containing the GST A1 ARE (−833 to −533 from the start codon) was prepared by PCR from the mouse GST A1 promoter (−1094 to −10), which was isolated from mouse brain cDNA and inserted into a luciferase reporter vector (ARE-TATA Luc$^+$) (35). The JB6 promotion-sensitive (P$^+$) mouse epidermal cell line and JB6 cells, stably transfected with either AP-1-luciferase reporter plasmid (4) or a NF-κB-luciferase reporter plasmid (a gift from Dr. Chuanshu Huang, New York University School of Medicine), were cultured in Eagle's minimal essential medium containing 5% FBS, 2 mM L-glutamine, and 50 μg/ml gentamicin. The human lung cancer epithelial cell line, A549, was obtained from the American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium containing 10% FBS. The cells were grown at 37 °C in a 5% CO_2 atmosphere. In all experiments, exponentially growing cells were used.

Assay of the Antioxidant Capacity of Chlorogenic Acid—The antioxidant capacity of chlorogenic acid was determined using a 2,2′-azio-diethylbenzthiazoline sulfonate test set (Randox Laboratories Ltd.). The principle of the assay depends on production of the radical cation 2,2′-azio-diethylbenzthiazoline sulfonate$^+$ in incubation medium containing the substrates (H_2O_2 and peroxidase), which is a blue-green color and can be detected at 600 nm. Antioxidants in the sample cause the suppression of this color production to a degree that is proportional to their concentrations. The assays were calibrated against standards and expressed as μmol/liter.

Assay of AP-1 or NF-κB Activity—Confluent monolayers of JB6 stable transfectants were trypsinized, and 2×10^4 viable cells suspended in 0.5 ml of Eagle's minimal essential medium supplemented with 5% FBS were seeded to each well of a 48-well plate. Plates were incubated overnight at 37 °C in a humidified atmosphere of 5% CO_2. The medium was then switched to 0.1% FBS Eagle's minimal essential medium and culture for 24 h to minimize basal activity of AP-1 or NF-κB. The cells were pretreated with or without chlorogenic acid for 1 h and then exposed to TPA (20 ng/ml) or UVB (4 kJ/m^2) irradiation. The cells were extracted with 100 μl of 1× lysis buffer provided in the luciferase assay kit by the manufacturer. Luciferase activity was measured using a Monolight luminometer, model 3010. The results are presented as relative AP-1 or NF-κB activity compared with untreated controls (36).

Cell Proliferation Assay by Electric Cell-Substrate Impedance Sensing (ECIS)—The ECIS model 1600R (Applied BioPhysics, Troy, NY) was used to measure the influence of chlorogenic acid on the proliferation of human cancer A549 cells or JB6 cells. The ECIS assay has been used for continuous measurement of cell micromotion, attachment, spreading, and growth (37, 38). The cells (1×10^4) were suspended in 400 μl of Dulbecco's modified Eagle's medium with or without chlorogenic acid and seeded on electrodes. The cells were equilibrated in the incubator for 15 min. The rate of cell proliferation on the microelectrode was monitored for 72 h as real-time changes in resistance.

Anchorage-independent Transformation Assay—The effect of chlorogenic acid on TPA-induced cell transformation was investigated in JB6 P$^+$ cells using the soft agar assay as described previously (4, 39). The cells (1×10^4) were exposed to TPA (20 ng/ml) in the presence or absence of different concentrations of chlorogenic acid, in 1 ml of 0.33% basal medium Eagle agar containing 15% FBS over 3.5 ml of 0.5% agar containing 15% FBS Eagle's minimal essential medium. The cultures were maintained in a 37 °C, 5% CO_2 incubator for 2 weeks, and the anchorage-independent colonies were counted.

Protein Kinase Phosphorylation Assay—The cells were extracted with 1× SDS sample buffer. Immunoblots for phosphorylation of ERKs, JNKs, and p38 kinase were carried out as described in the protocol of the manufacturer, using phosphospecific antibodies against phosphorylated sites of ERKs, JNK, and p38 kinase. Nonphosphospecific antibodies against ERKs and p38 kinase proteins provided in the assay kits were used to normalize the phosphorylation assay, using the same transferred membrane blot (36).

Enzyme Activity Assay—The activities of the typical phase 2 enzymes, NQO1 and GST, were measured spectrophotometrically as described previously (35, 40). For the NQO1 assay, the cells were grown in 96-well plates, treated with chlorogenic acid, and lysed by 0.8% digitonin. The reaction solution (25 mM Tris-HCl (pH 7.4), 0.06% bovine serum albumin, 5 μM FAD, 1 mM glucose 6-phosphate, 30 μM NADP, 300 units of glucose-6-phosphate dehydrogenase, 725 μM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, and 50 μM menadione) was added into the wells, and the reduced 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was measured at 610 nm. NQO1 induction by chlorogenic acid was expressed as ratios of treated over vehicle control (35). For total GST assay, JB6 cells were incubated with chlorogenic acid and then lysed with 200 μl of lysis buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100) for 30 min. Cytosolic fractions were prepared by centrifugation and stored at −70 °C until use. Assays were conducted in a thermostated compartment at 25 °C, using 1-chloro-2,4-dinitrobenzene as the substrate. Cytosolic protein (45 μg) was added to 800 μl of reaction mixture containing 100 mM KH_2PO_4 (pH 6.5) and 1 mM glutathione. The reaction was initiated by adding 1 mM 1-chloro-2,4-dinitrobenzene, and the formation of thioether at 5 min was measured at 340 nm. Total enzymatic activity of GST was expressed as nmol/min/mg protein.

Immunocytochemistry of Nrf2—After treatment with chlorogenic acid for 3 h, JB6 cells grown on Lab-TEK chamber slides were fixed in 100% methanol for 30 min at room temperature. The cells were then washed with phosphate-buffered saline followed by blocking with 5% bovine serum albumin in phosphate-buffered saline for 1 h. The cells were incubated with polyclonal rabbit anti-Nrf2 antibody

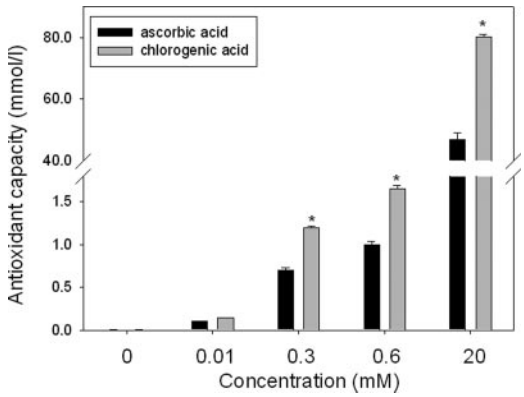

FIG. 1. **The antioxidant capacity of chlorogenic acid.** The antioxidant capacity of chlorogenic acid and ascorbic acid was determined as described under "Experimental Procedures." Chlorogenic acid or ascorbic acid was added into the reaction mixture and incubated at 37 °C. The absorbance at 600 nm was recorded according to the protocol of the RANDOX kit. The results were calculated as total antioxidant capacity and presented as mmol/liter. Data are means ± S.E. of three assays. *, antioxidant activity significantly greater than that of ascorbic acid.

(1:100) in phosphate-buffered saline containing 0.5% bovine serum albumin overnight at 4 °C. After they were washed four times with PBS, the cells were incubated with fluorescein isothiocyanate-conjugated secondary antibody (Santa Cruz Biotechnology) for an additional 1 h in the dark. Counterstaining with 4′,6-diamidino-2-phenylindole verified the location and integrity of nuclei. Stained cells were washed and examined using a laser-scanning confocal microscope, Zeiss LSM 510 (Thornwood, NY).

Preparation of Nuclear Extracts and Nrf2 Nuclear Translocation Assay—Nuclear extracts from JB6 cells were prepared as described previously (15). Briefly, cells were treated with or without chlorogenic acid and harvested. The cells were suspended in hypotonic buffer A (10 mM HEPES (pH 7.6), 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) for 10 min on ice and then vortexed for 10 s. Nuclei were pelleted by centrifugation at 12,000 × *g* for 20 s and were resuspended in buffer C (20 mM HEPES (pH 7.6), 25% glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) for 30 min on ice. The supernatants containing nuclear proteins were collected after centrifugation at 12,000 × *g* for 2 min and stored at −70 °C. Proteins that were extracted from either whole cell lysate (30 μg) or nuclei (30 μg) were separated by SDS-PAGE, transferred to nitrocellulose membranes, and detected with an Nrf2 antibody.

Transfection of Plasmids and Measurement of Luciferase Activity—A DNA sequence containing mouse GST A1 ARE (−833 to −533 from the start codon) was inserted into a luciferase reporter pGL3-Basic vector (ARE-TATA Luc$^+$) as described previously (35). JB6 cells were plated on 24-well plates at density of 50–60% confluence. The transfection complex containing 0.5 μg of plasmid DNA in Opti-MEM I medium was added to each well. The cells were treated with chlorogenic acid for 18 h following transient transfection of ARE-TATA Luc$^+$. Luciferase activity was measured using the luciferase assay kit as described above.

Statistical Analysis—Data are presented as means ± S.E. of *n* experiments/samples as noted in the figure legends. Significant differences were determined using Student's *t* test. Significance was set at $p \leq 0.05$.

RESULTS

The Total Antioxidant Capacity of Chlorogenic Acid—Reactive oxygen species (ROS) have been known to be mutagenic and associated with many diseases. In light of the important role of ROS in tumor promoter-induced AP-1 activation, transformation, and tumor promotion, we measured the antioxidant capacity of chlorogenic acid using the Randox reagent set. As shown in Fig. 1, chlorogenic acid displayed stronger antioxidant activity than that of ascorbic acid over the same concentration range, indicating that chlorogenic acid can effectively scavenge reactive oxygen radicals.

Chlorogenic Acid Inhibits the Proliferation of Cancer Cells—The effect of chlorogenic acid on proliferation of a human lung

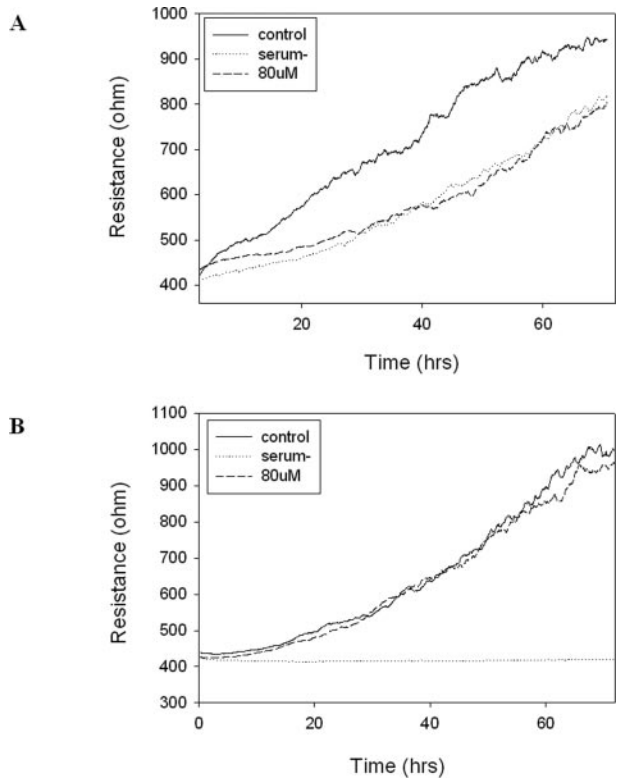

FIG. 2. **Effect of chlorogenic acid on proliferation of A549 and JB6 cells by ECIS assay.** A549 cells (*A*) or JB6 cells (*B*) were incubated in the electrode array wells with or without 80 μM chlorogenic acid (indicated as 80 μM) for 72 h. The ECIS resistance, indicating the cell growth rate, was monitored for the duration of the experiment. Data shown are representative of two independent experiments. Cells in serum-free (serum−) medium were used as a negative control.

cancer cell line, A549, was determined using the ECIS assay. Cells were grown in the electrode array wells and treated with chlorogenic acid for 72 h. The ECIS resistance, indicating the number of cells in the wells, was monitored for the duration of the experiment. At a concentration of 80 μM, proliferation of A549 cells was significantly suppressed (Fig. 2*A*). Interestingly, chlorogenic acid had little effect on the proliferation of JB6 cells at the same dose (Fig. 2*B*). This result suggests that chlorogenic acid may preferentially inhibit tumor cell growth.

Effects of Chlorogenic Acid on TPA- or UVB-induced AP-1 and NF-κB Activation—Previous studies have shown that either AP-1 or NF-κB activation is required for neoplastic transformation in JB6 cells (4, 5, 41) and that these two transcription factors play an important role in carcinogenesis. We thus tested the effects of chlorogenic acid on TPA- or UVB-induced AP-1 and NF-κB activity, using a reporter gene assay. Pretreatment of cells with chlorogenic acid markedly inhibited TPA-induced AP-1 and NF-κB activity over a similar concentration range (Fig. 3, *A* and *B*). At a concentration of 40 μM chlorogenic acid, TPA-induced AP-1 or NF-κB activation was suppressed by 30 or 42%, respectively. Chlorogenic acid alone had no effect on AP-1 or NF-κB activity. These inhibitory effects were not due to the cytotoxicity of chlorogenic acid on JB6 cells, since the ECIS assay indicated that the proliferation of JB6 cells was not affected by chlorogenic acid even at a concentration of 80 μM (Fig. 2*B*).

It is well known that UVB irradiation acts both as a tumor initiator and tumor promoter, playing a major role in the development of skin cancer (42). Thus, we investigated the effects of chlorogenic acid on UVB-induced AP-1 and NF-κB activation. As shown in Fig. 3, *C* and *D*, pretreatment of JB6 cells with chlorogenic acid resulted in a significant inhibition of AP-1 and NF-κB transactivation induced by UVB irradiation. At the concentration

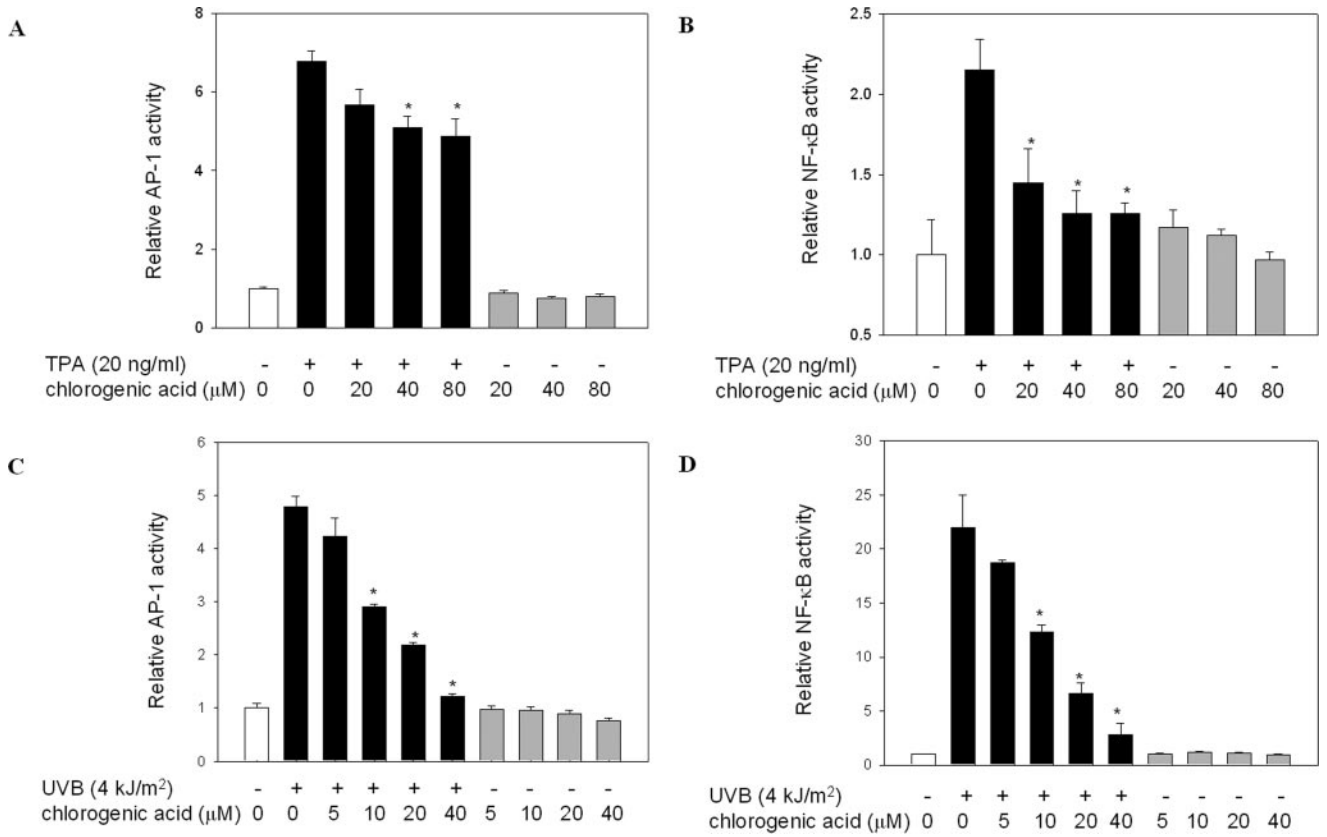

FIG. 3. **Inhibition of TPA- or UVB-induced AP-1 and NF-κB activation by chlorogenic acid.** JB6 cells were pretreated for 1 h with various concentrations of chlorogenic acid and then exposed to 20 ng/ml TPA (*A* and *B*) or 4 kJ/m^2 UVB radiation (*C* and *D*). After culturing the cells at 37 °C for 14 h (UVB) or 48 h (TPA), the AP-1 or NF-κB activity of the cell extract was measured by the luciferase activity assay. Results, presented as relative AP-1 or NF-κB induction compared with untreated control cells, are expressed as means and S.E. from three assay wells. Data shown are representative of three independent experiments. *, a significant inhibition of AP-1 or NF-κB activation by chlorogenic acid ($p < 0.05$).

of 40 μM of chlorogenic acid, the activation of AP-1 or NF-κB induced by UVB was almost completely blocked to the basal level.

Inhibition of TPA-induced Cell Transformation by Chlorogenic Acid—AP-1 and NF-κB activation are critical to TPA-induced cell transformation (4, 41). Based on the inhibitory effects of chlorogenic acid on tumor promoter-induced AP-1 and NF-κB activities, our next aim was to determine whether chlorogenic acid inhibited TPA-induced JB6 P$^+$ cell transformation. Cells (1×10^4) were exposed to 20 ng/ml TPA in soft agar for 14 days, and the colonies formed in the soft agar were recorded. The results indicated that TPA-induced cell transformation was significantly inhibited by chlorogenic acid over the same concentration range that repressed AP-1 and NF-κB activation (Fig. 4). The inhibition of cell transformation by chlorogenic acid could not be caused by growth inhibition, because the tested concentration range of chlorogenic acid did not suppress JB6 proliferation as measured by ECIS assessment. These results suggest that the inhibitory effects of chlorogenic acid on tumor promoter-induced AP-1 and NF-κB activation may contribute to the inhibition of TPA-induced cell transformation.

Effect of Chlorogenic Acid on TPA- or UVB-induced Activation of MAPKs—AP-1 has been identified as a target of MAPK signaling (5). MAPK pathways, including ERKs, JNKs, and p38 kinase, influence AP-1 transactivation by increasing the abundance of AP-1 components and/or altering the phosphorylation of their subunits (43). To determine which class of MAPK members might be involved in the inhibition of tumor promoter-induced AP-1 activation by chlorogenic acid, we analyzed the effects of chlorogenic acid on TPA- or UVB-induced phosphorylation of JNKs, ERKs, and p38 kinase. Previous studies indicated that

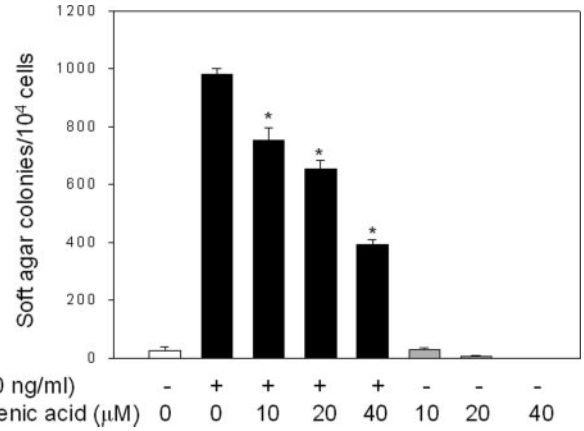

FIG. 4. **Inhibition of TPA-induced JB6 P$^+$ cell neoplastic transformation by chlorogenic acid.** JB6 P$^+$ cells (1×10^4) were exposed to TPA (20 ng/ml) with or without chlorogenic acid in 0.33% agar for 14 days and scored for colonies at the end of the experiments. Data are means ± S.E. of four experiments. *, a significant inhibition of transformation ($p < 0.05$).

ERK activation is an early event occurring at 0.5–4 h following TPA treatment, whereas activation of JNKs and p38 was a later event occurring at 6–24 h in JB6 cells (44). Western blot analysis was carried out using antibodies that recognize activated phosphorylated MAPK forms. Pretreatment of cells with chlorogenic acid resulted in a significant inhibition of UVB-induced JNKs and p38 activation even at the concentration of 15 μM, whereas a higher dose was required for inhibition of ERKs (Fig. 5, *A* and *B*). Chlorogenic acid also inhibited TPA-induced JNKs and p38 acti-

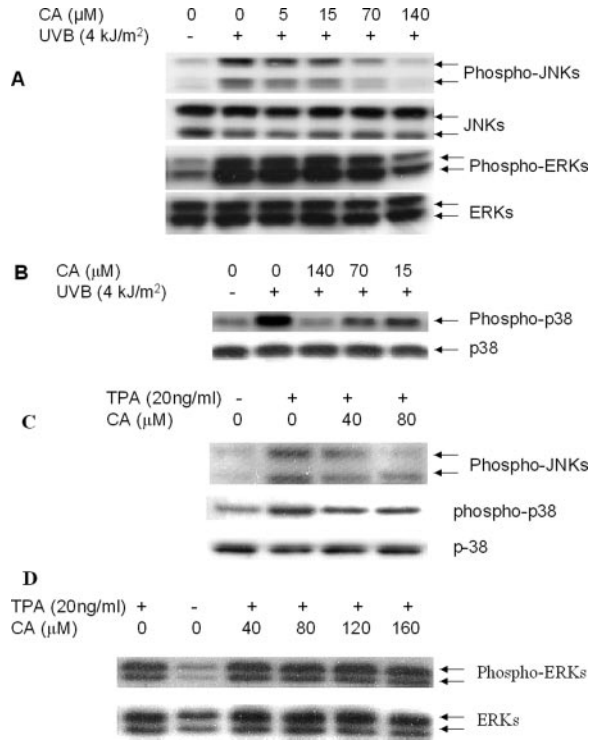

FIG. 5. **Influence of chlorogenic acid on UVB- or TPA-induced phosphorylation of MAPKs.** JB6 cells were pretreated with different concentrations of chlorogenic acid for 1 h and then were exposed to UVB (4 kJ/m^2) or TPA (20 ng/ml) for 30 min. For TPA-induced activation of JNKs and p38 kinase, the cells were treated with both of TPA and chlorogenic acid for 12 h. The phosphorylated and nonphosphorylated ERKs, JNKs, and p38 kinase proteins in the cell lysate were assayed using a PhosphoPlus MAPK kit from New England Biolabs. The phosphorylated proteins and nonphosphorylated proteins were detected, using the same transferred membrane blot following a stripping procedure. *A*, the effect of chlorogenic acid (*CA*) on UVB-induced phosphorylation of JNKs and ERKs. *B*, the effect of chlorogenic acid on UVB-induced phosphorylation of p38 kinase. *C*, the effect of chlorogenic acid on TPA-induced phosphorylation of JNKs and p38 kinase. *D*, the effect of chlorogenic acid on TPA-induced phosphorylation of ERKs.

vation but failed to show inhibition of ERKs even at a concentration of 160 μM (Fig. 5, *C* and *D*).

It is well known that in the cascade of signal transduction pathways of MAPKs, SEK1/MKK4 is a direct upstream signaling molecule (5, 10). Since chlorogenic acid displayed an inhibitory effect on the activation of MAPKs, we examined the effect of chlorogenic acid on MKK4 activation induced by UVB. We found that pretreatment of JB6 cells with chlorogenic acid blocked UVB-induced phosphorylation of MKK4 at the Thr^{261} site in a dose-dependent manner (Fig. 6). These results suggest that the inhibitory effects of chlorogenic acid on UVB-induced AP-1 activation may be modulated through the inhibition of MAPK and MAPKK activation.

Induction of NQO1 and GST Enzymatic Activities by Chlorogenic Acid in JB6 Cells—It was reported that chemopreventive agents could induce phase 2 gene expression, and this is considered as one of the important mechanisms for these agents to protect cells or organisms against environmental carcinogen insult (23, 26). To evaluate the possibility that chlorogenic acid possesses a similar induction of detoxifying enzymes, JB6 cells were incubated with the indicated concentrations of chlorogenic acid for 18 h, and the NQO1 and total GST activities were measured. Chlorogenic acid moderately increased both NQO1 and GST enzymatic activities in a dose-dependent manner (Fig. 7). After treatment of JB6 cells with chlorogenic acid at 40 and 80 μM, GST and NQO1 activities increased by 46.8 and 47.1%, respectively (Fig. 7).

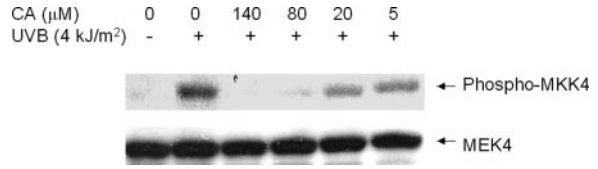

FIG. 6. **Chlorogenic acid inhibits UVB-induced threonine 261 phosphorylation of SEK1/MKK4.** JB6 cells were pretreated with different concentrations of chlorogenic acid (*CA*) for 1 h and exposed to UVB (4 kJ/m^2). Phosphorylated SEK1/MKK4 in cell lysate was determined, using a phosphospecific (Thr^{261}) antibody from Cell Signaling Technology. The phosphorylated proteins and nonphosphorylated proteins were detected, using the same transferred membrane blot following a stripping procedure.

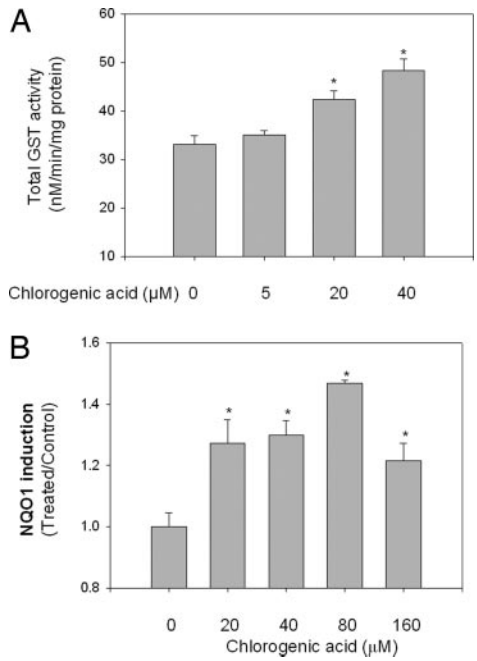

FIG. 7. **Chlorogenic acid treatment increases phase 2 enzyme activities.** JB6 cells were incubated with or without chlorogenic acid for 18 h. Total GST (*A*) and NQO1 (*B*) activities were measured as described under "Experimental Procedures." For total GST activity, values are mean ± S.E. from three experiments and are expressed as nmol/min/mg protein. For NQO1 induction, the values are means ± S.E. (n = 3) and are expressed as ratios of treated over vehicle control. *, a significant induction of GST activity by chlorogenic acid compared with the untreated control ($p < 0.05$).

Chlorogenic Acid Activates GST through the AREs—The mouse *GSTA1* promoter contains two AREs that might represent potential targets of regulation by chlorogenic acid. To investigate this possibility, JB6 cells were transfected with luciferase expression vectors carrying the wild-type mouse promoter ARE sequence version. The cells were stimulated with chlorogenic acid for 18 h. As shown in Fig. 8, at concentrations of 20 and 40 μM, chlorogenic acid increased the GSTA1 ARE-luciferase activity by 30 and 40%, respectively (Fig. 8). These results indicate that chlorogenic acid may stimulate *GST* gene expression by targeting the *GSTA1* promoter at the AREs.

Nuclear Translocation of Nrf2 by Chlorogenic Acid and the Involvement of the PI 3-Kinase Pathway—Nrf2 is a critical transcription factor for mediating amplification of the mammalian defense system against environmental stressors (31, 32). An increase in nuclear Nrf2 level is required for the activation of the ARE. To determine whether chlorogenic acid elicits the activation of the Nrf2, JB6 cells were treated with chlorogenic acid, and the Nrf2 nuclear translocation was measured by immunocytochemistry or Western blot as described under "Experimental Procedures." As shown in Fig. 9*A*, treatment of cells

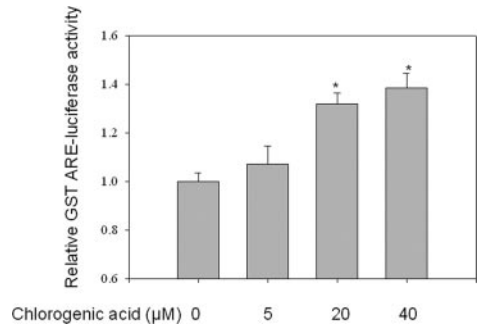

FIG. 8. **Chlorogenic acid targets the mouse *GSTA1* gene promoter at the AREs.** JB6 cells were transfected with expression vectors for luciferase under the control of the 5′-promoter region of mouse wild-type *GSTA1* comprising the AREs. The cells were then treated with or without chlorogenic acid (0–40 μM) for 18 h, and the luciferase activities were tested. Results, presented as relative ARE-luciferase induction compared with untreated control cells, are expressed as means and S.E. of three assay wells. The experiment was repeated three times. *, a significant induction of ARE-luciferase activity by chlorogenic acid compared with the untreated control ($p < 0.05$).

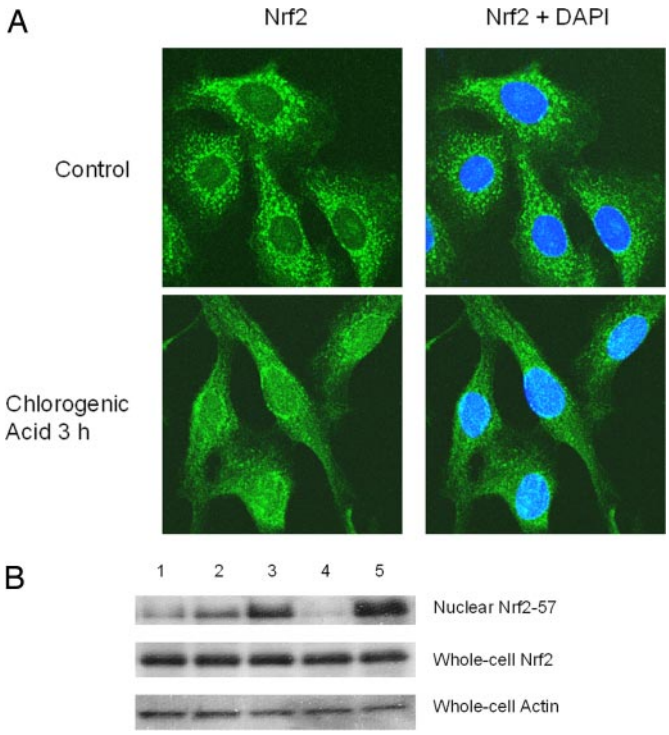

FIG. 9. **Chlorogenic acid induces Nrf2 nuclear translocation via PI 3-kinase pathway in JB6 cells.** *A*, translocation of Nrf2 to the nucleus by chlorogenic acid. JB6 cells were treated with chlorogenic acid for 3 h, and the standard immunocytochemical method was employed to determine the translocation of Nrf2 molecules. Immunocytochemical signal is observed principally in the nucleus at 3 h after chlorogenic acid administration. 4′,6-Diamidino-2-phenylindole was used to localize nuclei. *B*, JB6 cells were either incubated with or without chlorogenic acid (5–40 μM) for 3 h or pretreated with 25 μM of LY294002 for 30 min and then incubated with or without chlorogenic acid (5 μM) for 3 h. Nrf2 protein level in the nucleus or total cell lysate was determined by Western blot using an Nrf2 antibody (1:200). *Lane 1*, LY294002; *lane 2*, control; *lane 3*, chlorogenic acid 5 μM; *lane 4*, chlorogenic acid 5 μM plus LY294002; *lane 5*, chlorogenic acid 40 μM.

with chlorogenic acid for 3 h caused Nrf2 to have perinuclear and nuclear localization with reduced cytoplasmic staining. However, Nrf2 was relocalized in the cytoplasm with less intense nuclear staining at 6 h (data not shown). Immunoblot analysis revealed that the protein levels of Nrf2 in the nucleus were increased following the treatment of cells with chlorogenic acid for 3 h, whereas Nrf2 in total cell lysate remained unchanged (Fig. 9*B*). Furthermore, we found that chlorogenic acid-induced Nrf2 translocation was PI 3-kinase-dependent, since LY294002 (an inhibitor of the PI 3-kinase pathway) pretreatment could completely block Nrf2 translocation in JB6 cells (Fig. 9*B*). In addition, pretreatment of JB6 cells with LY294002 could also block chlorogenic acid-induced GST ARE-luciferase activity (Fig. 10). What is more, the basal levels of Nrf2 in the nucleus and the activity of transfected ARE-luciferase reporter were also inhibited by LY294002. These results indicated that chlorogenic acid induced the nuclear translocation of Nrf2.

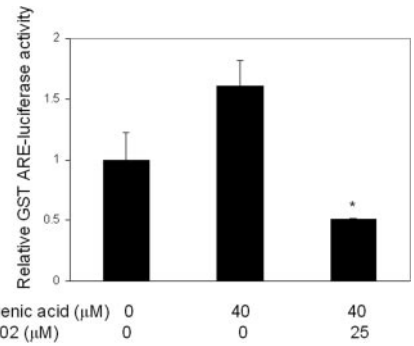

FIG. 10. **Effect of LY294002 on chlorogenic acid-induced GSTA1 ARE-luciferase reporter activity.** Luciferase reporter plasmids containing GSTA1 ARE were transiently transfected into JB6 cells. The cells were pretreated with LY294002 (25 μM) for 30 min and then treated with 40 μM of chlorogenic acid for 18 h, and the luciferase activities were measured. Results, presented as relative ARE-luciferase induction compared with untreated control cells, are expressed as means and S.E. of three assay wells. The experiment was repeated three times. *, a significant inhibition of ARE-luciferase activity by PI 3-kinase inhibitor compared with chlorogenic acid-treated cells ($p < 0.05$).

We want to note that chlorogenic acid may have no effect on the expression of Nrf2 itself because chlorogenic acid failed to increase the luciferase activity driven by the *nrf2* promoter in a luciferase reporter system containing the full-length promoter region of *nrf2* (data not shown). These results suggest that chlorogenic acid may stimulate Nrf2 transactivation via increasing the nuclear translocation of Nrf2 but not expression.

DISCUSSION

Chlorogenic acid, the ester of caffeic acid with quinic acid, is known to exist in coffee, fruits, and vegetables. This paper reports molecular evidence for anticarcinogenic potential for chlorogenic acid. Our findings suggested that chlorogenic acid displayed stronger antioxidant activity than that of ascorbic acid, a conventional antioxidant. Chlorogenic acid also inhibited proliferation of lung cancer cells and TPA-induced neoplastic transformation. The mechanistic studies suggested that the possible anticarcinogenic activity may due to the inhibitory effects on TPA- or UVB-induced NF-κB, AP-1, and MAPK signaling and the inductive effects on phase 2 gene activities.

Cell hyperproliferation is involved in the mechanism of carcinogenesis. It has been reported that oxidative stress induces the expression of several oncogenes, such as c-*myc* and c-*fos*, which enhance cell proliferation (45, 46). Compared with nonneoplastic cells, cancer cells constitutively generate large but nonlethal amounts of ROS that apparently function as signaling molecules in the MAPK pathway to constantly activate redox-sensitive transcription factors and responsive genes. These gene products are involved in the survival of cancer cells as well as their proliferation (47). From these perspectives, Toyokuni *et al.* (47) proposed the concept of "persistent oxidative stress in cancer cells." Reducing oxidative stress may suppress the proliferation of tumor cells (47, 48). The results of the

current study demonstrate that chlorogenic acid exhibited a stronger antioxidant capacity than ascorbic acid. The inhibitory effect of chlorogenic acid on the proliferation of A549 lung cancer cells may be due to its antioxidant properties by perturbing the favorable redox condition in cancer cells.

AP-1 has been implicated in tumor promotion because of its ability to alter gene expression in response to tumor promoters, such as epidermal growth factor, TPA, or UV irradiation. Polyphenolic phytochemicals, such as (−)-epigallocatechin gallate, may increase AP-1-associated responses via a MAPK signaling mechanism in normal human keratinocytes (49), which is helpful to promote keratinocyte differentiation in order to protect normal keratinocytes from carcinogen stimuli. On the other hand, inhibition of AP-1 and NF-κB activation by a variety of chemopreventive agents has been shown to reduce neoplastic transformation (36). *In vivo* studies in transgenic mice indicate that AP-1 transactivation is required for tumor promotion (7, 36). The blockade of TPA-induced cell transformation by chlorogenic acid might be through the inhibition of AP-1 activity. Therefore, the inhibitory effect of chlorogenic acid on AP-1 and NF-κB activation noted in this study may have a beneficial role in preventing carcinogenesis *in vivo*.

Although many mechanisms may be involved in the up- and down-regulation of AP-1 activity, MAPKs, including ERKs, JNKs, and p38 kinases, are known to be common signaling pathways mediating AP-1 activity (5, 43). Studies indicate that ERKs, JNKs, and p38 kinase are key molecules activated in response to oxidant injury (11). Both UVB and TPA can induce ROS generation in cells (13, 36). AP-1 is a downstream target of these three MAP kinases. We found that chlorogenic acid possesses strong antioxidant activity and inhibits UVB- or TPA-induced phosphorylation of ERKs, JNKs, and p38 kinase. These observations suggest that blocking of UVB- or TPA-induced AP-1 and MAPK activation with chlorogenic acid may also be due to its antioxidant properties.

The induction of phase 2 enzymes by chemicals or dietary factors to prevent carcinogenesis has been linked to cancer chemoprevention (22, 23, 50). The present study indicated that chlorogenic acid might stimulate NQO1 and GST enzymatic activities (Fig. 7). Further studies with GSTA1 indicated that this stimulation is related to the activation of promoter function of GSTA1, which results from the interaction of Nrf2 and AREs. Some chemopreventive agents, such as 3*H*-1,2-dithiole-3-thione and carnosol, could activate the promoter of *nrf2* and elicit the increase of Nrf2 expression (24, 51). Whereas chlorogenic acid has no effect on the activation of *nrf2* promoter and Nrf2 expression, it evidently induces the nuclear translocation of Nrf2 (Fig. 9, *A* and *B*), which may facilitate the interaction of Nrf2 and AREs. This translocation may not be caused by the dissociation of Nrf2 from Keap 1 repression through Nrf2 phosphorylation by MAPK cascade (52), because chlorogenic acid alone has no obvious influence on the MAPK-AP-1 pathway. Whether an alternative possibility, such as alterations in cellular redox balance, is involved in chlorogenic acid-induced Nrf2 translocation remains to be determined.

It has been reported that PI 3-kinase may regulate activation of the ARE-driven genes in tumor or primary mammal cells (51, 53). Our present data indicated that both the nuclear translocation of Nrf2 and GSTA1 ARE-luciferase reporter activity induced by chlorogenic acid were completely blocked by LY294002 (Figs. 9*B* and 10). These observations suggest that Nrf2 is downstream of PI 3-kinase in JB6 cells and that chlorogenic acid requires PI 3-kinase to induce GST expression in JB6 cells.

The nuclear translocation of Nrf2 induced by chlorogenic acid may be involved in the induction of GSTA1 ARE-luciferase activity as well as the increase in GST activity. In light of the essential physiological role of GST and its effective activity in detoxifying xenobiotics by conjugating glutathione to a range of electrophilic substrates, the present study demonstrated the potential function of natural antioxidant in modulating GST phase 2 enzymes and their context events. The potential for developing chemopreventive agents that work through induction of cytoprotective genes rather than simply as acting as direct antioxidants is extremely attractive (54). Chlorogenic acid displayed the induction of NQO1 and GST enzymatic activities, nuclear translocation of Nrf2, and GSTA1 ARE-luciferase activity, indicating that the possible chemoprotection of chlorogenic acid against carcinogenesis appeared to be at least partly due to modulatory effects on phase 2 detoxifying enzymes, such as GST and NQO1. Thus, our data together with other reports (2, 3) suggest a possibility that consumption of chlorogenic acid through the intake of vegetables or fruits may be linked with cancer prevention.

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Inhibition of Activator Protein-1, NF-κB, and MAPKs and Induction of Phase 2 Detoxifying Enzyme Activity by Chlorogenic Acid

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