N-Acetylcysteine Conjugate of Phenethyl Isothiocyanate Enhances Apoptosis in Growth-Stimulated Human Lung Cells

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Abstract

We previously showed that dietary treatment with the N-acetylcysteine conjugate of phenethyl isothiocyanate (PEITC-NAC) inhibited benz(a)pyrene-induced lung tumorigenesis in A/J mice, and that tumor inhibition was associated with induction of activator protein-1 (AP-1) activity and stimulation of apoptosis in the lungs of mice. In the present study, we show that PEITC-NAC also induces apoptosis and AP-1 activity in human lung adenocarcinoma A549 cells, and that activation of AP-1 is important in PEITC-NAC-induced apoptosis in these cells. PEITC-NAC induced AP-1 binding activity in A549 cells in a dose- and time-dependent manner; peak activity appeared at 10 μmol/L after 24 hours. At that time, flow cytometric analysis showed a sub-G₁ peak, indicating that ~4.5% of the cells had undergone apoptosis. When wild-type c-jun cDNA was transfected into A549 cells, PEITC-NAC–mediated apoptosis was greatly increased in the c-jun–transfected cells compared with the control vector–transfected cells, based on cell morphology and analysis of DNA fragmentation. Furthermore, cells that were pretreated with 100 nmol/L 12-O-tetradecanoyl phorbol-13-acetate, and then treated with 25 μmol/L PEITC-NAC, underwent enhanced apoptosis compared with cells that were treated with PEITC-NAC alone; cells treated with 12-O-tetradecanoyl phorbol-13-acetate alone showed active cell growth without apoptosis. Bivariate flow cytometric analysis of DNA strand breaks versus DNA content showed that apoptosis induced by PEITC-NAC occurred predominantly in the G₂-M phase. These findings suggest that growth-stimulated cells with an elevated basal AP-1 activity, i.e., A549 cells transfected with wild-type c-jun or treated with a tumor promoter, were more sensitive to PEITC-NAC–mediated apoptosis. The observation that PEITC-NAC induces apoptosis predominantly in growth-promoted cells, such as neoplastic cells, suggests a selective mechanism by which PEITC-NAC inhibits lung carcinogenesis. (Cancer Res 2005; 65(18): 8538-47)

Introduction

Isothiocyanates have been shown to have a broad chemopreventive activity against carcinogenesis in laboratory animals (1–7). Evidence obtained from epidemiologic studies also supports the protective role of dietary isothiocyanates against lung and colon cancers (8, 9). Therefore, certain isothiocyanates are potentially promising chemopreventive agents for human cancers (8, 10, 11). After administration of isothiocyanates, thiol conjugates of isothiocyanates are formed by nonenzymatic or enzymatic reaction with reduced glutathione (12). Stepwise enzymatic removal of glutamate and glycine from reduced glutathione yields l-cysteine–isothiocyanate conjugates, which are subsequently acetylated to yield N-acetyl-l-cysteine (NAC) conjugates of isothiocyanates (mercapturic acids) that are excreted in the urine (10, 12–14). NAC conjugates of some isothiocyanates also display chemopreventive activity against 4-(methylnitrosamino)-1-(pyridyl)-1-butane (NNK)- and benz(a)pyrene-induced lung tumors in A/J mice (15, 16), and they inhibit azoxymethane-induced colonic aberrant crypt foci formation in Fischer rats (17). Thiol conjugates of isothiocyanates have been reported to inhibit growth of human cancer cells in culture (18–20). Although the conjugates as chemopreventive agents seem to be less potent than corresponding parent isothiocyanates (16, 21), compared with the parent isothiocyanates, their slow release of isothiocyanates, and consequently, the lower toxicity, decreased pungency, and increased water solubility suggest that the thiol conjugates of isothiocyanates would be potentially more efficacious and a more desirable form for clinical trials.

Two major mechanisms have been proposed for the chemopreventive effects of isothiocyanates. One mechanism involves isothiocyanate binding and inactivation of phase I enzyme cytochrome P450s (7, 22) and induction of phase II enzymes, e.g., glutathione S-transferase, quinone reductase, and glucuronosyltransferases, possibly through activation of activator protein (AP-1; refs. 23–25). The other mechanism emerging in recent years involves the induction of apoptosis (16, 18, 20), which results in the deletion of genetically damaged cells, so that clonal expansion is aborted. Isothiocyanates and their conjugates have been shown to induce apoptosis or block proliferation in cultured cells (26–33). A/J mice treated with the N-acetylcysteine conjugate of phenethyl isothiocyanate (PEITC-NAC) had an increased apoptotic rate in the lung, which seemed to underlie the inhibition of benz(a)pyrene-induced lung tumorigenesis by PEITC-NAC (16). Our recent study showed that PEITC, sulforaphane, and their NAC conjugates inhibit the progression of lung adenomas to adenocarcinomas in A/J mice, possibly through increasing apoptosis (34). Therefore, induction of apoptosis seems to play an important role in chemoprevention by isothiocyanates.

In various cell types, PEITC induced a sustained c-Jun NH₂-terminal kinase (JNK) activation in a dose-dependent manner, and the JNK activation caused by isothiocyanates was associated with the induction of apoptosis (27, 35). PEITC-induced apoptosis was suppressed by interfering with the JNK pathway using dominant-negative mutants of JNK1 or MEKK1, implying that the JNK pathway is required for apoptotic signaling. That
isothiocyanate-induced JNK activation was blocked by the antioxidants, 2-mercaptopethanol and NAC, suggests that cell death signaling was triggered by oxidative stress. Furthermore, reactive oxygen- or nitrogen-induced AP-1 binding was associated with apoptosis in rat lung epithelial cells (36, 37). Our study showed that the induction of JNK activation and AP-1–binding activity by PEITC–NAC were accompanied by apoptosis in lung of A/J mice (16). However, AP-1 activation caused by PEITC–NAC in human lung cells has not been investigated and its relationship with apoptosis is not presently well-understood. In this study, we have shown that PEITC–NAC induces apoptosis in human lung cancer cells and established the role of AP-1 in apoptosis induced by PEITC–NAC by using c-jun-transfected and 12-O-tetradecanoyl phorbol-13-acetate (TPA)-treated human lung cells. A proapoptotic condition is predisposed by AP-1 activity in A549 cells, i.e., preexisting AP-1 activation sensitizes cells to apoptosis induced by PEITC–NAC.

**Materials and Methods**

**Cell lines.** The human lung adenocarcinoma cell line A549 was obtained from the American Type Culture Collection (Bethesda, MD), and maintained in DMEM supplemented with 10% fetal bovine serum. Normal human lung cells (small airway epithelial cells) were purchased commercially (Bio Whittaker, Inc., Walkersville, MD), and maintained in small airway epithelial growth medium provided by the same company. All cells were grown at 37 °C under a 5% CO2 atmosphere.

**Reagents.** PEITC was obtained from Aldrich (Milwaukee, WI) and its NAC conjugate was synthesized using the method described previously (38). Unless specified otherwise, A549 cells were treated with 10 or 25 μmol/L of PEITC–NAC. TPA was purchased from Sigma (St. Louis, MO).

**SuperArray analysis.** GEArray Q Series Mouse Cancer Pathway Finder Gene Arrays (SuperArray Inc., Bethesda, MD) were employed for analysis of gene expression in lung tissue of mice treated with dietary PEITC–NAC during the progression stage of tumor development. Total RNA isolated from individual mouse lung lobes was pooled according to various treatment groups. There were two pooled RNA samples used for this experiment: (a) control RNA was pooled from mice treated with benzo(a)pyrene and NNK alone, whereas (b) PEITC–NAC treated RNA was pooled from mice treated with benzo(a)pyrene, NNK plus PEITC–NAC (34). cDNA probe synthesis was done using a 32P-RT-Labeling Kit (SuperArray) that converts pooled RNA samples into labeled probes. The hybridization and the signal detection steps were done according to the instructions provided commercially. The quantification of gene expression was obtained using a digital imaging system (Chenlmager 5500, Alpha Innotech, San Leandro, CA). Software provided by SuperArray was used for data analyses.

**Electrophoretic mobility shift assay.** Double-stranded oligonucleotides containing the consensus-binding site for AP-1 were purchased (Santa Cruz Biotechnology Inc., Santa Cruz, CA). The oligos were labeled with γ-32P-ATP (6,000 Ci/mm, Amersham Pharmacia Biotech Inc., Piscataway, NJ) using polynucleotide kinase (Promega, Madison, WI) according to standard procedures. The labeled DNA was incubated with 5 μg of total proteins as specified in Results for 10 minutes at room temperature in the presence of 1 μg of poly(dI)-d(C) oligomer (Boche Molecular Biochemicals, Indianapolis, IN) and DNA-binding buffer as described previously (39). The complexes were then separated on a 7.5% polyacrylamide gel and autoradiographed.

In supershift reactions, 5 μg of total proteins from cells treated with 25 μmol/L PEITC–NAC for 24 hours was incubated with radiolabeled probe. After 10 minutes, specific antibodies were added to the DNA-binding reaction mixtures and incubated for another 2 hours. The reaction mixtures were then separated on a 4% polyacrylamide gel containing 0.7% glycerol.

**Cell cycle analysis.** A549 cells were harvested 24 hours after treatment with various concentrations of PEITC–NAC by fixation in 70% ethanol. Cellular DNA was determined following staining with 1 μg/mL of 4′,6-diamidino-2-phenylindole (DAPI, Molecular Probes, Eugene, OR) dissolved in PBS. Cellular blue fluorescence was measured using the Elite ESP flow cytometer/cell sorter (Coulter, Miami, FL) following excitation with a Nl/Cad UV light–emitting laser. The data were collected and DNA histograms were deconvoluted using Multicycle software (Phoenix Flow Systems, San Diego, CA).

**Transfection.** The mammalian cell transfections were done using a standard method previously described (40). Twenty micrograms of pMEK MTH-jun plasmid or the parent pMEK MTH plasmid (41) were transfected into 5 × 105 A549 cells using electroporation with 230 V. 960 mF (RTX electroporation system) in 2× HEPEs buffer. After transfection, cells were maintained in normal growth medium for 24 hours, followed by the addition of G418 (800 μg/mL). For selection of stable neomycin-resistant transfectants, the cells were cultured in G418 selection medium for 10 days, then maintained in medium with 400 μg/mL G418. Two transfected cell lines were generated: (a) A549/c-jun was transfected with wild-type c-jun and (b) A549/control vector was transfected with the empty vector, pMEK MTH.

**Annexin V apoptotic cell staining.** The cells were harvested and cyt centrifuged on slides; slides were then stained using an Annexin V-Cy3 Apoptosis Detection Kit (Sigma), which provides Annexin V (red) and 6-carboxyfluorescein diacetate (6-CFDA, green) dual staining. Two transfected A549 cell lines, A549/control vector and A549/c-jun, were treated with 25 μmol/L PEITC–NAC for 16 hours, followed by fixation to slides and staining. The staining was done by following the manufacturer's recommendations.

**Northern blot analysis.** RNA extraction, electrophoresis, gel transfer to nylon membranes and blot hybridization were done as described previously (42). The blots were washed twice at 65 °C in 2× SSC and 0.1% SDS for 15 minutes. The c-jun cDNA insert (1.2 kb) of the pMEK MTH-jun plasmid was excised as a probe for hybridization and labeled by incorporation of α-32P-dCTP (6,000 Ci/mm, Amersham) into the c-jun sequence using a Random Primed DNA Labeling Kit (Roche Molecular Biochemicals). The amount of RNA loaded was monitored using 28 S and 18 S RNA stained by ethidium bromide.

**Analysis of DNA fragmentation.** To confirm the appearance of apoptotic cells, nuclear DNA fragmentation was analyzed by agarose gel electrophoresis (43). The ethanol-fixed cells were centrifuged at 800 × g for 5 minutes; the cell pellets were resuspended in 40 μL of phosphate-citrate buffer, consisting of 192 parts of 0.2 mol/L Na2HPO4 and 8 parts of 0.1 mol/L citric acid (pH 7.8), at room temperature for at least 30 minutes. After centrifugation at 1,000 × g for 5 minutes, the supernatant was concentrated in a Speed Vac concentrator. A 3 μl aliquot of 0.25% NP40 (v/v in H2O) and 3 μl of RNase A solution (1 mg/ml) were added. After 30 minutes of incubation at 37 °C, 3 μl of proteinase K solution (1 mg/ml) was added and the extract was incubated for an additional 30 minutes at 37 °C. After adding the loading buffer, the extract was subjected to electrophoresis on 0.8% agarose gel. The presence of the characteristic “ladder” pattern of discontinuous DNA fragments was visualized by ethidium bromide staining.

**Assay for single-cell DNA strand breaks.** A549 cells treated with or without TPA, PEITC–NAC, or TPA plus PEITC–NAC were collected at the times specified in the text, cyt centrifuged, and stained with DAPI. Photographs were taken using an Olympus AX 70 microscope (Melville, NY) with fluorescence and SPOT RT Slider digital image system (Diagnostic Instruments Inc., Sterling Heights, MI).

**Detection of cell cycle phase-specific DNA strand breaks (terminal nucleotidyl transferase–mediated nick end labeling).** A549 cells were fixed with 1% formaldehyde for 15 minutes and then permeabilized by post-fixation in 70% ethanol. The presence of in situ DNA strand breaks, a characteristic feature of apoptosis, was detected by labeling with a fluorochrome-conjugated nucleotide in the reaction catalyzed by the terminal deoxynucleotidyl transferase; cellular DNA was counterstained with propidium iodide. The kit provided by Phoenix Flow Systems was used in this assay. This method is described in detail elsewhere (44). Green and red fluorescence of cells probed for DNA strand breaks and DNA content was measured using a FACScan flow cytometer (Becton Dickinson, San Jose, CA) with standard settings of green (strand breaks) and DNA fluorescence detection.
respective phases of the cell cycle, DNA content frequency histograms were deconvoluted using CELLQuest software.

**Protein isolation.** Cells were incubated at 37°C with 5% CO₂ followed by mock treatment or by treatments specified in Results. Total proteins were then isolated using radioimmunoprecipitation assay buffer. The protease inhibitors aprotonin (1 μg/ml), leupeptin (1 μg/ml), pepstatin (1 μg/ml), phenylmethylsulfonyl fluoride (0.1 mmol/l), the phosphatase inhibitor Na₂VO₄ (1 mmol/l), and NaF (1 mmol/l) were added to all buffers. Protein concentrations were determined by means of the Coomassie Plus protein assay reagent (Pierce, Rockford, IL); aliquots of the proteins were stored at −80°C.

**Western blot analysis.** Western blot analysis was carried out as described previously (16). Briefly, total proteins were prepared from the A549 cells with sham treatment or PEITC-NAC stimulation. Fifty micrograms of the total proteins were boiled for 5 minutes in the presence of Laemmli sample buffer, and then separated by 10% SDS-PAGE. The proteins on the gel were transferred to nitrocellulose membranes. The anti-phospho-JNK 1/2, anti-Bax (Santa Cruz Biotechnology), anti-p53 (Oncogene Research Products, Cambridge, MA), and anti-Bcl-2 (Cell Signaling Technology, Inc., Beverly, MA) antibodies were diluted to the concentration according to commercial recommendations. Immunoreactive bands were detected with an enhanced chemiluminescence kit (Amersham). The loading amount and transfer quality were carefully monitored by Ponceau S staining of each blot. The quantification of the gene expressions was done using a gel documentation system (ChemiImager 5500, Alpha Innotech). The apoptotic results were normalized by dividing the densitometer reading for each band by the corresponding housekeeping gene. The apoptotic index at each treatment of the cell line is the normalized expression level of Bax divided by the normalized expression level of Bcl-2.

**Results**

**Evidence of c-Jun Induction after PEITC-NAC Treatment in the Lungs of A/J Mice**

We reported previously that PEITC-NAC, when administrated in the diet, induced AP-1 activity in lung tissue of A/J mice at a dose that inhibited tumorogenesis in the tumor promotion stage (16). More recently, we investigated the chemopreventive activity of PEITC and sulforaphane and their NAC conjugates during the tumor progression stage in lungs of A/J mice exposed to NNK and benzo(a)pyrene (34). In an effort to determine the underlying mechanism(s) for the tumor inhibition by PEITC-NAC, mouse lung tissues from the tumor progression study were used to detect possible gene expression alterations by employing a SuperArray technique. Pooled total RNA from the carcinogen-treated group was labeled as the hybridization probe for the control blot (Fig. 1A), whereas corresponding RNA from the carcinogens plus PEITC-NAC–treated group was used as the hybridization probe for the treatment blots (Fig. 1B). When control and treatment blots were compared (for details, see Materials and Methods), gene expression analysis showed that among 96 detected genes, which are related to carcinogenesis pathways, cyclin E, c-jun, Akt, Akt₂, and Bak were up-regulated, whereas CD44, Muc1, FGFR2, Myc, Plaur, CD3, cyclin D1, Bad, Atm, and Bcl-2 were down-regulated in lung tissues of PEITC-NAC–treated mice. The spectrum of alterations of gene expression in the treatment group suggests possible mechanisms for inhibition of tumor development by PEITC-NAC. Oncogenes (Myc, cyclin D1, Atm, and Bcl-2), invasion- and metastasis-related genes (Muc1 and Plaur), and the angiogenesis-related gene, FGFR2, exhibited decreased expression levels in the PEITC-NAC treatment group. Unfortunately, three genes that are very important in the apoptotic pathway, p53, JNK, and Bax were expressed at levels too low to be reliably compared by our method. Because Akt was up-regulated, it is quite probable that apoptosis induced by PEITC-NAC is not mediated through the phosphatidylinositol 3-kinase/AKT pathway. These data provide suggestive evidence for the molecular mechanism of the chemopreventive activity of isothiocyanates, even though most of the alterations seen in the SuperArray blots were not confirmed by other methods. However, a transcriptional factor, c-Jun, drew our attention, not only because its expression was increased 2.5-fold (after being normalized by expressions of housekeeping genes) by treatment of PEITC-NAC, but also because it confirmed our previous observation that the AP-1 activity was up-regulated in lung tissue of mice treated with PEITC-NAC during the tumor promotion stage (16). We observed consistently that PEITC-NAC induces c-Jun expression and AP-1 activity in vivo, which was accompanied by an increased apoptotic ratio in mouse lung tissue. Therefore, in this study, we investigated the role of c-Jun and AP-1 in PEITC-NAC–induced apoptosis in human lung cell lines because these evidences were observed in both of our animal bioassays.

**PEITC-NAC Induces AP-1 Activity and Apoptosis in the Human Lung Cell Line**

PEITC-NAC induces AP-1 activity in human lung cell line A549 in a dose- and time-dependent manner. To determine whether PEITC-NAC induces AP-1 activity in human lung cells, total proteins were isolated from A549 human lung cells treated with PEITC-NAC. The binding activity of these proteins to AP-1 target sequences was assayed with electrophoretic mobility shift assay (EMSA). Figure 2. I–A shows that 24 hours after treatment, AP-1 activation was stimulated by PEITC-NAC at a concentration as low as 1 μmol/L. The peak binding activity was observed with...
PEITC-NAC enhances apoptosis in promoted human lung cells

Figure 2. I, dose-dependence and kinetics of induction of AP-1 activity in A549 cells by PEITC-NAC. Total proteins (5 μg) prepared from A549 cells treated with and without PEITC-NAC were incubated with the ³²P-labeled AP-1 sequence in the presence of DNA-binding buffer for 10 minutes at room temperature, followed by separation on 7.5% PAGE and analysis by autoradiography. A, cells treated with different concentrations of PEITC-NAC for 24 hours. B, cells treated with 10 μmol/L PEITC-NAC for different times. C, binding competition assay. Cells were treated with 10 μmol/L PEITC-NAC for 24 hours or DMSO. Cold AP-1, protein preincubated with 10-fold (2 ng) of unlabeled AP-1 for 10 minutes before adding the ³²P-labeled probe. Nonspecific DNA, protein preincubated with 10-fold nonspecific DNA sequences for 10 minutes. The last lane is obtained from preincubation with the mutant AP-1. AP-1, position of the free probe (no protein); control cells were treated only with the vehicle DMSO. Arrows, position of the protein-AP-1 complexes. II, DNA fragmentation analysis by flow cytometry. A549 cells were treated with DMSO for 24 hours as a vehicle control (A) or with 10 μmol/L PEITC-NAC (B). Arrow, the peak contributed by apoptotic cells with hypo-diploid DNA content.

10 μmol/L, showing a clear double band. However, further increasing concentrations of PEITC-NAC (25-100 μmol/L) reduced the AP-1 activity, which is probably attributable to cell death. AP-1 activation appeared as early as 30 minutes after the treatment with 10 μmol/L PEITC-NAC; a double band appeared after 6 hours, and the activity remained elevated up to 24 hours (Fig. 2, I-B). At the 24-hour point, the double band appeared again as shown in Fig. 2, I-A, lane "10 μmol/L," which was under identical treatment conditions. As shown in Fig. 2, I-C, the binding activity in Fig. 1A and B was specific for the AP-1 element, as it was competed by the unlabeled AP-1 probe, but not by the nonspecific DNA fragment or the mutant AP-1 probe.

PEITC-NAC induces apoptosis in A549 cells. To examine whether PEITC-NAC causes apoptosis in human lung cells as it did in lungs of A/J mice, we did flow cytometry on the cells treated with 10 μmol/L PEITC-NAC for 24 hours. When compared with control cells (Fig. 2, II-A), a distinct sub-G₁ peak (Fig. 2, II-B, arrow) appeared; it represents 4.5% of the cells that had undergone apoptosis. This result shows that the treatment with 10 μmol/L PEITC-NAC induces apoptosis in a small fraction of cells without affecting the survival of the majority of cells. A549 cells without PEITC-NAC treatment did not show any detectable apoptosis. Although 4.5% is a small number, however, considering the dynamics, that small proportion will be a significant effect in the human body.

The dose 10 μmol/L is important, as it shows the effects of PEITC-NAC at a dose that is close to physiologic concentrations in humans. At this concentration, as shown in Fig. 2, I and II, we observed a high AP-1 binding activity, and ~4.5% cells underwent apoptosis after 24 hours of PEITC-NAC treatment. This mild effect of PEITC-NAC on apoptosis may well reflect its chemopreventive activity, because of the large amount of cell death caused by high-dose PEITC-NAC in the culture dish, such as that shown in Fig. 3 below, is less probable in vivo. The dose-dependent curve in Fig. 2, I-A shows that the AP-1 activities were down-regulated at 50 and 100 μmol/L; this is due to the fact that at 100 μmol/L of PEITC-NAC, no lung cells appeared to survive after 24 hours. Actually, 24 hours after 50 to 100 μmol/L PEITC-NAC treatment, all of the A549 cells were detached from culture dishes. Consequently, most of these cells did not respond to AP-1 induction.

PEITC-NAC–Induced Apoptosis Enhanced in Actively Growing Cells

Apoptosis induced by PEITC-NAC is enhanced in c-Jun–transfected cells. To identify the constituents of the PEITC-NAC-induced AP-1 complex, supershift assays were done. Antibody to c-Jun, but not Jun B and Jun D transcription factors supershifted on AP-1 binding complex, the antibody anti-Fos family (including c-Fos, Fos B, fra-1, and fra-2) also shifted the AP-1 complex (Fig. 3, I-A), indicating that c-Jun and Fos are involved in the AP-1 complex. Because c-Jun is a component of the AP-1 binding complex induced by PEITC-NAC, to determine the relationship of c-Jun and AP-1 activity with apoptosis induction by PEITC-NAC in A549 cells, a full-length c-jun CDNA construct, pMEX MTH-jun and its empty vector pMEX MTH were transfected into A549 cells via

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electroporation. Twenty-four hours after transfection, the cells were selected by G418 (800 μg/mL) for 10 days to generate two cell lines: A549/control vector and A549/c-jun. Figure 3, I-B shows that the A549/c-jun cell line overexpresses c-jun mRNA (1.35 kb). AP-1 binding activity was elevated in the A549/c-jun cell line (Fig. 3, I-C).

To evaluate the role of AP-1 in apoptosis, several techniques were employed. Figure 3, II shows the morphology (A and B) and DNA fragmentation (C) of the two transfected cell lines after incubation with 25 μmol/L PEITC-NAC. Compared with the control vector, c-jun transfected cells with high AP-1 activity showed enhanced apoptosis (Fig. 3, II-B and C).

In addition, dual staining with Annexin V and 6-CFDA was done on the control vector- and c-jun–transfected cell lines to show the membrane evidence of apoptosis. Annexin V binds to phosphati-

Figure 3. I, AP-1 activity in A549 transfectants. Two cell lines, A549/c-jun and A549/control vector, were generated by electroporation of corresponding plasmid. A, super-shift shows that c-Jun and Fos are in the AP-1 complex. AP-1, probe only; No antibody, AP-1 complex from total protein of PEITC-NAC-treated A549 cells. Each of the other four lanes was loaded with total protein from PEITC-NAC-treated A549 cells plus the antibody as labeled. Hollow arrow, AP-1 protein-DNA complex; solid arrow, super-shift bands. B, Northern blot analysis of c-Jun cDNA expression. Total RNA was prepared from the two transfectants followed by separation on 1% agarose gel and transfer to nylon membrane. The blot was hybridized with the c-Jun probe (details in Materials and Methods). Top, Northern blot autoradiograph; arrow, endogenous and exogenous c-Jun mRNA expressed in these transfectants. Bottom, ethidium bromide–staining of the same RNA gel (top) as a control for the loading amounts. The two bands are the 28S and 18S rRNA. C, AP-1 binding activity. Total proteins were isolated from the transfectants A549/c-jun, and A549/control vector. EMSA was done as described in Fig. 1; arrow, AP-1 complex. II, increased apoptosis in c-jun transfectants treated with PEITC-NAC. A549 transfectants, A549/control vector (A) and A549/c-jun (B) were treated with 25 μmol/L PEITC-NAC for 24 hours. A and B, cell morphology by phase-contrast microscope. C, DNA fragmentation analysis by DNA ladders. The transfectants were treated with 25 μmol/L PEITC-NAC for 24 hours. Cellular DNA was extracted and analyzed by agarose gel electrophoresis. III, Annexin V staining in A549 transfectants treated with PEITC-NAC. A549/control vector (A and B) and A549/c-jun (C and D) were treated with 25 μmol/L PEITC-NAC for 16 hours, each cell line was then fixed and fastened on a slide by cytocentrifuge. Each slide was stained using an Annexin V-Cy3 apoptosis detection kit with dual staining (red/Annexin V, left; and green/6-CFDA, right). The kit allows the differentiation among apoptotic cells (Annexin V-positive, 6-CFDA-positive), necrotic cells (Annexin V-positive, 6-CFDA-negative), and viable cells (Annexin V-negative, 6-CFDA-positive).
dylerine moieties that become exposed on the outer surface of the cell membrane during apoptosis, whereas 6-CFDA staining serves as the marker for viable cells. This combination allows the detection of apoptotic cells (Annexin V-positive, 6-CFDA-positive), necrotic cells (Annexin V-positive, 6-CFDA-negative), and viable cells (Annexin V-negative, 6-CFDA-positive). Cells with green stain only are viable; those with red stain only (Annexin V) are necrotic cells, and those showing both red and green stains are apoptotic cells. The results showed that c-jun–transfected A549 cells showed enhanced apoptosis (Fig. 3, III-C and D) compared with the control vector–transfected cells. In the control vector transfectants, even though apoptosis had already begun 16 hours after PEITC-NAC treatment, it was not predominant (Fig. 3, III-A and B). These results indicate that in the cells with high AP-1 activity, the sensitivity to apoptosis induced by PEITC-NAC was enhanced, which is agreement with the data presented in Fig. 3, II.

Apoptosis induced by PEITC-NAC is enhanced in TPA-treated cells. A549 cells transfected with c-jun cDNA have an increased potential for apoptosis; therefore, we investigated the responses to PEITC-NAC treatment in TPA-pretreated cells that had elevated AP-1 activity. The results presented in Fig. 3, IV-A (cell morphology) or Fig. 3, IV-B (DAPI staining) were obtained from A549 cells incubated with TPA (100 nmol/L) for 12 hours prior to the addition of PEITC-NAC (25 μmol/L) for another 24 hours. A549 cells treated with TPA and subsequently with PEITC-NAC (Fig. 3, IV-A-d) had a much higher incidence of apoptosis compared with the cells treated with PEITC-NAC alone (Fig. 3, IV-A-c), whereas cells treated with control-vehicle (Fig. 3, IV-A-a) or treated with TPA alone (Fig. 3, IV-A-b) showed active growth. The results shown in Fig. 3, IV-B, were treated identically to those in Fig. 3, IV-A, but were stained with the DNA fluorochrome DAPI. The cells showed characteristics of apoptosis, i.e., DNA strand breakage and nuclear fragmentation after PEITC-NAC or TPA plus PEITC-NAC treatment. These results clearly indicate that apoptosis induced by PEITC-NAC was enhanced in the TPA-pretreated cells.

Apoptosis induced by PEITC-NAC occurs predominantly in dividing cells. To explore the mechanism that may be involved in the enhancement of apoptosis in growth-stimulated cells, the cell cycle phase specificity in PEITC-NAC-induced apoptosis was investigated. Toward this end, A549 cells were treated with 25 or 50 μmol/L PEITC-NAC for 24 hours. DNA strand breaks during apoptosis were detected by end-labeling, which was combined with the analysis of cellular DNA content. This method allows the correlation of apoptotic cells with the specific phase of the cell cycle. Figure 3, V shows that 24 hours after treatment with 25 μmol/L PEITC-NAC, a substantial portion of the cells accumulate in the G2-M phase. In cells treated with 50 μmol/L PEITC-NAC for 24 hours, the G2-M fraction was significantly reduced, accompanied by a distinct population of apoptotic cells. These cells were identified by incorporation of BrdUTP into DNA strand breaks at a specific time point.
position consistent with those cells having originated from the 
G2-M population. The slight shift to the left of the apoptotic 
population is a result of DNA degradation; small molecular weight 
DNA removed during cell washing decreased the total stainable 
population is a result of DNA degradation; small molecular weight 
apoptosis following exposure to PEITC-NAC.

In an effort to determine the underlying mechanism for PEITC-
NAC-induced apoptosis, A549 cells transfected with c-jun or with 
control vector were analyzed by Western blot. The expressions of the 
genes JNK, p53, Bcl-2, and Bax, which are important in the apoptotic 
pathway, were detected. The results show that phosphorylation of 
JNK 1 and 2 in both transfectants were induced by 25 μmol/L PEITC-
NAC treatment at 3 hours, and reduced near baseline level after 24 
hours (Fig. 4, I-A). The accumulations of p53 were induced by PEITC-
NAC treatment at 3 and 24 hours in these two cell lines, although 
A549/c-jun cells showed higher induction compared with A549/
control vector cells (Fig. 4, I-A). A549/c-jun cells exhibited a higher 
level of p53 than control cells at 3 hours after treatment, and a lower 
level at 24 hours. This decrease of p53 at 24 hours in A549/c-jun cells 
could be caused by extensive cell death as shown in Fig. 3, II-B; or the 
cells which survived at 24 hours had already entered the next cycle. 
The differences in the expression of Bcl-2 and Bax in these two cell 
lines were weak, but were very constant. We observed repeatedly 
that the expression of Bcl-2 was increased after PEITC-NAC 
treatment; and increased expression of Bcl-2 was less pronounced 
in A549/c-jun cells than in control cells (Fig. 4, I-A and B). In 
contrast, both cell lines showed elevated expression levels of Bax at 
3 hours and Bax remained elevated at 24 hours (Fig. 4, I-A and B). It is 
interesting to note that the expression of Bax was higher in untreated 
A549/c-jun cells than in untreated A549/control vector cells; the 
expression of Bax in A549/c-jun remained at high levels after PEITC-
NAC treatment when compared with A549/control vector cells. On 
the other hand, expression of Bcl-2 level was slightly lower in 
untreated A549/c-jun cells than in control cells (Fig. 4, I-B), however, 
this deficiency became remarkable 3 hours after PEITC-NAC 
treatment. Because these changes were not especially dramatic, 
we quantified the expression of Bax and Bcl-2 from a representative 
gel by densitometry (Fig 4, I-B). If we set the ratio of the expression 
of Bax versus Bcl-2 as the apoptotic index and then assigned the 
apoptotic index from untreated A549/control vector cells as "1", the 
apoptotic indices in the control cells 3 and 24 hours after treatment 
were 1.25 and 0.48, respectively; the apoptotic indices in A549/c-jun 
cells: untreated, 3 and 24 hours after treatment with PEITC-
NAC treatment. A549 

Figure 4. I-A, c-Jun transfectants show increased JNK phosphorylation 
and an activated p53 apoptotic pathway after PEITC-NAC treatment. A549 
transfectants were treated with 25 μmol/L PEITC-NAC for 0, 3, or 24 hours, 
followed by total protein isolation. Western blot analysis was done with 
tumor human lung cells (small airway epithelial cells) treated with and without 
PEITC-NAC were incubated with the 32P-labeled AP-1 (A) and p53 (B) 
conservative elements in the presence of DNA-binding buffer for 10 minutes 
at room temperature, followed by separation on 7.5% PAGE and analysis 
by autoradiography. The cells were treated with 10 μmol/L PEITC-NAC for 
different times.
c-jun–transfected cells (Fig. 3, II-A). The time course of AP-1 activation in normal lung cells was very similar to that in A549 cells. p53 activity was strongly induced in small airway epithelial cells 0.5 hours after PEITC-NAC and further elevated at 6 hours, whereas within 24 hours this activity was decreased (Fig. 4, II-B). It was not surprising to observe that p53 activation was more dramatically induced in small airway epithelial cells than in A549 cells, because the p53 pathway is often attenuated in cancer cells. This accelerated and remarkable response to PEITC-NAC in normal lung cells may possibly be attributed to both accumulation and phosphorylation of p53.

Discussion

Concordant with our previous observation in lung tissue of A/J mice treated with PEITC-NAC, in this study, we showed that PEITC-NAC induced apoptosis in human lung cancer cell line A549 and the induction of apoptosis was associated with induction of AP-1 binding activity. We observed that PEITC-NAC enhanced apoptosis in c-Jun-transfected cells; it also enhanced apoptosis in TPA-treated cells; moreover, it enhanced apoptosis in cells at G2-M phase of the cell cycle. These observations indicated that PEITC-NAC enhanced apoptosis in cells that were growing more actively because the cells that are not actively growing would stay in G1 phase most of the time, and actively growing cells have more opportunities to pass through the G2-M phase, thus, PEITC-NAC-induced apoptosis has more chance to occur in actively growing cells.

c-jun expression and AP-1 activation have been associated with apoptosis induced by various agents, such as ionizing radiation, SV40 T antigen, vitamin E succinate, and antitumor drugs, etc. (45–48). It has been reported that c-Jun expression and its NH2-terminal phosphorylation can promote neuronal apoptosis (49–51). Experiments with sympathetic neurons cultured in vitro, as well as with cerebellar granule neurons and differentiated PC12 cells, have shown that JNK/c-Jun signaling can promote apoptosis following survival factor withdrawal. When c-Jun phosphorylation site mutants were expressed in cerebellar granule neurons, c-Jun phosphorylation has been shown to be necessary for apoptosis. c-Jun[asp], a constitutively active c-Jun mutant in which the known and potential serine and threonine phosphoacceptor sites in the transactivation domain have been mutated to aspartic acid, induces apoptosis under all conditions tested. In contrast, c-Jun[ala], which cannot be phosphorylated because the same sites have been mutated to alanine, blocks apoptosis caused by survival signal withdrawal. In nonneuronal tissues, however, the role of AP-1 activation in apoptosis was reportedly very different (52–54). In apoptosis induced by H2O2 and certain antitumor agents, cells transfected with the dominant-negative c-jun mutant (TAM67) exhibited delayed apoptosis and increased overall cell survival (55, 56). The studies in 3T3 mouse fibroblasts and human umbilical vein endothelial cells showed that increased c-Jun activity was sufficient to trigger apoptotic cell death (53, 56). On the other hand, anticancer drugs induced apoptosis in human acute leukemia cells without inducing of c-jun expression (57). Furthermore, it has been shown that c-Jun protects cells from UV-induced cell death and cooperates with nuclear factor-κB in the prevention of apoptosis induced by tumor necrosis factor-α (54). Our observation with c-jun–transfected cells (Fig. 3, II and III), and TPA-stimulated cells (Fig. 3, IV), indicate that if the elevated AP-1 activity is a preexisting condition, either by overexpression of the c-Jun oncogene or by treatment with a tumor-promoting agent, PEITC-NAC enhances the apoptotic effect on these growth-stimulated cells.

Certain compounds derived from fruits and vegetables, such as all-trans-retinoic acid and grape seed proanthocyanidin, have antiapoptotic action that occurs via JNK and AP-1 activation (58, 59). On the other hand, selenium compounds induced apoptosis through AP-1 activation, because TAM67-transfected cells treated with selenodiglutathione showed reduced apoptosis (60). Induction of apoptosis by various isothiocyanates, including PEITC, has been previously shown to be mediated by JNK (27, 61). We observed that PEITC-NAC induced a clear stress-response in A549 cells. Stress-induced genes such as JNK (Fig. 4, I, A549/control vector, 0 and 3 hours) was stimulated after treatment with PEITC-NAC. c-Jun is one of the direct substrates of JNK, so AP-1 activity induced by PEITC-NAC is attributed to the JNK pathway. In contrast to our previous results in mouse lung tissue (16), the accumulations of p53 were elevated 3 and 24 hours after PEITC-NAC treatment in A549 cell lines. Huang et al. (30) showed that p53 is essential for PEITC-induced apoptosis in mouse epidermal cells. The present study showed that the p53 pathway may be involved in isothiocyanate-induced apoptosis in A549 cells. Because p53 phosphorylated by JNK had been described in various p53 activation systems (62–65), our hypothesis is that PEITC/NAC induced JNK activity, which enhanced apoptosis, perhaps through the p53 pathway. The difference in PEITC-NAC–mediated p53 accumulation between this study and the previous study in A/J mice might be attributed to the time-dependent accumulation in cultural cells versus a continuous stimulation for p53 in animals fed a diet containing the isothiocyanate conjugates. No remarkable differences between control and c-jun–transfected cell lines for PEITC-NAC–induced JNK1/2 phosphorylation and p53 accumulation were observed, indicating that JNK and p53 are upstream or independent from effects of AP-1 activity. On the other hand, basal expression levels of Bax were increased in c-jun-transfected cells. This finding suggests that AP-1 might be involved indirectly in the regulation of Bax expression in A549 cells, and that elevated intrinsic AP-1 activation confers the cells a proapoptotic status, that is related to higher Bax expression. At 3 hours after PEITC–NAC treatment, the expression of Bax was increased in both cell lines, and the apoptotic protein was dominant compared with antiapoptotic protein in both cell lines at the time. Afterwards, in control cells (A549/control vector), the expression ratio of Bax versus Bcl-2 showed a remarkable change: the Bcl-2 level was elevated and that of Bax was reduced 24 hours after PEITC-NAC treatment. This self-regulation prevented further apoptosis in certain control cells. In AP-1-activated cells (A549/c-jun) this self-rescue regulation did not appear; 24 hours after PEITC-NAC treatment, the level of Bax was still predominant. As a result, the majority of c-jun-transfected cells underwent apoptosis.
Phorbol esters have been used to mimic the action of the second lipid messenger diacylglycerol by activating PKC. Although phorbol esters induce apoptosis in certain cell lines (66, 67), they are well known as the promoters of mitogenesis in most cells. For example, it has been reported that TPA protects HL-60 cells from taxol-induced apoptosis and blocks Fas receptor–induced apoptosis in Jurkat and U937 cells (68–70). In the present study, TPA alone had a different effect on A549 cells, as it neither induces apoptosis in these cells nor protects the cells from PEITC-NA-induced apoptosis, but instead enhanced PEITC-NA–induced apoptosis. The observation that PEITC-NA enhanced the apoptotic process in TPA-promoted A549 cells, combined with the results that PEITC-NA enhance the apoptotic process in A549/c-jun, the oncogene-transfected cells, suggests an important role for PEITC-NA as a chemopreventive agent by selectively enhancing apoptosis in promoted cells. We are currently investigating the apoptotic pathway induced by PEITC-NA in human cell lines. These studies will eventually provide more detailed mechanism insights to help identify molecular targets in the chemopreventive activity of isothiocyanates in humans.

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N-Acetylcysteine Conjugate of Phenethyl Isothiocyanate Enhances Apoptosis in Growth-Stimulated Human Lung Cells

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