Advances in Brief

Inhibition of Benzo(*a*)pyrene-induced Lung Tumorigenesis in A/J Mice by Dietary *N*-Acetylcysteine Conjugates of Benzyl and Phenethyl Isothiocyanates during the Postinitiation Phase Is Associated with Activation of Mitogen-activated Protein Kinases and p53 Activity and Induction of Apoptosis[1]

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Abstract

Recent studies in cell culture have shown that isothiocyanates (ITCs) induce apoptosis via activation of mitogen-activated protein (MAP) kinases and p53 pathways, suggesting a potential for ITCs or their conjugates to inhibit tumorigenesis during the postinitiation phase. To evaluate whether ITC compounds administered after carcinogen treatment inhibit lung tumorigenesis, we investigated in A/J mice the effects of the *N*-acetylcysteine (*N*AC) conjugates of benzyl (BITC-*N*AC) and phenethyl ITC (PEITC-*N*AC) in the diet (15 μmol/g) administered after a single dose of 20 μmol benzo(*a*)pyrene [B(*a*)P]. The formation of lung adenomas was examined 140 days after B(*a*)P dosing. Both the BITC-*N*AC and PEITC-*N*AC-treated groups showed a significant reduction in lung tumor multiplicity from 6.1 ± 3.1 tumors/mouse in the B(*a*)P group fed the control diet to 3.7 ± 2.9 and 3.4 ± 2.7 tumors/mouse ($P = 0.018$ and 0.006, respectively). To investigate the mechanisms of tumor inhibition, lung tissues were obtained at 21, 84, and 140 days at interim sacrifices during the bioassay. These tissues showed a significant increase in apoptosis as determined by *in situ* end-labeling for both ITC-*N*AC-treated groups. The MAP kinase pathway was activated in the ITC-*N*AC-treated groups. The activation of c-Jun NH$_2$-terminal kinase was higher in the BITC-*N*AC and PEITC-*N*AC groups when compared with B(*a*)P-treated control. The phosphorylation of p38 and extracellular signal-regulated kinases (ErKs) 1 and 2 was also induced by these treatments. To determine the downstream target of MAP kinases, activator protein-1 (AP-1) and nuclear factor-κB activities were evaluated by gel shift assay. The AP-1 binding activity was remarkably increased in lung tissue from both the BITC-*N*AC and PEITC-*N*AC groups. No change in nuclear factor-κB binding activity was found, however. Phosphorylation of p53 was also higher than the constitutive levels in both ITC-*N*AC-treated groups, but no induction of p53 expression was detected. This study demonstrates the chemopreventive efficacy of the *N*AC conjugates of PEITC and BITC administered in the diet after a single dose of B(*a*)P for lung tumorigenesis and provides the first *in vivo* evidence that activation of MAP kinases, AP-1 transcription factors, p53 phosphorylation, and the induction of apoptosis may be involved in chemopreventive activity of these compounds.

Introduction

ITCs,[3] occurring as glucosinolates in cruciferous vegetables (1, 2), have been shown to have cancer chemopreventive activity in laboratory animals. Studies indicate that ITCs are versatile anticarcinogenic compounds for various organ sites, including lung, esophagus, mammary gland, liver, small intestine, colon, pancreas, and bladder (3–9). The widely investigated mechanisms by which ITCs inhibit tumorigenesis are the inhibition of cytochrome P-450s involved in the activation of carcinogens and/or the induction of the phase II detoxifying enzymes, including glutathione *S*-transferases, quinone reductase, and UDP-glucuronosyltransferases (7, 8, 10). Our previous studies have shown that pretreatment with ITC-thiol conjugates, similar to that with parent ITCs, inhibits lung tumorigenesis induced by the tobacco carcinogen 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanone (11). Studies also suggest that thiol conjugates of ITCs exert their activities by releasing ITCs and thiols via deconjugation (12, 13). We hypothesize, on the basis of these studies, that ITC conjugates act as carriers of ITCs with improved efficacy, because they are less toxic and more stable than the parent ITCs.

In addition to the activities of ITCs on phase I and phase II enzymes, recent studies in cell culture have shown that ITCs and their conjugates induce apoptosis (14–18), a protective mechanism against neoplastic development in which genetically damaged or improperly divided cells are eliminated. *In vitro* and *in vivo* studies have demonstrated that suppression of apoptosis is involved in tumor promotion caused by chemical agents. It has been reported that ITCs induce JNK activation in cultured cells, and that this activation is associated with induction of apoptosis. Other studies have demonstrated that PEITC induces p53 transactivation in a dose- and time-dependent manner in a mouse epidermal cell line with accompanying apoptosis. In contrast, PEITC did not induce apoptosis in p53 (−/−) mouse embryo fibroblasts, suggesting that a p53-mediated mechanism is involved in ITC-induced apoptosis (14). Results of these *in vitro* studies suggest that ITCs may exhibit chemopreventive potential when administered during the postinitiation phase of tumorigenesis. Indeed, a recent study showed that the *N*AC conjugates of ITCs given p.o. after the administration of azoxymethane inhibit aberrant crypt foci formation in the colon of F344 rats (19).

In this study, the chemopreventive potential of the *N*AC conjugates of two widely occurring ITCs, BITC and PEITC, administered in the diet during the postinitiation phase of B(*a*)P-induced lung tumorigenesis in A/J mice, was investigated. The molecular mechanisms involving apoptosis and changes in related genes, including MAP kinases, AP-1, and p53, were examined using the mouse lung tissues obtained during the tumor bioassay. This study is part of our long-range goal to discover mechanism-based chemopreventive agents for ex-smokers who remain at an increased risk of lung cancer even after smoking cessation.

Materials and Methods

Diets, Chemicals, and Reagents. PEITC, BITC, and *N*AC were purchased from Aldrich (Milwaukee, WI). The *N*AC conjugates of BITC and PEITC were prepared using a method published previously (11). The purity was verified by their proton nuclear magnetic resonance spectra and by high-performance liquid chromatography (>98%). B(*a*)P (purity >97%) and cottonseed oil were obtained from Sigma Chemical Co. (St. Louis, MO). Other reagents used were obtained from commercial sources at the highest purity available.

The ITC conjugates were incorporated (15 μmol/g diet) into AIN-76A diets (5% corn oil) by mixing with dextrose prior to diet preparation. The conjugates, 9.36 g of BITC-*N*AC (15 mmol) or 9.79 g of PEITC-*N*AC (15 mmol), were dissolved in 50 ml of ethyl acetate and then mixed with 200 g of dextrose to ensure even coating of dextrose particles. After mixing with dextrose, the solvent was removed using a rotary evaporator and further dried using a vacuum pump (2–3 h). Diets were prepared in 1–2-kg batches and were stored at 4°C in a container purged with nitrogen. PEITC-*N*AC and BITC-*N*AC were stable for at least 1 month. Stability was determined by extraction of 0.5-g portions of the diet with 2.5 ml of methanol (2×). The methanol extracts were combined, and a 1-ml aliquot was filtered using a 0.47-μm syringe filter. A 10-μl sample was then analyzed on high-performance liquid chromatography (12).

Tumor Bioassay. Female strain A mice (Jackson Laboratories, Bar Harbor, ME) of 4 weeks of age were housed under quarantine in polycarbonate cages (5 mice/cage) and provided modified AIN 76A diet (5% corn oil) and acidified drinking water *ad libitum.* The mice were maintained on a 12-h light:12-h dark regimen at 22 ± 5°C and 50 ± 20% relative humidity. After 1 week, the mice were weighed and distributed into four groups containing 30–35 mice on the basis of body weight. At 7 weeks of age, the mice in groups 1–3 were gavaged with a single dose of 20 μmol of B(*a*)P in 0.2 ml of cottonseed oil; group 4 received the vehicle only. Two days after dosing with the carcinogen, diets containing BITC-*N*AC (15 μmol/g, group 2) and PEITC-*N*AC (15 μmol/g, group 3) were provided. Groups 1 [B(*a*)P control] and 4 (vehicle only) remained on the modified AIN-76A diet with 5% corn oil. Tap water was provided *ad libitum* during the course of the bioassay. Mice were observed daily; diets were replenished on the fourth day of the week and completely replaced on the seventh day. Mice were weighed weekly for 4 weeks and then monthly and at termination. At 84 days after B(*a*)P dosing, 4 mice/group were killed (CO_2, cervical dislocation) to harvest lung tissues for molecular and immunohistochemical studies and to quantify lung adenomas, if present. At 140 days after B(*a*)P, the remaining mice in each group were killed. The number of lung tumors was recorded, and lobes of lungs were placed in 10% phosphate-buffered formalin for histological and immunohistochemical analysis. The remainder of the lung lobes was snap frozen in liquid nitrogen.

In the second bioassay, A/J mice of the same age from The Jackson Laboratory were treated using an identical protocol and maintained under the same conditions as described above for 21 days only. Diet consumption was measured twice weekly, and body weights were determined weekly until termination. Mice were sacrificed at termination for harvesting lung tissues to be used in molecular studies. Mean tumor multiplicity and body weights at each time point were compared between groups using Student's *t* test.

ISEL. Formalin-fixed, paraffin-embedded sections from the lungs of mice of the four experimental groups were prepared. ISEL (or terminal deoxynucleotide transferase dUTP nick end-labeling) was performed using an apoptosis detection kit (Enzo Diagnostics, Farmingdale, NY) according to the manufacturer's instructions with the following exceptions: (*a*) endogenous peroxidase was blocked using 3% hydrogen peroxide in methanol for 15 min; (*b*) labeling solution was made up of 45 μl of label reagent, 0.3 μl (10 μ/μl) of terminal deoxynucleotide transferase and 4.7 μl of sterile, distilled water. Sections were incubated in a 37°C water bath for 15 min; (*c*) the color reaction with 3,3′-diaminobenzidine was completed in 10 min on a 37°C heating block; and (*d*) counterstaining was done with Gill's 2 hematoxylin (Shandon-Lipshaw, Pittsburgh, PA) at a 1:10 dilution for 30 s. Sections of liver were used as controls. Cells undergoing apoptosis identified by ISEL were counted under a microscope; a total of 1500 cells of alveolar epithelium from 20 to 24 visual fields/slide were tallied. Three slides/treatment group were analyzed.

Western Blot Analysis. Western blot analysis was performed as described previously (20). Briefly, total proteins were prepared from each group of pooled mouse lungs. Lung samples were removed and immediately placed in 1× PBS with 2 mM DTT, 0.1 mM EDTA, and a protease inhibitor mixture. The samples were then immediately transferred and homogenized in a radio-immunoprecipitation assay buffer with the protease inhibitors, aprotinin (1 μg/ml), leupeptin (1 μg/ml), pepstatin (1 μM), phenylmethylsulfonyl fluoride (0.1 mM), and the phosphatase inhibitors Na_3VO_4 (1 mM) and NaF (1 mM). Samples were centrifuged at 16,500 × *g* for 30 min at 4°C. The supernatants were collected as the total proteins. Equal amounts (30 μg) of the total proteins were boiled for 5 min in the presence of Laemmli sample buffer, loaded on each lane, and separated by 10% SDS-PAGE. The gels were then transferred to nitrocellulose membranes. Equal amounts of protein loading for each lane was checked by Ponceau (Sigma Chemical Co., St. Louis, MO) staining. The anti-JNK1, anti-phospho-JNK1/2, anti-Bax (Santa Cruz Biotechnology, Santa Cruz, CA), anti-p53, anti-p21[WAF1/CIP1] (Oncogene Research Products, Cambridge, MA), anti-phospho-p38, anti-phospho-Erk1/2, and anti-phosphor-p53: Ser-6, Ser-9, Ser-15, Ser-20, and Ser-392 (Cell Signaling, Beverly, MA) antibodies were diluted to the concentration according to commercial recommendations. Immunoreactive bands were detected with an enhanced chemiluminescence kit (ECL; Amersham Pharmacia Biotech, Piscataway, NJ).

EMSA. Double-stranded oligonucleotides containing the consensus binding site for AP-1, mutated AP-1, NF-κB, and mutated NF-κB were purchased commercially (Santa Cruz Biotechnology, Santa Cruz, CA). EMSA was performed as described previously (21). Briefly, all oligonucleotides were labeled with [γ-^{32}P]ATP (6000 Ci/mmol; Amersham Pharmacia Biotech) using polynucleotide kinase (Promega Corp., Madison, WI) according to standard procedures. The labeled DNA (0.4 ng, 4400 cpm) was incubated with 10 μg of total proteins for 10 min at room temperature in the presence of 1 μg of poly(deoxyinosinic-deoxycytidylic acid) oligomer (Boehringer Mannheim, Indianapolis, IN) and DNA-binding buffer. The complexes were then separated on a 7.5% polyacrylamide gel and autoradiographed.

Results

Inhibition of Tumor Multiplicity. At termination of the bioassay, lung adenomas were quantified and expressed as tumor multiplicity (number of tumors/mouse). Mice in group 1 treated with B(*a*)P and fed the control diet had 6.1 ± 3.1 tumors/mouse. Mice in groups 2 and 3 treated with B(*a*)P followed by feeding diets containing BITC-*N*AC and PEITC-*N*AC developed only 3.7 ± 2.9 and 3.4 ± 2.7 tumors/ mouse, corresponding to a 39 and 44% of tumor reduction, respectively (Table 1). Animals were observed throughout the bioassay and showed no signs of toxicity. However, mice fed the diet containing ITC compounds gained less body weight than control mice. At termination, the average body weight of groups 2 (22.9 g) and 3 (22.8 g) showed approximately 10–12% less weight compared with groups 1 (25.8 g) and 4 (25.7 g). The food consumption records showed that during the bioassay, all mice gained ~0.1 g of weight/gram of food consumed. The reduction in body weight gains in these groups were, therefore, consistent with the reduced food consumption of mice in groups 2 and 3 compared with groups 1 and 4. These results suggest that the reduction in body weight gains for mice in the ITC-treated groups was mainly attributable to palatability.

Increase in Apoptotic Rate. Apoptosis in lung tissues obtained 84 and 140 days after administration of ITC diets was determined by ISEL. Results showed that the apoptotic indices were elevated ~2-fold in the BITC-*N*AC- and PEITC-*N*AC-treated groups at 84 days (Fig. 1), just before the tumors appeared. Similar results were obtained

Table 1 *The multiplicity and incidence of lung adenoma in treatment groups*

Treatment group	No. of mice	Tumor multiplicity (no. of tumors/mouse)	Tumor incidence (%)
1. B(*a*)P	23	6.13 ± 3.13[a]	96
2. B(*a*)P + BITC-*N*AC	18	3.72 ± 2.90[b]	94
3. B(*a*)P + PEITC-*N*AC	18	3.39 ± 2.71[b]	89
4. Untreated control	18	0.11 ± 0.31	11

[a] Mean ± SD.

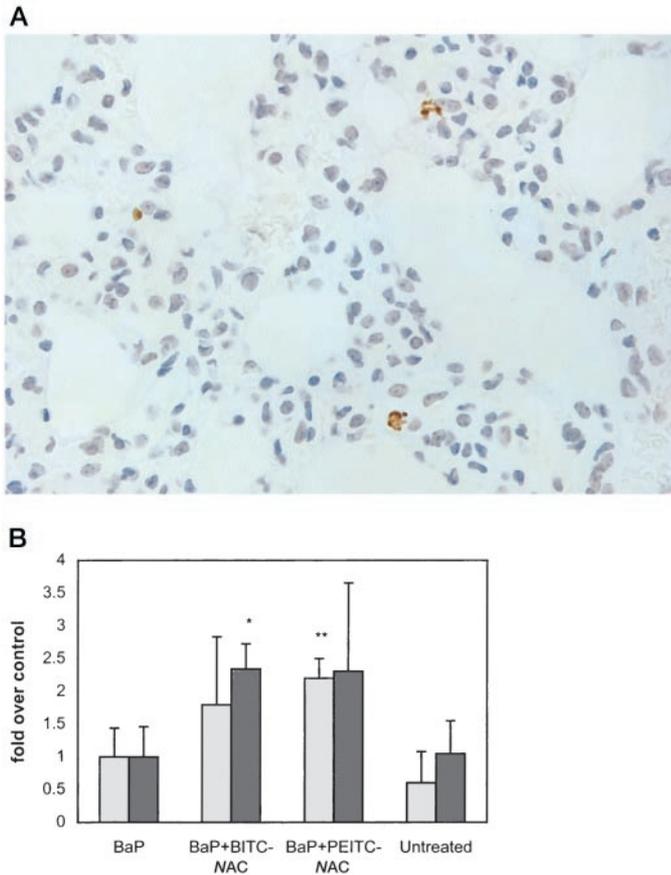[b] $P < 0.05$, compared with positive control group.

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A

B

Fig. 1. *A,* typical *in situ* end-labeling of apoptosis in mouse lung tissue. The nuclear chromatin of apoptotic cells stained by terminal deoxynucleotide transferase dUTP nick end-labeling is irregularly condensed. *B,* changes in apoptotic rate of lung tissue obtained from each group. Tissues were obtained at 84 and 140 days after the bioassay began. The *Y-axis* is the fold amount over B(*a*)P-treated control. ∗ (P = 0.01) and ∗∗ (P = 0.04) as compared with the B(*a*)P group (group 1) using 2-tailed student *t* test; *bars,* SD. ▨, 84 days; ■, 140 days.

from BITC-*N*AC and PEITC-*N*AC groups after 140 days, when tumors had developed. Nontumorous lung tissue of these two groups had a >2-fold increase in apoptosis (2.4-fold for BITC-*N*AC and 2.3-fold for PEITC-*N*AC). Because alveolar epithelial cells are mostly quiescent, a 2-fold increase of the apoptotic cells may critically result in reduction of tumor multiplicity.

Activation of MAP Kinase Activity. To study whether the activities of MAP kinases were altered by dietary treatment of ITC-*N*AC compounds, the total proteins from the lungs of the controls and ITC-treated mice were isolated. The activities of JNK in the lysates were determined by Western blot analysis. As shown in Fig. 2*A,* the phosphorylation levels on Ser-185 and Ser-188 of JNK1 and JNK2, detected by the phospho-specific antibody, were increased in lung tissue of ITC-*N*AC-treated mice obtained 21 days after B(*a*)P administration. B(*a*)P (Fig. 2*A, Lane 2*)-treated groups showed no significant change in JNK1 and JNK2 phosphorylation levels from the untreated group (Fig. 2*A, Lane 5*). Groups treated with B(*a*)P plus BITC-*N*AC and PEITC-*N*AC (Fig. 2*A, Lanes 3* and *4*) showed an increase of JNK1 (p46) phosphorylation levels, whereas JNK2 (p54) phosphorylations were only slightly induced, compared with JNK1. The total proteins isolated from NIH 3T3 cells 15 min after UV (20 J) treatment served as a positive control for phospho-JNK1 and JNK2 (Fig. 2*A, Lane 1*). Fig. 2*B* shows that equal amounts of JNK1 were expressed in all groups. The results indicate that ITCs induce JNK1 phosphorylation but not its expression. When the total proteins in

mouse lung tissue obtained 140 days after B(*a*)P treatment were examined, similar results were obtained showing an approximately 2–3-fold increase of phosphorylation of JNK1 and JNK2 by ITC-*N*AC conjugate treatment (data not shown).

The same blots used for phospho-JNK were stripped and reprobed with anti-phospho-p38 antibody. Fig. 2*C* indicates that p38 phosphorylation levels did not change in mice treated with B(*a*)P (group 1, *Lane 2*) compared with the untreated mice in group 4 (*Lane 5*). However, the ITC-*N*AC-treated groups 2 and 3 showed a significant increase in p38 phosphorylation. The UV-treated NIH 3T3 cells as a positive control showed a strong phospho-p38 band. Erk1 and Erk2 activities were detected in the same blot (Fig. 2*D*). The mice treated with B(*a*)P fed the control diet had a low level of phospho-Erk2 (p42), whereas the groups fed the diets containing ITC conjugates showed an elevated phosphorylation level of Erk2. However, Erk1 phosphorylations were barely detectable in all groups.

Activation of p53 Phosphorylation. To investigate the possible role of p53 in apoptosis induced by BITC-*N*AC or PEITC-*N*AC, we analyzed the expression of p53 and its phosphorylation level in lungs obtained at termination of the bioassay by Western blot using specific antibodies. Although treatment with ITC compounds did not cause apparent accumulation of p53 or change the level of p53 expression (Fig. 3*A, lower panel*), the level of phosphorylation of p53 at Ser-15 appeared to be enhanced (Fig. 3*A, upper panel*). BITC-*N*AC caused only a moderate increase, whereas the PEITC-*N*AC treatment resulted in a stronger increase in the phosphorylation. The phosphorylation levels of p53 Ser-6, Ser-9, Ser-20, and Ser-392 were also assayed, and the results are summarized in Fig. 3*B*. Phosphorylation at Ser-9, Ser-20, and Ser-392 was induced in the ITC-*N*AC-treated groups when compared with the B(*a*)P-treated (group 1) and untreated groups (group 4). The phosphorylation at Ser-6 remained unchanged.

Expression of p53 Effector Genes *p21*[WAF1/CIP1] and *Bax*. The activation of p53 by phosphorylation is expected to enhance the expression of p21[WAF1/CIP1] and Bax (22, 23). The proteins from lung
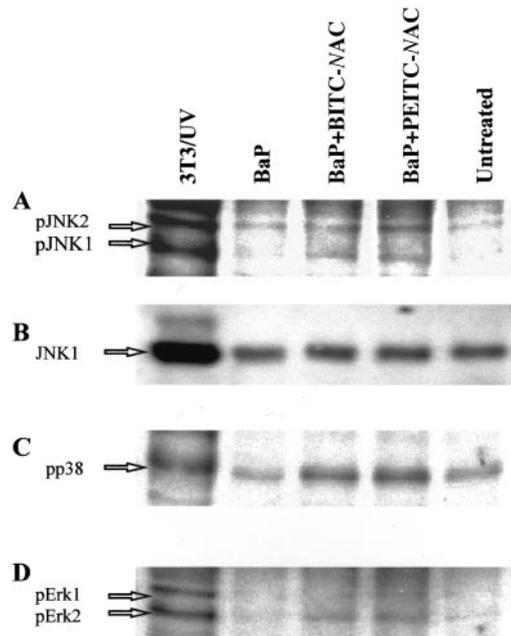

Fig. 2. Increased level of MAP kinase phosphorylation in lung tissue of mice treated with ITC-*N*AC. Lung tissues were obtained on day 21 of the bioassay. Western blot analysis was performed with total proteins obtained from lung tissue of each treatment group. Antibodies used for *A* was anti-phospho-JNK1 and 2; *B* was anti-JNK 1; *C* was anti-phospho-p38 MAP kinase; and *D* was anti-phospho-Erk1 and 2. The *first lane* (*3T3/UV*) is the total protein isolated from NIH 3T3 cells 15 min after UV irradiation as a positive control.
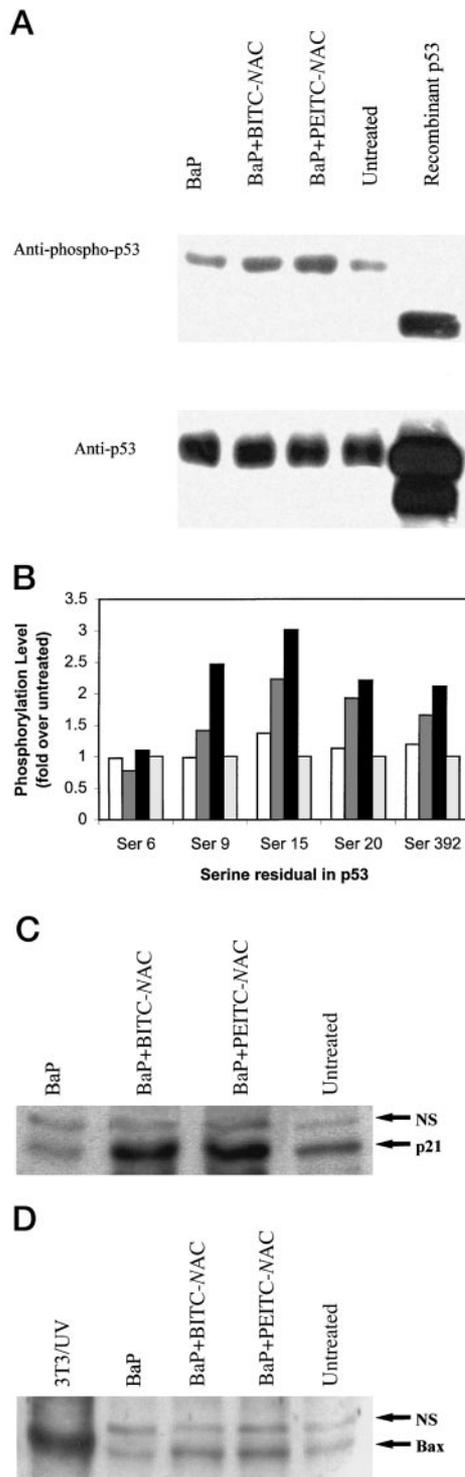
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Fig. 3. Activation of p53 phosphorylation and increased expression of p21[WAF1/CIP1] and Bax in lung tissue from mice treated with ITC-*N*AC. Western blot analysis was performed with anti-phospho-p53 and anti-p53 antibodies using total proteins of lungs. *A*, phosphorylation on Ser-15. The *upper blot* was probed with anti-phospho-p53 (Ser-15) antibody, and the *lower blot* shows same membrane probed with anti-p53 antibody. Recombinant p53 serves as a positive control for p53 and a negative control for phospho-p53. *B*, phosphorylation levels of the serine residues 6, 9, 15, 20, and 392. The *Y-axis* is the fold of phosphorylation level over that of untreated control. The measurements were based on densitometry of phospho-p53 bands on Western blot. □, B(*a*)P; ▨, B(*a*)P + BITC-*N*AC; ■, B(*a*)P + PEITC-*N*AC; ▢, untreated. Western blot analysis was performed with anti-p21[WAF1/CIP1] (*C*) and anti-Bax (*D*) antibodies using total proteins of lungs. *NS*, a nonspecific band. UV-treated 3T3 cells in *D* serve as a positive control.

homogenate used for the p53 analysis were also assayed for p21[WAF1/CIP1] and Bax by Western blot analysis. Our results show that, indeed, the activation of p53 in the lung tissues of ITC-*N*AC-treated groups was accompanied by an increase in expression of p21[WAF1/CIP1] and Bax. Fig. 3*C* shows that p21[WAF1/CIP1] expression in the ITC-*N*AC-treated groups was significantly higher than that in groups 1 and 4. Although the induction of Bax expression was not as strong as p21[WAF1/CIP1], it is still clear that the treated groups showed increased expression of Bax (Fig. 3*D*).

Activation of Transcription Factors AP-1 but not NFκB. The increase of AP-1 and NFκB binding activity has been demonstrated in cultured human colon cancer cells treated with BITC, and BITC is believed to be involved in the induction of phase II enzymes (24). To determine whether dietary ITC-*N*AC compounds affect the transcription of genes regulated by AP-1 and NFκB, total protein extracts were prepared from the lung tissue of mice from all four groups 21 days after B(*a*)P administration. The binding activity of these proteins to AP-1 and NFκB was determined using the EMSA. AP-1 binding activities were strongly induced by ITC-*N*AC treatments (Fig. 4*A-I*). However, NFκB binding activities in ITC-*N*AC-treated groups were not significantly different from the control (Fig. 4*B-I*). The binding activity induced by PEITC-*N*AC is specific for the AP-1 sequence, because the addition of a 10× unlabeled AP-1 sequence completely abolished binding activity (Fig. 4*A-II*). Similarly, the sustained NFκB binding activity is specific for the NFκB target sequence because a 10× unlabeled NFκB sequence effectively competed with the binding activity of the proteins from the untreated control group (Fig. 4*B-II*). Furthermore, a 10× unlabeled mutant AP-1 or NFκB sequence and a 10× extra nonspecific DNA sequence did not alter the binding activity of AP-1 or NFκB.

Discussion

The discovery of agents with the potential to reduce the risk of lung cancers that are effective when administered after exposure to tobacco carcinogens is an important step toward chemoprevention trials in ex-smokers. There are only a limited number of such agents thus far identified from animal bioassays, with little known regarding their mechanisms of action *in vivo* (25). The present study not only demonstrated that the *N*AC conjugates of BITC and PEITC can inhibit B(*a*)P-induced lung tumorigenesis during the postinitiation stages but also shed light on the molecular mechanisms of inhibition *in vivo* by these agents.

In the tumor bioassay, body weight disparities between the groups fed diets containing ITC compounds and the control groups were noted. These differences were probably caused by the reduction of food consumption in the treated groups attributable to palatability, as indicated by the food consumption data. It raises questions as to whether the inhibition of lung tumorigenesis is a result of lowered caloric intake. Several lines of evidence suggest that this is not the case: (*a*) Little is known on the relationship of caloric restriction and lung tumorigenesis, but the extent of the decreases in body weight gain is probably too small to cause such a sizable reduction in tumor multiplicity based on published data for some other organ sites (26). (*b*) Although it is known that caloric restriction could influence gene expression (27), the molecular responses characterized in this study seem to be opposite to those found in animals on the calorie-restricted diet. For example, our studies showed that JNK1, p38, and Erk1 phosphorylation levels were induced by ITC compounds after 21 days of treatment, and AP-1 activity was also strongly induced. Liu *et al.* (28) reported that the calorie restriction inhibits TPA-induced AP-1 binding activity and also inhibits TPA-induced Erk activity but not p38 and JNK in the epidermis of SENCAR mice. Furthermore, our

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results demonstrated an induction of *p53, p21,* or *Bax* genes, yet others have shown that p53 phosphorylation and p27, p21, and p16 expression are not affected by calorie restriction in F344 rats (29).

Numerous studies in cell culture have shown that ITCs induce MAP kinase activity, AP-1, NFκB activity, and p53 activity (14–17, 24). Our study is the first to demonstrate that oral administration of ITC compounds at the doses that inhibit lung tumorigenesis induces MAP kinase phosphorylation, AP-1 binding activity, and p53 activity in the target tissue of tumor inhibition. Compared with the results obtained from studies in cell culture, the activation of JNK activity in mouse lung was less pronounced, yet the induction of the AP-1 activity was comparable. We did not detect any changes in NFκB binding activities in the mouse lung after treatments. The differences in molecular responses between *in vitro* and *in vivo* may be attributable to factors such as the concentrations of ITCs in culture medium *versus* tissue-, cell-specific responses to ITCs, and/or uptake and metabolism *in vivo*. The activation of p53 is known to play a key role in the protection against tumorigenesis. Consistent with this, we have shown that BITC-*N*AC and PEITC-*N*AC activated p53 activity in mouse lungs by inducing phosphorylation and, subsequently, induced the expression of its effector genes, *Bax* and *p21*$^{WAF/CIP1}$. However, questions re-
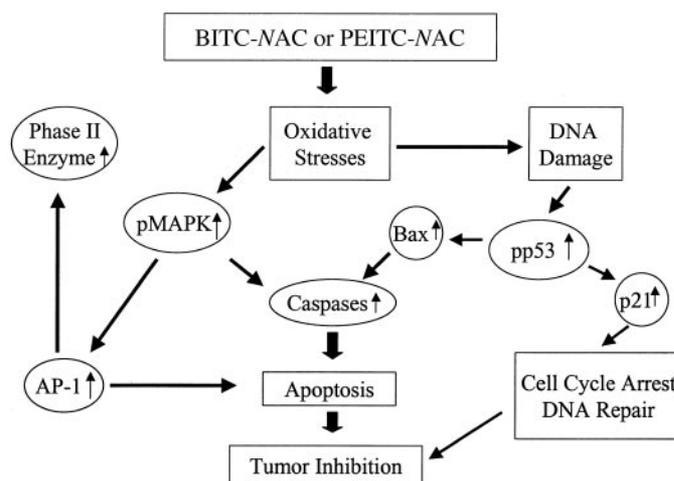

Fig. 5. A proposed mechanism for the inhibition of lung tumorigenesis by ITC conjugation.

garding how these ITC compounds activate p53 phosphorylation still remain to be investigated. Taken together, we have shown that dietary ITC conjugates induce molecular responses in mouse lung similar to those seen in ITC-treated cells *in vitro*, supporting the contention that the effects we see in the lung are caused by ITCs released by deconjugation.

The cellular and molecular responses in mouse lungs after treatment with BITC-*N*AC and PEITC-*N*AC are known to be associated with oxidative stress. Although these compounds have not been shown specifically to cause oxidative DNA damage, some ITCs are cytotoxic, weakly mutagenic, and can stimulate lipid peroxidation in cultured cells (30, 31). It is possible that ITC conjugates via deconjugation may cause oxidative DNA damage by depleting GSH and/or an alteration of the redox potential in lung cells by *N*AC (32, 33). To respond to the changes in oxidative status, lung cells may go into apoptosis through activation of MAP kinases and p53. A proposed molecular mechanism for the inhibition of lung tumorigenesis by ITC conjugates via apoptosis is shown in Fig. 5. Clearly, more studies are needed to substantiate this mechanism. Regardless of the biochemical nature of the cellular stress caused by them, these ITC compounds are apparently not sufficient to induce tumorigenesis, because all of our previous bioassays in A/J mice or F344 rats showed that ITCs administered alone did not cause any tumors (11, 34). The results of this study show that administration of ITC conjugates in the diet during the postinitiation stages of B(a)P-induced lung tumorigenesis can elicit a series of stress-related molecular responses leading to cell death that ultimately is manifested in the reduction of lung tumor formation.

Acknowledgments

We thank Eric Larios and Jie Chen for technical assistance and Victoria Baker for performing the immunohistochemical assays. We also thank Dr. Meena Jhanwar-Uniyal and Dr. Despina Komninou for valuable discussions regarding the manuscript.

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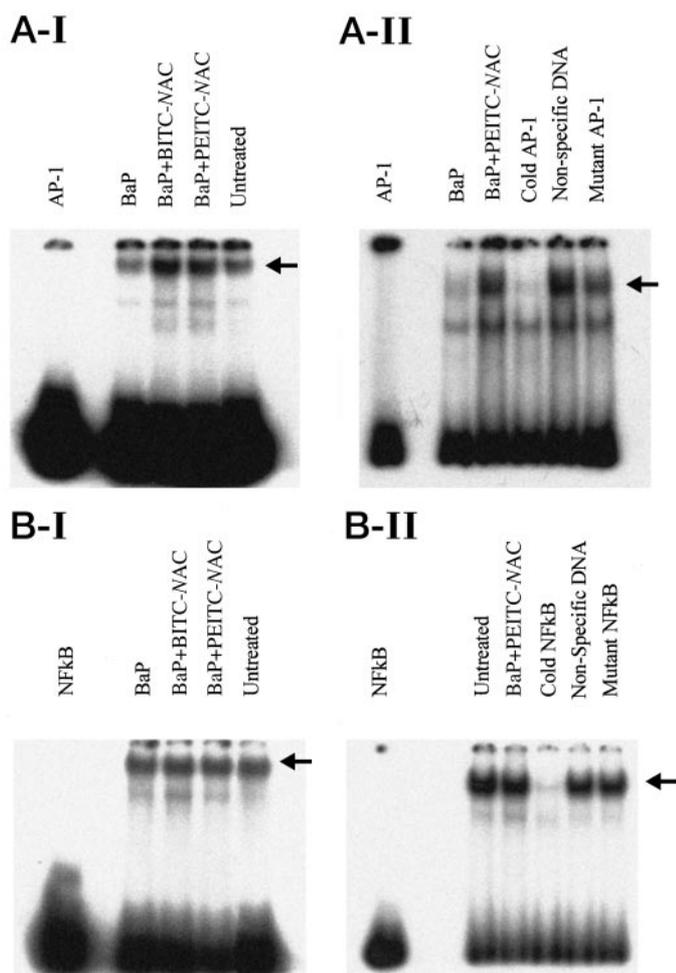

Fig. 4. ITC-*N*AC treatment activates binding of AP-1 target sequence. EMSA was performed with AP-1 and NFκB target sequences using total proteins of lungs from each group. *A-I* and *B-I* demonstrate binding activity to AP-1 and NFκB, respectively. In the binding competition, assays used the total protein from the PEITC-*N*AC group (*A-II*) and the protein from untreated group (*B-II*). The protein in *Lane Cold AP-1* or *Lane Cold NFκB* was preincubated with 10-fold amounts (4 ng) of unlabeled AP-1 (*A-II*) or NFκB (*B-II*) for 10 min before addition of ^{32}P-labeled probe. Proteins in *Lane Non-specific DNA* were preincubated with 10× nonspecific DNA sequences for 10 min. The *last lane* was obtained from the preincubation with mutant AP-1 (*A-II*) or mutant NFκB (*B-II*).

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Inhibition of Benzo(*a*)pyrene-induced Lung Tumorigenesis in A/J Mice by Dietary *N*-Acetylcysteine Conjugates of Benzyl and Phenethyl Isothiocyanates during the Postinitiation Phase Is Associated with Activation of Mitogen-activated Protein Kinases and p53 Activity and Induction of Apoptosis

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