Inhibition of Benzo(a)pyrene-induced Lung Tumorigenesis in A/J Mice by Dietary N-Acetylcytisteine 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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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 (NAC) conjugates of benzyl (BITC-NAC) and phenethyl ITC (PEITC-NAC) in the diet (15 μmol/g) administered after a single dose of 20 μmol benzo(a)pyrene [Ba(P)]. The formation of lung adenomas was examined 140 days after Ba(P) dosing. Both the BITC-NAC and PEITC-NAC-treated groups showed a significant reduction in lung tumor multiplicity from 6.1 ± 3.1 tumors/mouse in the Ba(P) group fed the control diet to 3.7 ± 2.9 and 3.4 ± 2.7 tumors/mouse (P = 0.01 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-NAC-treated groups. The MAP kinase pathway was activated in the ITC-NAC-treated groups. The activation of c-Jun NH2-terminal kinase was higher in the BITC-NAC and PEITC-NAC groups when compared with Ba(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. AP-1 binding activity was remarkably increased in lung tissue from both the BITC-NAC and PEITC-NAC 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-NAC-treated groups, but no induction of p53 expression was detected. This study demonstrates the chemopreventive efficacy of the NAC conjugates of PEITC and BITC administered in the diet after a single dose of Ba(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 the chemopreventive activity of these compounds.

Introduction
ITCs, 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 cytotoxic P-450 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 nitrosamine)-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 NAC 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 NAC conjugates of two widely occurring ITCs, BITC and PEITC, administered in the diet during the postinitiation phase of Ba(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 NAC were purchased from Aldrich (Milwaukee, WI). The NAC 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-NAC (15 mmol) or 9.79 g of PEITC-NAC (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-NAC and BITC-NAC 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°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-NAC (15 μmol/g, group 2) and PEITC-NAC (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 (CO2, cervical dislocation) to harvest lung tissues for Western Blot Analysis. The multiplicity and incidence of 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-NAC and PEITC-NAC 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).

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-NAC and PEITC-NAC 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-NAC- and PEITC-NAC-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

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. of mice</th>
<th>Tumor multiplicity (no. of tumors/mouse)</th>
<th>Tumor incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. B(a)P</td>
<td>23</td>
<td>6.13 ± 3.13</td>
<td>96</td>
</tr>
<tr>
<td>2. B(a)P + BITC-NAC</td>
<td>18</td>
<td>3.72 ± 2.96</td>
<td>94</td>
</tr>
<tr>
<td>3. B(a)P + PEITC-NAC</td>
<td>18</td>
<td>3.39 ± 2.71</td>
<td>89</td>
</tr>
<tr>
<td>4. Untreated control</td>
<td>18</td>
<td>0.11 ± 0.31</td>
<td>11</td>
</tr>
</tbody>
</table>

*Mean ± SD.

*P < 0.05, compared with positive control group.
from BITC-NAC and PEITC-NAC 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-NAC and 2.3-fold for PEITC-NAC). 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-NAC 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. 2A, 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-NAC-treated mice obtained 21 days after B(a)P administration. B(a)P (Fig. 2A, Lane 2)-treated groups showed no significant change in JNK1 and JNK2 phosphorylation levels from the untreated group (Fig. 2A, Lane 5). Groups treated with B(a)P plus BITC-NAC and PEITC-NAC (Fig. 2A, 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. 2A, Lane 1). Fig. 2B 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-NAC conjugate treatment (data not shown).

The same blots used for phospho-JNK were stripped and reprobed with anti-phospho-p38 antibody. Fig. 2C 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-NAC-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. 2D). 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-NAC or PEITC-NAC, 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. 3A, lower panel), the level of phosphorylation of p53 at Ser-15 appeared to be enhanced (Fig. 3A, upper panel). BITC-NAC caused only a moderate increase, whereas the PEITC-NAC treatment resulted in a stronger increase in the phosphorylation. The phosphorylation levels of p53 Ser-6, Ser-9, Ser-20, and Ser-392 were barely detectable showing an approximately 3-fold increase of the phosphorylation of JNK1 and JNK2 by ITC-NAC conjugate treatment (data not shown). However, the ITC-NAC-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. 2D). 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.

**Expression of p53 Effector Genes p21\textsuperscript{WAF1/CIP1} and Bax.** The activation of p53 by phosphorylation is expected to enhance the expression of p21\textsuperscript{WAF1/CIP1} and Bax (22, 23). The proteins from lung tissues were obtained at 84 and 140 days after the bioassay began. The Y-axis bars, 84 days. The left y-axis is the fold amount over B(a)P-treated control. A, Lane 2, B(a)P; A, Lane 5, B(a)P plus BITC-NAC; A, Lane 3, BITC-NAC; A, Lane 4, PEITC-NAC; A, Lane 1, untreated group. Antibodies used for Western blot analysis were anti-phospho-JNK1 and 2; anti-phospho-p38; 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.
Homogenate used for the p53 analysis were also assayed for p21\(^{\text{WAF1/CIP1}}\) and Bax by Western blot analysis. Our results show that, indeed, the activation of p53 in the lung tissues of ITC-NAC-treated groups was accompanied by an increase in expression of p21\(^{\text{WAF1/CIP1}}\) and Bax. Fig. 3C shows that p21\(^{\text{WAF1/CIP1}}\) expression in the ITC-NAC-treated groups was significantly higher than that in groups 1 and 4. Although the induction of Bax expression was not as strong as p21\(^{\text{WAF1/CIP1}}\), it is still clear that the treated groups showed increased expression of Bax (Fig. 3D).

**Activation of Transcription Factors AP-1 but not NF\(\kappa\)B.** The increase of AP-1 and NF\(\kappa\)B 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-NAC compounds affect the transcription of genes regulated by AP-1 and NF\(\kappa\)B, total protein extracts were prepared from the lung tissue of mice from all four groups 21 days after B(\(\alpha\))P administration. The binding activity of these proteins to AP-1 and NF\(\kappa\)B was determined using the EMSA. AP-1 binding activities were strongly induced by ITC-NAC treatments (Fig. 4A-I). However, NF\(\kappa\)B binding activities in ITC-NAC-treated groups were not significantly different from the control (Fig. 4B-I). The binding activity induced by PEITC-NAC is specific for the AP-1 sequence, because the addition of a 10\(^{-6}\) unlabeled AP-1 sequence completely abolished binding activity (Fig. 4A-II). Similarly, the sustained NF\(\kappa\)B binding activity is specific for the NF\(\kappa\)B target sequence because a 10\(^{-6}\) unlabeled NF\(\kappa\)B sequence effectively competed with the binding activity of the proteins from the untreated control group (Fig. 4B-II). Furthermore, a 10\(^{-6}\) unlabeled mutant AP-1 or NF\(\kappa\)B sequence and a 10\(^{-6}\) extra nonspecific DNA sequence did not alter the binding activity of AP-1 or NF\(\kappa\)B.

**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 NAC conjugates of BITC and PEITC can inhibit B(\(\alpha\))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
results demonstrated an induction of p53, p21, or Bax genes, yet others have shown that p53 phosphorylation and p21, 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 action 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-NAC and PEITC-NAC activated p53 activity in mouse lungs by inducing phosphorylation and, subsequently, induced the expression of its effector genes, Bax and p21WAF/CIP1. However, questions regarding 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-NAC and PEITC-NAC 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 NAC (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 Br(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.

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References


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