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The chemopreventive agent phenethyl isothiocyanate sensitizes cells to Fas-mediated apoptosis

Juliet M.Pullar[1], Susan J.Thomson, Monica J.King, Christopher I.Turnbull, Robyn G.Midwinter and Mark B.Hampton

Free Radical Research Group, Department of Pathology, Christchurch School of Medicine and Health Sciences, PO Box 4345, Christchurch, New Zealand

[1]To whom correspondence should be addressed
Tel: +64 3 364 1524; Fax: +64 3 364 1083;
Email: juliet.pullar@chmeds.ac.nz

The chemopreventive properties of the isothiocyanates have been attributed to their ability to inhibit phase I enzymes that activate procarcinogens, induce phase II protective enzymes and trigger apoptosis in transformed cells. In this study we provide evidence for a new mechanism of chemoprevention, wherein sublethal doses of phenethyl isothiocyanate (PEITC) sensitize cells to Fas-mediated apoptosis. The phenomenon was observed in the Fas-resistant T24 bladder carcinoma cell line and in Jurkat T cells overexpressing the anti-apoptotic protein Bcl-2. Caspase-3-like activity was increased up to 20-fold of that observed with either PEITC or anti-Fas antibody alone. While PEITC activated ERK, JNK and p38, inhibitors of these MAP kinases did not block apoptosis. PEITC transiently depleted cellular glutathione, providing a putative mechanism for sensitizing the cells to apoptosis. However, lowering glutathione with buthionine sulfoximine did not mimic the effect of PEITC. Instead, we propose that PEITC promotes apoptosis by directly modifying intracellular thiol proteins. The ability of PEITC to sensitize cells to receptor-mediated apoptosis provides an additional mechanism to explain its chemopreventive properties.

Introduction

Fas (CD95/Apo-1) is a cell surface death receptor involved in the transduction of apoptotic signals in both normal and transformed cells. Ligation of the receptor with Fas ligand or an agonistic antibody results in structural rearrangement of the receptor complex and recruitment of the adapter molecule Fas-associated death domain and pro-caspase-8 to a death-inducing signal complex (DISC). Pro-caspase-8 undergoes autocatalytic cleavage at the DISC to release active caspase-8 into the cytoplasm, which directly (type I) or via mitochondrial factors (type II), initiates activation of the downstream caspases and apoptosis (1,2). Resistance to Fas-mediated apoptosis has been described during different stages of normal cell development and also in many types of tumor cells (3–9). In these cells, down-regulation of signaling through Fas has been proposed to negate the pro-apoptotic effect of some oncogenes (10), promote evasion of cytotoxic T cells and facilitate resistance to chemotherapeutic treatment (8,11,12). Mechanisms of resistance identified to date include decreased receptor levels, expression of decoy receptors, increased expression of inactive caspase-8 mimics, such as FLICE-like inhibitory protein, and post-receptor modulation of cell death by endogenous caspase inhibitors and anti-apoptotic proteins of the Bcl-2 family (3,13–15).

Isothiocyanates derived from cruciferous vegetables have strong chemopreventive activities against carcinogen-induced cancers in experimental animal models (16,17). In most cases the isothiocyanates were administered either before or during exposure to the carcinogen. The chemopreventive properties of these compounds have been attributed to the suppression of carcinogen bioactivation by cytochrome P450 isozymes (phase I) and increased detoxification and elimination of carcinogens via induction of enzymes such as quinone reductase and glutathione *S*-transferases (GSTs) (phase II) (16). However, isothiocyanates were also able to reduce tumor numbers in a mouse model of lung carcinogenesis when added in the post-initiation phase, implying that they had chemopreventive properties independent of effects on the phase I and phase II responses (18). It has been proposed that the anticarcinogenic activity of isothiocyanates is also associated with their ability to induce apoptosis of tumor cells (19,20). The mechanism of induction is unknown, but there is evidence that the mitogen-activated protein kinases (MAP kinases), in particular activation of c-Jun N-terminal kinase (JNK), is involved in isothiocyanate-induced apoptotic signaling (19,21).

Under physiological conditions, isothiocyanates react with reduced glutathione (GSH) either directly or via GSTs to form dithiocarbamates. These conjugates are exported from the cell, rapidly lowering intracellular levels of the tripeptide (22). The tripeptide glutathione (γ-glutamyl-cysteinyl-glycine) is present inside cells at 1–10 mM, depending on cell type and the stage of the cell cycle (23,24), where it acts as a redox buffer (25). Elevated levels of GSH are linked to increased proliferation and impaired apoptosis (26). Indeed, a number of studies suggest that an increase in intracellular reductants, whether GSH or thioredoxin, promote resistance to apoptosis, including the Fas pathway (27–32). Lowering intracellular GSH by an efflux mechanism has been reported during apoptosis, and while this can occur in a caspase-dependent manner (33), others propose that GSH efflux is a critical early event in the initiation of apoptosis (34).

The flavoprotein inhibitor diphenyleneiodonium (DPI) sensitizes T24 bladder carcinoma cells to Fas-mediated apoptosis (35,36). We have reported that DPI triggers rapid efflux of intracellular GSH out of the cells via a canalicular GSH transporter (36), providing a potential mechanism for

Abbreviations: BSO, buthionine sulfoximine; DEVD-AMC, Ac-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin; DISC, death-inducing signal complex; DMSO, dimethylsulfoxide; DPI, diphenyleneiodonium; ERK1/2, extracellular signal-regulated kinase; GSH, reduced glutathione; GSTs, glutathione *S*-transferases; JNK, c-Jun N-terminal kinase; MAP kinases, mitogen-activated protein kinases; MBB, monobromobimane; PBS, phosphate-buffered saline; PEITC, phenethyl isothiocyanate; PI, propidium iodide; PS, phosphatidylserine.

its pro-apoptotic activity. Given the chemopreventive properties of the isothiocyanates, we have tested whether they can sensitize cells to Fas-mediated apoptosis and determined what role GSH plays in sensitization.

Materials and methods

Materials

Human anti-Fas IgM (clone CH-11) was purchased from Upstate Biotechnology (Lake Placid, NY) and the caspase substrate Ac-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (DEVD-AMC) was purchased from Peptide Institute Inc (Osaka, Japan). PEITC, propidium iodide, dansyl chloride, buthionine sulfoximine (BSO) and the goat anti-rabbit peroxidase antibody were from Sigma Chemical Co. (St Louis, MO). Working solutions of PEITC were prepared in dimethylsulfoxide (DMSO) and diluted in growth medium just prior to addition to cells. The final concentration of DMSO in the medium was kept constant at 0.1%. Sulforaphane was obtained from LKT Laboratories Inc. (St Paul, MN) and dissolved in equal parts of dimethylformamide, acetonitrile and DMSO prior to addition to the culture medium. Anti-phospho-extracellular signal-regulated kinase 1/2 (ERK1/2), phospho-JNK, phospho-p38 and MEK1/2 inhibitor (UO126) were purchased from Cell Signaling Technology (Beverly, MA). The JNK activity kit, anti-JNK antibody, JNK inhibitor II, p38 inhibitor SB203580 and monobromobimane (MBB) were from Calbiochem (San Diego, CA). Antibodies directed against total ERK1/2 and p38 were purchased from Sigma (St Louis, MO). Mouse anti-Bcl-2 antibody (clone Bcl-2-100) was from Zymed Laboratories Inc. (San Francisco, CA) and mouse anti-rabbit GAPDH antibody from Research Diagnostics Inc. (Flanders, NJ). Complete protease inhibitor cocktail tablets and geneticin were obtained from Roche Diagnostics (Mannheim, Germany). Lipofectamine 2000 was obtained from Stratagene (La Jolla, CA) and pCIneo from Promega (Madison, WI). Phosphatidylserine (PS) exposure was measured with an Apotest-FITC kit from Nexins Research (Hoeven, The Netherlands). Cell culture materials were from Gibco BRL, supplied by Invitrogen New Zealand Ltd (Auckland, NZ).

Cell culture

The human bladder carcinoma cell line T24 and the Jurkat T lymphocyte cell line were obtained from American Type Culture Collection (Rockville, MD). T24 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine. T24 cells were used as subconfluent cultures in 15 mm diameter wells, except when assayed for MAP kinase activity, when 34 mm wells were used. Jurkat cells were cultured in RPMI-1640 containing 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Both cell types were maintained at 37°C in a humidified atmosphere with 5% CO_2. Fresh medium was added to the cell lines 1 h prior to treatment.

Generation of Bcl-2 overexpressing Jurkat cells

Human Bcl-2 cDNA, pICbcl-2, was obtained from Professor Suzanne Cory (Melbourne, Australia). The Bcl-2 coding region was subcloned from pCIbcl-2 into pCIneo and sequenced. Jurkat cells were transfected using Lipofectamine 2000. After 24 h cells were selected using geneticin at an active concentration of 315 μg/ml. Stable lines were selected by serial dilution. Resistant clones were analyzed for Bcl-2 expression by western blotting, with GAPDH levels determined as a loading control.

Assessment of cell viability

Plasma membrane integrity was monitored using propidium iodide (PI) staining. After treatment, cells were harvested either by centrifugation or following incubation with trypsin/EDTA. Cells were resuspended in phosphate-buffered saline (PBS) containing 2 μg PI, incubated in the dark for 10 min and the cell fluorescence measured using a bivariate flow cytometer (Becton Dickinson, Mountain View, CA). PI-positive cells were expressed as a percentage of the total cells analyzed. In some circumstances cells were co-stained with Annexin V–FITC to detect phosphatidylserine exposure on apoptotic cells, according to the manufacturer's instructions.

Determination of caspase activity

The measurement of DEVD-AMC cleavage was modified from Nicholson *et al*. (37). After treatment, cells were harvested either by centrifugation or following incubation with trypsin/EDTA for adherent cells and stored as pellets at −80°C. Immediately prior to assay, the pellets were thawed by the addition of 100 μl of buffer (100 mM HEPES, 10% sucrose, 5 mM dithiothreitol, 10^{-4}% NP-40 and 0.1% CHAPS at pH 7.25) containing 50 μM DEVD-AMC. The rate of release of fluorescent AMC was monitored at 37°C (excitation 370 nm, emission 445 nm) and the amount of AMC liberated calculated from a standard curve generated with the free compound.

Glutathione determination

Cellular GSH levels were measured by HPLC following derivatization with dansyl chloride (36,38). Briefly, the cells were washed in PBS, and lysis buffer containing iodoacetate was added to the wells. Cells were transferred to microcentrifuge tubes, the pH corrected to 8–8.5 with lithium hydroxide and the extracts incubated for 30 min. Dansyl chloride was then added and the extracts were incubated for a further 60 min. Following extraction with chloroform, HPLC analysis was performed as described previously (38). Alternatively, intracellular GSH was measured using the MBB method. After treatment, cells were resuspended in 400 μl of PBS, the pH brought to 8 with 0.1 M potassium hydroxide, and 1 mM MBB in acetonitrile was added. The samples were incubated for 20 min in the dark. The protein was then precipitated with trichloroacetic acid (5% w/v final concentration), removed by centrifugation and the supernatants analyzed by HPLC with fluorescent detection (excitation 394 nm, emission 480 nm) (39).

MAP kinase activity

After treatment, cells were washed in ice-cold PBS, scraped and extracted for 15 min on ice in lysis buffer (25 mM HEPES, pH 7.4, containing 100 mM NaCl, 2 mM EDTA, 1.6 mg/ml Complete™ protease inhibitors, 1 mM sodium vanadate and 0.5% NP-40). Insoluble material was removed by centrifugation at 13 000 *g* for 5 min at 4°C. Cell lysates were separated by SDS–PAGE (12% gel) and transferred to PVDF membrane. The blots were probed with antibodies against the phosphorylated and unphosphorylated forms of p38, ERK1/2 and JNK according to the manufacturer's instructions. A peroxidase-conjugated secondary antibody and chemiluminescence were used to detect the immunoreactive bands.

Active JNK was also assayed using a Calbiochem kit. Briefly, JNK was immunoprecipitated, a kinase reaction with c-Jun as substrate performed and JNK activity detected by western blotting with a phospho-c-Jun-specific antibody.

Results

Effect of PEITC on T24 cells

PEITC has been shown previously to temporarily lower intracellular GSH, activate MAP kinases and trigger apoptosis in a dose-dependent manner (19–22,40). We initially investigated whether the same biochemical events occurred in Fas-resistant T24 bladder carcinoma cells and, if so, what concentration of PEITC was required. PEITC caused a dose-dependent loss in viability of the cells after a 24 h period (Figure 1A). The morphological appearance of the T24 cells treated with PEITC resembled apoptosis, with extensive contraction, membrane blebbing and detachment of cells. To confirm apoptosis was occurring, caspase-3-like activity was measured 6 h after addition of isothiocyanate to the cells. Caspase activation was detectable with 10 μM PEITC, with caspase activity being 2.5-fold above that seen in untreated cells (Figure 1B). A 4.5-fold increase was observed at 15 μM PEITC, before declining at higher doses (Figure 1B).

After 90 min treatment with PEITC, the T24 cells were harvested and western blots performed with antibodies directed against the dually phosphorylated active forms of JNK, ERK1/2 and p38. We detected a phosphorylated isoform of JNK at 10 and 20 μM PEITC, which returned to basal levels at higher doses (Figure 2). Another isoform was detectable at 20 μM PEITC and it remained phosphorylated as the concentration of PEITC increased. JNK activation was confirmed at 10 and 30 μM PEITC by use of an immune complex kinase assay that measures phosphorylation of c-jun, the substrate for active JNK (see Figure 6). PEITC induced ERK1/2 phosphorylation, with bands detectable at 5 μM PEITC and maximal activity achieved between 20 and 100 μM. The active form of p38 was also detected in T24 cells upon exposure to 20 μM PEITC (Figure 2).

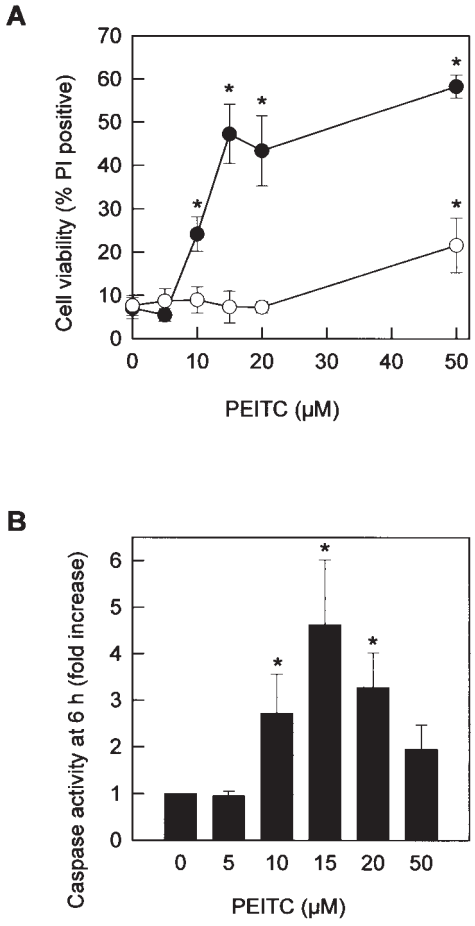

Fig. 1. PEITC induces T24 cell death. Subconfluent T24 cells were transferred to fresh medium for 1 h and then incubated with PEITC. (**A**) PI-positive cells were measured 6 (open circle) and 24 h (solid circle) after the addition of isothiocyanate and (**B**) caspase activity was assayed 6 h after PEITC was added. Four 15 mm wells were combined per treatment condition. The results are the means ± SD of three experiments. Treatments that are significantly different ($P < 0.05$, one way repeated measures ANOVA) from control cells are indicated (*).

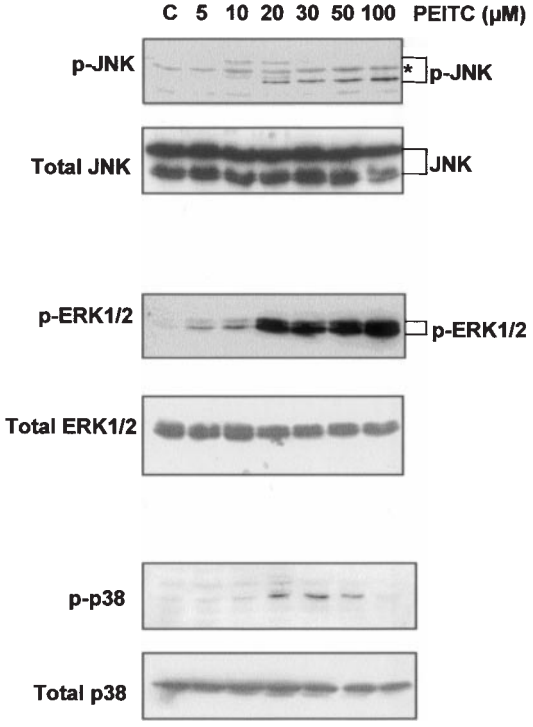

Fig. 2. PEITC induces MAP kinase activation. T24 cells were treated with increasing concentrations of PEITC for 90 min in medium. The cells were lysed, run on SDS–PAGE and blotted with antibodies to phosphorylated JNK, ERK1/2 or p38. A non-specific band in the p-JNK western blot is indicated (*). Antibodies to JNK, ERK1/2 and p38 were used to show that changes in the phosphorylation of the MAP kinases were not due to alterations in protein levels.

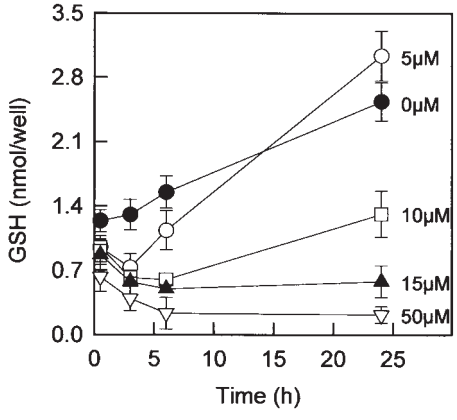

Fig. 3. PEITC induces GSH loss. Subconfluent T24 cells were transferred to fresh medium for 1 h and then incubated with PEITC. At the indicated times, control cells (solid circle) and those treated with 5 (open circle), 10 (open square), 15 (solid triangle) and 50 μM (open triangle) PEITC were derivatized with MBB and the samples separated by HPLC. GSH levels have been expressed as nmol/well and are the means ± SD of three to four experiments performed in duplicate.

Addition of PEITC to T24 cells also resulted in a rapid dose-dependent depletion of intracellular GSH (Figure 3). The loss was apparent within 30 min of adding PEITC and maintained over 24 h at the higher isothiocyanate concentrations (Figure 3). In contrast, incubation with 5 μM PEITC for 24 h resulted in recovery of cellular GSH to levels above the controls. Toxicity was not evident at 6 h, except at 50 μM PEITC, indicating that the loss of GSH preceded a loss in cell viability (Figures 1A and 3).

PEITC sensitizes T24 cells to Fas-mediated apoptosis

As previously established (35), T24 cells were relatively resistant to Fas-induced apoptosis (Figure 4A and B). However, when cells were pre-treated with sublethal concentrations of PEITC and then anti-Fas antibody added, toxicity was significantly elevated above that seen with Fas alone (Figure 4A and B). Indeed, treatment of cells with 10 μM isothiocyanate in combination with the anti-Fas antibody resulted in elevation of caspase activity almost 70-fold above control levels (Figure 4A). PI staining after 24 h showed a dose-dependent increase in Fas-mediated apoptosis with increasing concentration of the isothiocyanate. Anti-Fas antibody in combination with 10 μM PEITC caused an almost complete loss of viability, whereas either of these agents alone was <30% effective (Figure 4B).

Sulforaphane, an isothiocyanate produced in broccoli (41), was also able to sensitize T24 cells to Fas-mediated apoptosis, but not as effectively as PEITC (Figure 4C). After 4 h

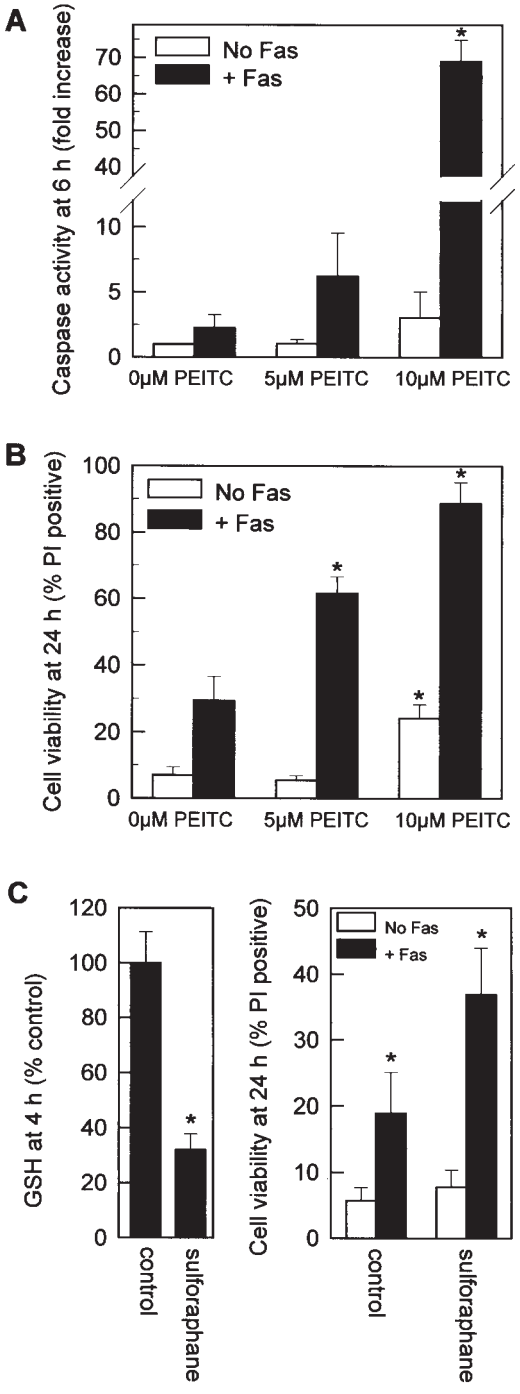

Fig. 4. PEITC sensitizes T24 cells to Fas-mediated cell death. Subconfluent T24 cells were transferred to fresh medium for 1 h and incubated with increasing concentrations of PEITC (5 or 10 μM). After 30 min, anti-Fas antibody (0.25 μg/ml) was added (solid bars) or the cells were left with PEITC alone (open bars). (**A**) Caspase activity was measured 6 h after addition of the anti-Fas antibody. Four 15 mm wells were combined per treatment condition. (**B**) PI-positive cells were measured 24 h after anti-Fas was added. Results are the means ± SD of three experiments. (**C**) Subconfluent T24 cells were transferred to fresh medium for 1 h and either left untreated or incubated with sulforaphane (20 μM). GSH changes: after 4 h with sulforaphane, cells were derivatized with dansyl chloride and the lysates separated by HPLC. The results are the means ± SD of three experiments. PI-positive cells: following a 30 min incubation with sulforaphane, anti-Fas antibody was added (0.25 μg/ml; solid bars) or the cells were left with the compound alone (open bars). PI-positive cells were measured 24 h later and are the means ± SD of four experiments. Treatments that are significantly different from control cells ($P < 0.05$, one way repeated measures ANOVA) are indicated (*).

treatment with 20 μM sulforaphane T24 cells had 30% of their GSH remaining (Figure 4C).

We also tested the ability of PEITC to sensitize Jurkat cells overexpressing the anti-apoptosis protein Bcl-2. Two stably transfected clones expressing moderate (Jurkat/Bcl-2 #11) and high (Jurkat/Bcl-2 #9) levels of Bcl-2 were selected (Figure 5A). The Jurkat cells responded rapidly to anti-Fas antibody with a substantial increase in caspase activity observed within 1 h of treatment, and pre-treatment with PEITC did not alter the sensitivity of these cells to Fas (Figure 5B). In contrast, caspase activation in the Jurkat/Bcl-2 #9 cells was 12% of Jurkat cell levels after 4 h incubation with anti-Fas (Figure 5C). However, treatment of the Bcl-2 transfectant with sublethal doses of PEITC increased responsiveness to Fas, as shown by a dramatic increase in caspase activity (Figure 5C and 5E). The elevated caspase activity was mirrored by increased levels of apoptotic and necrotic cells at 24 h, confirming the ability of PEITC to augment Fas-mediated apoptosis (Figure 5E). The Jurkat/Bcl-2 #11 clone had an intermediate response to the anti-Fas antibody. In these cells caspase activity could also be enhanced by the addition of PEITC (Figure 5D).

MAP kinase activation is not involved in sensitization

We used specific inhibitors of the MAP kinases in order to establish whether PEITC-induced activation of JNK, ERK1/2 or p38 has a role in the sensitization of T24 cells to Fas-mediated apoptosis. We first confirmed the ability of these compounds to inhibit MAP kinase activity in T24 cells (Figure 6). PEITC-induced activation of JNK, ERK1/2 and p38 was substantially reduced by their respective inhibitors (JNK inhibitor II, UO126 and SB203580).

When cells were treated with the MAP kinase inhibitors before addition of PEITC and Fas, none of the inhibitors were able to prevent the sensitizing effects of PEITC (Figure 7A). Indeed, inhibitors of p38 and JNK appeared to enhance killing in response to PEITC and Fas. The JNK and p38 inhibitors also sensitized cells to Fas (Figure 7B) and to PEITC alone (Figure 7C), while the ERK pathway inhibitor, UO126, did not alter levels of T24 cell death under any of the conditions tested (Figure 7A–C). The MAP kinase inhibitors themselves had no effect on basal toxicity (data not shown).

Lowering of GSH is not sufficient to sensitize cells to apoptosis

To investigate the role of the loss of GSH in the sensitization to Fas by PEITC, T24 cells were incubated with BSO, an inhibitor of GSH synthesis. This compound lowered intracellular GSH to 60–70% of the control after 4 h incubation (Figure 8A), similar to the effect of PEITC (Figure 3). Subsequent addition of anti-Fas antibody to these cells did not decrease cell viability below that seen with Fas alone (Figure 8B). If cells were incubated with BSO for longer periods (16 h) to more substantially reduce GSH to $<25\%$ of control values, there was still no observable sensitization of the T24 cells to Fas (not shown). These results suggest that the lowering of intracellular GSH levels by PEITC is not sufficient to sensitize the cells to apoptosis.

Discussion

In this study we have shown that treatment of cells with the isothiocyanate PEITC promotes Fas-mediated apoptosis.

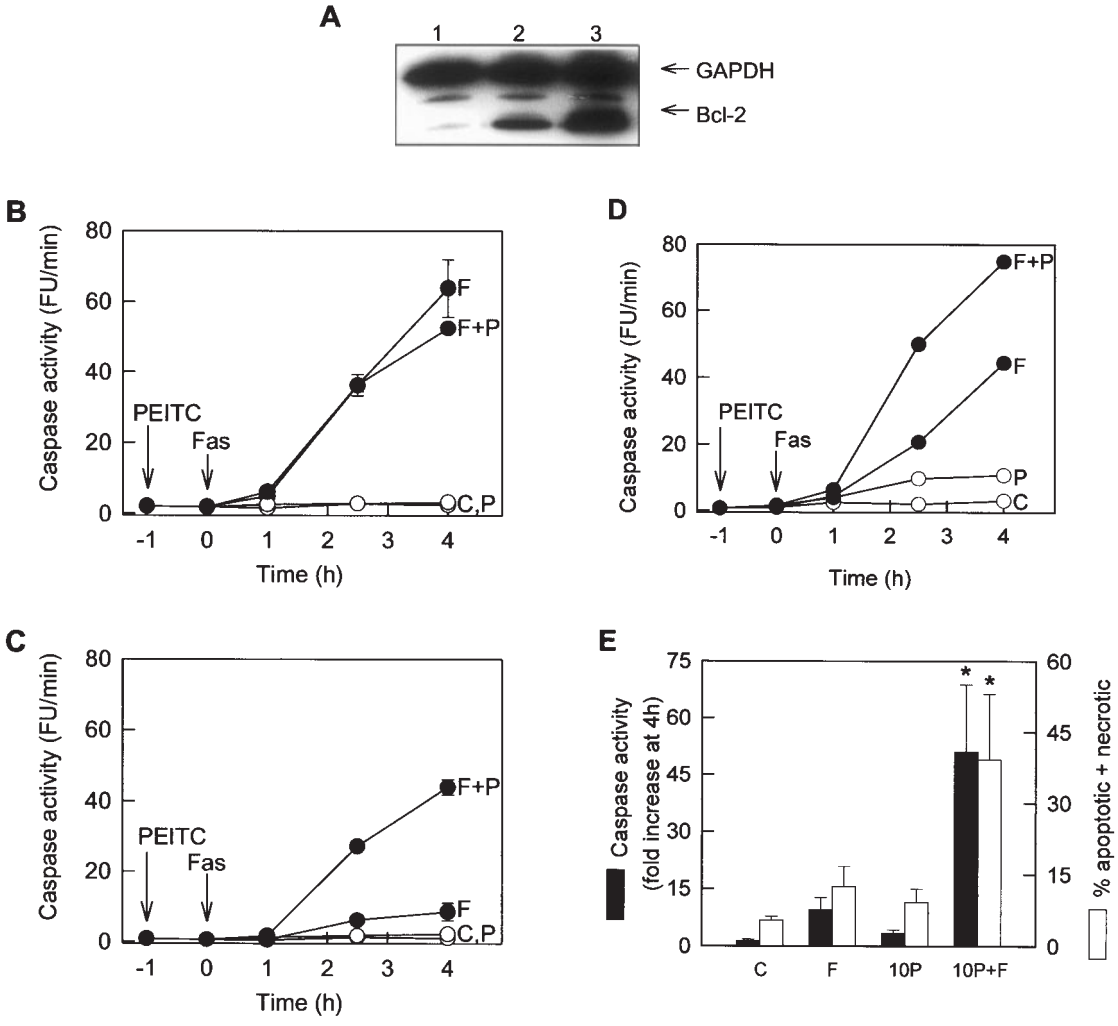

Fig. 5. PEITC sensitizes Jurkat cells overexpressing Bcl-2 to Fas-mediated apoptosis. (**A**) Bcl-2 and GAPDH levels in the Jurkat cell clones were assayed by western blotting. Lane 1, Jurkat cells; lanes 2 and 3, clones 11 and 9, respectively. Jurkat (**B**), Jurkat/Bcl-2 #9 (**C**) and Jurkat/Bcl-2 #11 (**D**) cells were transferred to fresh medium (5×10^6 cells/ml) for 1 h and then left untreated or incubated with PEITC (2 μM for Jurkat cells and 10 μM for clones 9 and 11) and/or anti-Fas antibody (50 ng/ml) as described in the figure (10 μM PEITC caused significant caspase activation by itself in the Jurkat cells). At the indicated times, 0.5×10^6 cells were removed for analysis of caspase activity. Results are duplicate determinations from one of three representative experiments. (**E**) Data from three to four independent experiments on Jurkat/Bcl-2 #9 cells treated with or without 10 μM PEITC and 50 ng/ml anti-Fas antibody. Caspase activity (solid bars) was measured 4 h after treatment with Fas, and is expressed as the fold increase above that of untreated Jurkat/Bcl-2 #9 cells (means ± SD of three to four experiments). Apoptotic and necrotic cells were measured and combined (open bars) 24 h after the addition of Fas using the PS/PI FACS assay (means ± SD of three experiments). Treatments which are significantly higher ($P < 0.05$, one way repeated measures ANOVA) than cells treated with Fas alone are indicated (*).

The effect was observed in T24 bladder carcinoma cells, one of the many well-characterized cell lines that show resistance to activation of apoptosis via the Fas death receptor, and in Jurkat T-lymphoma cells made resistant by overexpression of the anti-apoptosis protein Bcl-2. Sensitization occurred at concentrations below where PEITC by itself was able to trigger apoptosis. The most dramatic effect, however, was the magnitude of caspase activation that was achieved. In the T24 cells there was a >10-fold elevation in caspase-3-like activity over that achieved with either PEITC or Fas stimulation alone at any concentration, indicating a strong synergistic effect.

The isothiocyanates are known to induce the phase II enzymes that protect against carcinogenic oxidants and electrophiles, inhibit the carcinogen-activating phase I enzymes and cause apoptosis of transformed cells (16,20). The ability of this class of compounds to also sensitize transformed cells to receptor-mediated apoptosis could make an important contribution to their chemopreventive activity. Resistance to Fas-mediated apoptosis has been widely described in tumors, providing a mechanism for evading surveillance of the immune system (7–9,12). Studies of autoimmune lymphoproliferative syndromes in mice and humans, resulting from genetic defects in Fas or the Fas ligand, have shown an increased incidence of some malignancies and a faster rate of tumor progression in others (reviewed in 42). On such evidence, Fas is described as a tumor-suppressor gene, and the ability of PEITC to revert cells from a Fas-resistant to a Fas-sensitive phenotype provides a feasible mechanism for chemoprevention. Further investigations into the link between the pro-apoptotic properties of the isothiocyanates and chemoprevention is warranted.

Reports indicate a role for MAP kinases in phase II activation by isothiocyanates (43) and in direct induction of apoptosis by PEITC (19,44,45). Therefore, it was possible that MAP kinases played a role in the ability of PEITC to sensitize cells

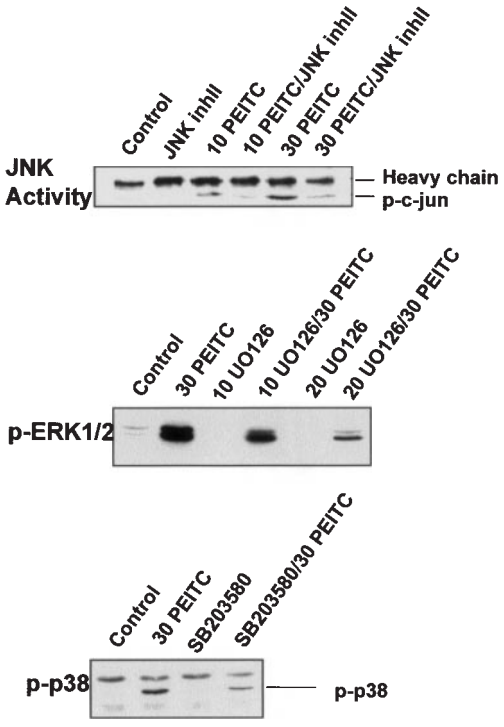

Fig. 6. MAP kinase inhibition. T24 cells were preincubated in fresh medium for 1 h with specific MAP kinase inhibitors of JNK, MEK1/2 and p38 (30 μM JNK inhibitor II, 10 and 20 μM UO126 and 30 μM SB203580, respectively). PEITC was added at 10 or 30 μM as indicated and the cells incubated for 90 min. ERK1/2 and p38 activation was assayed by western blotting as described above. JNK activity was measured by assaying the phosphorylation of its substrate c-jun. Results are representative of two to three experiments.

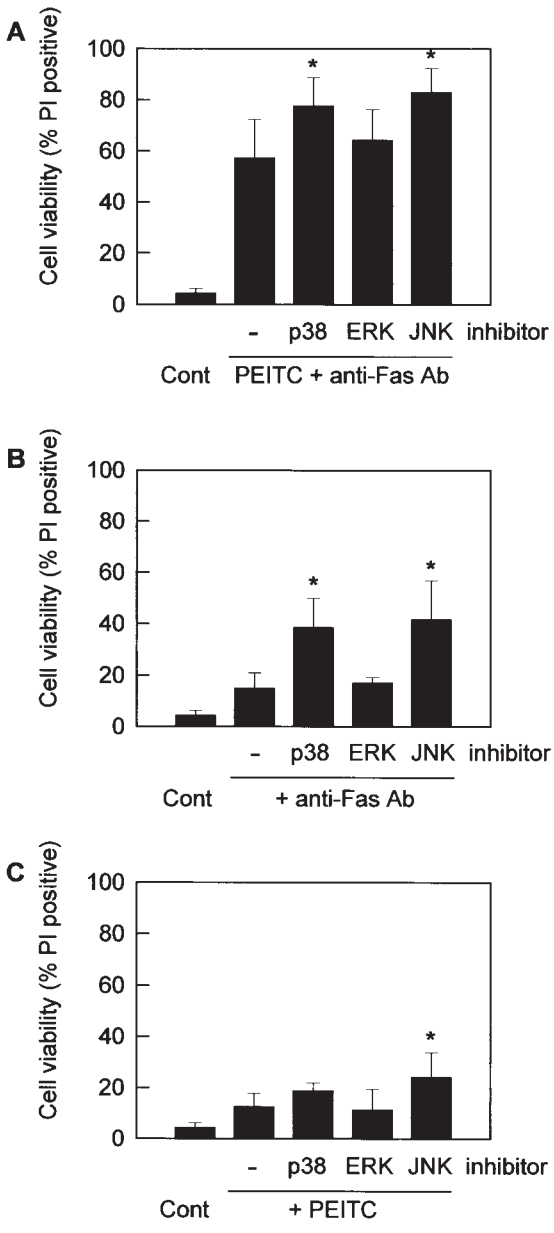

Fig. 7. Effect of MAP kinase inhibitors on PEITC sensitization to Fas. Subconfluent T24 cells were transferred to fresh medium for 1 h and left untreated or incubated with MAP kinase inhibitors (30 μM SB203580, 20 μM UO126 and 30 μM JNK inhibitor II, respectively). Cells were then treated with PEITC for 30 min and then anti-Fas antibody was added. PI-positive cells were assayed 24 h after addition of anti-Fas. T24 cells incubated with PEITC and anti-Fas antibody (**A**), with anti-Fas antibody alone (**B**) or with PEITC alone (**C**). The results are the means ± SD of three to four experiments. Kinase inhibitor treatments which are significantly different ($P < 0.05$, one way repeated measures ANOVA) from cells without inhibitors are indicated (*).

to Fas-mediated apoptosis. While we observed activation of ERK1/2, JNK and p38 upon treatment of T24 cells with PEITC, inhibitors of the respective kinases did not block sensitization. Indeed, the inhibition of JNK and p38 actually appeared to promote apoptosis. This contrasts with reports for a role for JNK activation in the induction of apoptosis by PEITC alone (19) and a recent study showing that PEITC-induced apoptosis of PC3 human prostate cancer cells is dependent on ERK1/2 activation (45). As is common in the MAP kinase field, we can only ascribe variances to differences between cell types.

The primary reaction of isothiocyanates at physiological pH is with thiols (16,22). Post-translational modification of protein thiols is recognized as a cell signaling mechanism that may regulate sensitivity to apoptosis. A variety of proteins, including transcription factors, membrane channels, cysteine-dependent proteases, phosphatases and kinases, or proteins that bind and regulate the activity of these cellular constituents, have cysteine residues that can switch between reduced and oxidized forms (46–48). This causes active site or conformational modifications, thereby altering catalytic activity or protein–protein interactions. Transient thiol oxidation is proposed to be a routine and fundamental event during the initiation of apoptosis. Redox-sensitive targets include a thiol protein involved in opening of the mitochondrial permeability transition pore (49) and proteins such as thioredoxin and glutaredoxin bound to the apoptosis-signaling kinase ASK-1 (48). Alternatively, apoptosis could progress without the need for thiol oxidation, but oxidation of redox-sensitive signaling proteins, such as protein kinases and phosphatases, or transcription factors could promote a pro-apoptotic environment in cells that are normally resistant.

Disruption of the thiol redox balance in cells can result from an increase in exogenous or endogenous oxidant exposure or alterations to the cellular network of thiol reductants. An elevated concentration of intracellular GSH is linked to impaired apoptosis (27–30), and the ability of PEITC to conjugate with this intracellular redox buffer provided a mechanism for sensitization. We were able to exclude this possibility

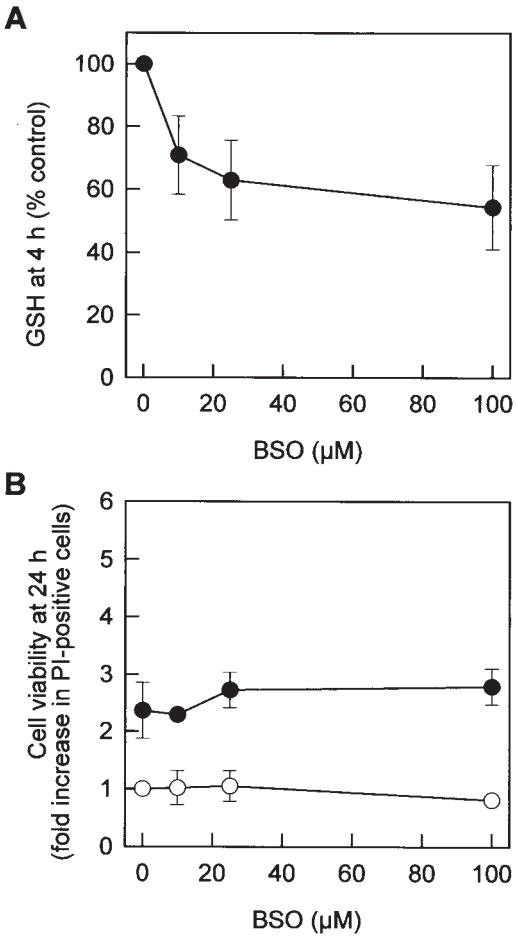

Fig. 8. BSO does not alter T24 cell sensitivity to Fas-mediated apoptosis. Subconfluent T24 cells were transferred to fresh medium and incubated with increasing concentrations of BSO (10–100 μM). After 4 h, anti-Fas antibody (0.25 μg/ml) was added or the cells were left with PEITC alone. (**A**) GSH was measured 4 h after the addition of BSO using the MBB assay. (**B**) PI-positive cells were measured 24 h after anti-Fas was added (solid circle) or the cells left with BSO alone (open circle). The results are the means ± SD of two to three experiments. The viability of cells treated with BSO was not significantly different from untreated cells (one way repeated measures ANOVA, $P > 0.05$).

because sensitization did not occur in cells treated with BSO, an inhibitor of glutathione synthesis. Isothiocyanates are also able to react directly with protein thiols to form a dithiocarbamate (22). If the target cysteine residue is critical for catalysis, metal binding, structure or protein–protein interactions, then covalent modification by an isothiocyanate will have a similar net result as thiol oxidation. Indeed, it has recently been shown that activation of the phase II transcription factor Nrf-2 by sulphoraphane is associated with direct modification of Keap-1, the actin-binding thiol protein that restrains Nrf-2 in the cytoplasm (50). Identification of the intracellular protein targets of PEITC will be crucial in elucidating the mechanism of its pro-apoptotic activity.

The ability of PEITC to sensitize Bcl-2 overexpressing cells to Fas-mediated apoptosis suggests that PEITC is acting downstream of receptor oligomerization and DISC formation. The exact mechanism(s) of Bcl-2 action remains unclear, but appears to be linked to the release of pro-apoptotic factors from the mitochondria and/or regulation of caspase activation (51). In addition to allowing Fas-mediated apoptosis to proceed, PEITC triggers apoptosis by itself in Bcl-2 positive cells (at higher concentrations than those used in Figure 5), suggesting that it either provides an alternative route to bypass the Bcl-2 block or that it may directly interfere with the anti-apoptotic properties of Bcl-2. Deciphering the mechanism by which PEITC influences the Fas pathway will provide novel insight into the regulation of death receptor-mediated apoptosis, as well as increased understanding of the biological activity of the isothiocyanates.

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