The roles of JNK and apoptotic signaling pathways in PEITC-mediated responses in human HT-29 colon adenocarcinoma cells

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Phenethyl isothiocyanate (PEITC) is a potential chemopreventive agent that is present naturally in widely consumed vegetables, especially in watercress. It has been extensively investigated for its anticancer activities against lung, fore-stomach and esophageal tumorigenesis. Here we investigated the pro-apoptotic effect of PEITC in HT-29 human colorectal carcinoma cell line, and the mechanism of apoptosis induced by PEITC. PEITC-induced apoptosis was determined by DNA fragmentation assay and diamidino-2-phenylindole (DAPI) staining technique. To understand the mechanisms of apoptosis induced by PEITC, we studied the role of caspases, mitochondria-cytochrome c release, and mitogen-activated protein kinase (MAPK) signaling pathways involved in PEITC-induced apoptosis in HT-29 cells. Both the caspase-3 and -9 activities were stimulated by PEITC. The release of cytochrome c from the mitochondrial inter-space was time- and dose-dependent, with a maximal release at 50 μM after 10 h treatment. Three MAPKs [JNK (c-Jun N-terminal kinase), extracellular signal-regulated protein kinase (ERK) and p38 kinase] were activated shortly after PEITC treatment in HT-29 cells. Importantly, the SP600125 compound, an anthrapyrazolone inhibitor of JNK, but not the ERK and p38 inhibitor, suppressed apoptosis induced by PEITC. Similarly, this JNK inhibitor attenuated both cytochrome c release and caspase-3 activation induced by PEITC. In summary, this study shows that PEITC can induce apoptosis in HT-29 cells in a time- and dose-dependent manner via the mitochondria caspase cascade, and the activation of JNK is critical for the initiation of the apoptotic processes. This mechanism of PEITC may play an important role in the killing of cancerous cells and offer a potential mechanism for its anticancer action in vivo.

Introduction

Colon cancer is one of the leading causes of cancer death in the US (1). Epidemiological studies have demonstrated an inverse association of colon cancer with intake of cruciferous vegetables (2,3). Dietary isothiocyanates, such as PEITC, are present in large quantities in cruciferous vegetables including watercress, broccoli and cabbage (4). Many isothiocyanates are effective chemopreventive agents against carcinogen-induced cancers in experimental animals. Apart from the colorectum, isothiocyanates inhibit cancer formation in various tissues such as rat lung, esophagus, mammary gland, liver, small intestine and bladder (5–9). Previous studies suggested that chemopreventive activity of isothiocyanates against chemically induced cancers is due to their ability to induce Phase II drug metabolizing enzymes such as glutathione S-transferase and quinone reductase, which detoxify the activated carcinogenic metabolites (10,11). It has also been shown that certain isothiocyanates may inhibit enzymes such as cytochrome P-450 involved in the bioactivation of carcinogens (12). Furthermore, previous studies suggested that isothiocyanates could induce apoptosis in certain types of cancer cells. For example, it has been shown that PEITC and AITC (allyl isothiocyanate) can induce apoptosis in human leukemia HL-60 cells (13,14). PEITC has also been shown to induce apoptosis in HeLa cervical cancer cells and PC-3 prostate cancer cell line (15,16).

The mitogen-activated protein (MAP) kinase family is activated in response to a wide variety of extracellular stimuli and mediates signal transduction cascades that play an important regulatory role in cell growth, differentiation and apoptosis (17). In the mammalian system, the biochemical properties of three MAP kinases have been characterized in detail, the extracellular signal-regulated kinase (ERK), the c-Jun N-terminal kinase (JNK) also referred to as stress-activated protein kinase and the P38 MAP kinase (18). The ERK subgroup of MAP kinases is activated primarily by mitogenic stimuli such as growth factors (19). In contrast, the JNK and P38 pathways are activated primarily by a diverse array of cellular stress including UV irradiation, hydrogen peroxide, DNA damage, heat and osmotic shock (18).

It was reported that PEITC induced JNK activation in HeLa and HT-1080 cells (20). In Jurkat cells, PEITC was found to induce a sustained activation of JNK1, and this was associated with the activation of MEKK1 (16). It was also reported that in p53-deficient PC-3 cells PEITC induced the activation of ERK and P38 but not JNK (21). In addition, Yang et al. (22) showed that N-acetylcysteine conjugate of PEITC activated JNK, p38 and ERK in benzo(a)pyrene-treated A/J mice.

To better understand the anticancer mechanism of isothiocyanates, we examined the effect of PEITC, a predominant isothiocyanate in cruciferous vegetables, on HT-29 human colon carcinoma cells. Here, we report that PEITC is capable of inducing apoptosis by releasing cytochrome c from the mitochondria and activation of caspase-9 and -3, and this mitochondrial caspase-mediated apoptosis is related to activation of JNK.

Materials and methods

Cell culture

Human colorectal cancer cell line HT-29 was obtained from American Type Culture Collection (ATCC). The cells were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 2.2 g/l sodium

Abbreviations: DAPI, diamidino-2-phenylindole; ERK, extracellular signal-regulated protein kinase; JNK, c-Jun N-terminal kinase; MAPKs, mitogen-activated protein kinases; PEITC, phenethyl isothiocyanate.
bicarbonate, 100 U/ml penicillin and 100 µg streptomycin. Before treatment with chemicals, the medium was removed when cells were ~80% confluent and starved overnight in MEM containing 0.5% serum. This would allow the cells to be maintained and acclimatized in the same environment, before comparing the effect of PEITC on these cells.

**Reagents**

Phenelzine isothiocyanate (PEITC) and diamidino-2-phenylindole (DAPI) were obtained from Sigma (St Louis, MO). The fluorogenic tetrapeptide substrates of caspases (Ac-DEVD-MCA and Ac-LEHD-MCA) were purchased from Peptides International (Louisville, KY). Mouse anti-cytochrome c monoclonal antibody was obtained from Pharmingen (San Diego, CA). Rabbit anti-phospho-JNK, -ERK1/2 and -p38 polyclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA). Rabbit anti-JNK1(FL) and -p38(Chi-20) polyclonal antibodies and mouse anti-ERK2 (D-2) antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). SP600125, PD98059 and SB203580 were purchased from Calbiochem (San Diego, CA).

**Nuclear staining assay**

After treatments, floating cells were collected by centrifugation at 2000 g for 15 min, and attached cells were first trypsinized and then harvested by centrifugation. Apoptotic cells with condensed or fragmented nuclei were visualized by DAPI staining. Briefly, cells were washed once with ice cold PBS before fixing in a solution of methanol:acetic acid (3:1) for 30 min. Fixed cells were placed on slides. After evaporation of fixing solution, cells were stained with 1 µg/ml DAPI for 15 min. The nuclear morphology of cells was examined by fluorescence microscopy.

**DNA fragmentation assay**

After treatment, cells were harvested and lysed in a buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA and 0.5% Triton X-100 for 30 min on ice. Cells lysates were vortexed and cleared by centrifugation at 12 500 g for 20 min. DNA in the supernatant was extracted with an equal volume of neutral phenol:chloroform:isoamyl alcohol mixture (25:24:1) at room temperature for 15 min and precipitated with 2 vol of 100% ethanol and one-tenth volume of 3 M sodium acetate (pH 5.2) overnight at −20°C. The DNA precipitates were spun down at 12 500 g for 20 min and washed once with 70% ethanol. The air-dried DNA pellets were incubated with 5 mg/ml DNAse-free RNase in a 40 µl Tris–EDTA buffer (pH 8.0) at 37°C for 2 h. Fragmented DNA was resolved on 1.5% agarose gel in the presence of 0.5 µg/ml ethidium bromide.

**Caspase activity assay**

After treatment, HT-29 cells were washed twice with ice-cold PBS and lysed for 30 min at 4°C in a buffer containing 50 mM Tris–HCl (pH 7.4), 50 mM β-glycerophosphate, 15 mM MgCl₂, 15 mM EDTA, 100 µM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT) and 150 µg/ml digitonin. Cell lysates were homogenized by passing through a 23-G needle three times. Homogenates were kept on ice for 30 min, and then centrifuged at 12 500 g for 20 min at 4°C. The supernatants were collected and protein concentrations were determined by Bio-Rad protein assay, was mixed with 4× loading buffer, and pre-heated at 95°C for 3 min. The samples were then loaded on a 10% mini SDS–polyacrylamide gel, and run at 200 V. The proteins were transferred onto PVDF membrane for 1.5 h using semi-dry transfer system (Fisher, Pittsburg, PA). The membrane was blocked in 5% bovine serum albumin solution for 1 h at room temperature, then incubated overnight at 4°C with indicated primary antibody (1:1000 dilution). After hybridization with primary antibody, the membrane was washed with TBST (Tris buffered-saline Tween-20) three times, then incubated with HRP-labeled secondary antibody for 45 min at room temperature and washed with TBST three times. Final detection was performed with ECL™ (Enhanced Chemiluminescence) western blotting reagents (Amersham Pharmacia Biotech).

**Trypan Blue staining**

After drug treatments, floating and attached cells were harvested and centrifuged for 10 min at 1000 g. Cell pellets were resuspended in PBS, and incubated with 0.4% Trypan Blue solution for 15 min. The number of cells was counted by using a hemocytometer. Stained cells were read as dead cells and unstained cells as viable cells. The percentage of dead cells was calculated as the ratio between the number of stained cells and the total cell counts.

**Results**

**PEITC induced apoptosis in HT-29 colon cancer cells**

HT-29 cells were treated with various concentrations of PEITC for 24 h. Cell death was assayed by DAPI staining, which detects nuclear morphology (Figure 1). The number of

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**Fig. 1.** PEITC induces nuclear condensation in HT-29 cells. Cells were either treated with 0.1% DMSO as vehicle control or treated with different concentrations of PEITC for 24 h. Cells were harvested and washed with ice cold PBS followed by fixation for 30 min. Fixed cells were incubated with 1 µg/ml DAPI for 15 min and the nuclear morphology of cells was examined by a fluorescence microscopy. Condensed and fragmented nuclei were indicated by arrows.
the condensed and fragmented nuclei increased with the exposed concentrations of PEITC. Furthermore, DNA fragmentation, a hallmark event in apoptosis cells, was also detected in a dose-dependent manner in cells treated with PEITC (Figure 2). From this result, PEITC induced apoptosis in HT-29 cells in the concentration range of 10–50 μM.

**PEITC induced caspase activities**

To measure the activation of caspases in PEITC-treated cells, the specific fluorogenic tetrapeptide substrates for each caspase were used. First, the activity of caspase-3, the converging point of different caspase-dependent apoptosis pathways, was measured after cells were treated with different concentrations of PEITC. As shown in Figure 3A, PEITC strongly induced caspase-3-like activity at the concentration of 25 and 50 μM. And at the concentration of 50 μM, the activation of caspase-3 can be detected at ~7 h and peaks at 12 h. Caspase-9, the mitochondria damage-related caspase, was also activated [Figure 3B, the 12 h treatment was significantly different from control (Student’s t-test, \( P < 0.05 \)). This implied the possible involvement of mitochondrial damage in PEITC-induced apoptosis.

**PEITC induced cytochrome c release from the mitochondria**

To confirm the involvement of the mitochondrial apoptotic program, the release of cytochrome c into the cytosol was investigated. As shown in Figure 4, when cells were treated with PEITC for 10 h, the release of cytochrome c from mitochondria to the cytosol was detected at a 10 μM concentration and reached the maximum at 50 μM. We also performed a time course study and it appeared that the release of cytochrome c started after ~3 h and increased dramatically after 10 h of treatment with 50 μM of PEITC.

**Induction of MAP kinase activity**

To investigate the role of other signal transduction events induced by PEITC that could contribute to apoptosis, we studied the role of MAPK pathway. We found that exposure of HT-29 cells to PEITC resulted in a time-dependent phosphorylation and activation of all three major MAPKs, JNK, ERK and p38. Activation of ERK and p38 were evident as early as 15 min after 50 μM PEITC treatment and persisted for the duration of the experiment (Figure 5A). Phosphorylation of
JNK started at ~30 min and peaked at 1 h. We also investigated the dose-dependent activation of MAPK (Figure 5B). JNK and ERK activation at 1 h was observed after treatments with 5 μM of PEITC and reached the maximum level at 50 μM concentration. However, the activation of JNK declined when the concentration of PEITC reached 100 μM. The activation of p38 was initiated at a relatively higher concentration of PEITC (>10 μM).

The specific inhibitor of JNK, SP600125, attenuated PEITC-induced apoptosis

Studies using MAPK inhibitors were carried out to determine whether the activation of MAPKs contribute to PEITC-induced apoptosis in HT-29 cells. Figure 6 shows the effect of JNK inhibitor on PEITC-induced cell death. The cell viability of control (treated with DMSO) and 10 μM SP600125 alone were very similar. Cell viability was significantly decreased in cultures exposed to PEITC with an IC50 of ~25 μM. The PEITC-induced cell death at different concentrations of PEITC was analyzed by using respective antibodies against phosphorylated MAP kinases, and antibodies against non-phosphorylated proteins as controls. Molecular weights of the proteins are indicated in kilodaltons.

**Fig. 4.** PEITC induces cytochrome c release from mitochondria. Cytosolic fractions of HT-29 cells were treated with different concentrations of PEITC for 10 h (top) and HT-29 cells were treated with 50 μM PEITC for different time periods (bottom). Twenty micrograms of protein was subjected to immunoblot analysis using antibody against cytochrome c, and anti-β-actin was used as loading control.

**Fig. 5.** MAPKs activation by PEITC. Cells were treated with different concentrations of PEITC for 1 h (top) or with 50 μM for different time periods (bottom). Cells were then harvested as described in the Materials and methods. Activation of MAP kinases, JNK, ERK and p38 kinase were examined by using respective antibodies against phosphorylated MAP kinases, and antibodies against non-phosphorylated proteins as controls. Molecular weights of the proteins are indicated in kilodaltons.
Fig. 6. Effect of SP600125, a specific inhibitor of JNK, on HT-29 cell viability after treatment with PEITC. After overnight serum starvation, cells were either treated with PEITC for 24 h or pre-treated with 10 μM SP600125 for 1 h before PEITC treatment. Trypan Blue staining was used to determine the cell viability. The percentage of dead cells was calculated as the ratio between the number of stained cells and the total cells. The data shown are means ± SD of three separate experiments done in duplicate.

Fig. 7. The activation of caspase-3 by PEITC is blocked by SP600125. Cells were treated either with different concentrations of PEITC or pre-treated with 10 μM of SP600125 and then treated with PEITC for 12 h. Whole-cell lysates were prepared and caspase-3 activity was examined using Ac-DEVD-AMC as a fluorescent substrate. The data shown are means ± SD of three separate experiments done in duplicate.

Fig. 8. The effect of the MAPK inhibitors SP600125, SB203580 and PD98059, and caspase inhibitor zVAD, on the cytochrome c release from mitochondria induced by PEITC. SP600125, SB203580, PD98059 and zVAD were added as 1 h pre-treatment at the indicated concentrations. Cells were then treated with 50 μM of PEITC for 10 h. The cytosolic fractions were prepared and subjected to immunoblot analysis using monoclonal antibody against cytochrome c, and anti-β-actin was used as loading control.
concentrations was blocked in the presence of SP600125 ($P < 0.05$ at 5, 25, 50 and 100 μM). There was no protection against PEITC-induced cell death by either the ERK inhibitor PD98059 or the p38 inhibitor SB203580 as measured at 24 h (data not shown).

We further investigated the mechanism that accounts for the pro-apoptotic actions of JNK in PEITC-induced apoptosis in HT-29 cells. We tested the involvement of the caspase pathway, by examining the effect of JNK inhibitor on caspase-3 activation. As shown in Figure 7, when cells were treated with PEITC for 12 h, caspase-3 activity was induced starting with 10 μM of PEITC treatment. The effect was attenuated with the pre-treatment of the JNK inhibitor SP600125 at 10 μM concentration. We next studied the effect of the JNK inhibitor on the cytochrome c release. At 10 μM, SP600125 totally abolished the release of cytochrome c from mitochondria induced by PEITC, while the ERK and p38 inhibitors had no effect on PEITC-induced cytochrome c release or caspase-3 activity (Figure 8). The caspase inhibitor zVAD-fmk failed to block the release of cytochrome c, indicating that JNK-mediated activation of the mitochondrial apoptosis pathway is not a secondary consequence of caspase activation.

**Discussion**

Caspases are a family of cysteine proteases, which play a central role in the apoptosis machinery. These enzymes participate in a cascade that is triggered in response to pro-apoptotic signals and culminates in cleavage of a set of target proteins, with the specific cleavage site after aspartic acid, resulting in disassembly of the cell (23). Caspase-3 activity was shown to be induced by PEITC in HeLa cells (15). PEITC and its cytosine conjugate, PEITC-cys, induced caspase-3 and -8 activities in HL-60 cell line, and it was suggested that caspase-8 played a critical role in PEITC and PEITC-cys-induced apoptosis (13). However, in this study, caspase-8 activity was not induced by PEITC treatment in HT-29 cells (data not shown). Instead, caspase-3 and -9 were induced by PEITC in a similar pattern, which suggested that mitochondria might be involved. The mitochondrion caspase cascade is initiated by the release of cytochrome c from the intermembrane space into the cytosol. Cytochrome c then forms the apoptosome together with procaspase-9 and Apaf1, whereas caspase-9 is activated (24). The subsequent activation of caspase-3 then occurs and leads to the apoptotic processes (25). This is the first report to show that PEITC can induce cytochrome c release from the mitochondria as well as caspase-9 activation, thereby demonstrating that the mitochondria is involved in the PEITC-induced apoptosis in HT-29 cell line.

Several upstream signaling events have been suggested as activators for the mitochondrial caspase cascade, one of which is JNK activation (26,27). The mechanism that accounts for the pro-apoptotic actions of JNK has not been completely elucidated. One potential mechanism is that accounts for the pro-apoptotic actions of JNK is the release of the Bax subfamily of the Bcl-2-related proteins (28). Recently, the critical role of JNK in UV-induced apoptosis was shown using Jnk1 -/- Jnk2 -/- double null mouse embryonic fibroblast (26). It strongly suggested that JNK activation is necessary for stress-related release of cytochrome c, mitochondrial dysfunction and apoptosis, although the exact role of JNK in these processes is yet to be elucidated.

It was recently shown that SP600125, a reversible ATP-competitive inhibitor, specifically inhibits activation of the JNK MAP kinase in response to a variety of stress stimuli (29). In order to determine whether activation of JNK is directly associated with the pro-apoptotic activity of PEITC in HT-29 cells, we sought to block JNK activity using SP600125 and to determine the effect on the extent of apoptosis induced by PEITC. Our results showed that inhibition of the JNK pathway with SP600125 attenuated the effects of PEITC on mitochondrial cytochrome c release and caspase-3 activation. However, as inhibition of ERK by PD98059 and p38 by SB203580 did not prevent caspase activation, cytochrome c release or cell death induced by PEITC, the biological roles of ERK and p38 activation by PEITC in HT-29 cells might not be related to apoptosis (although PEITC can induce ERK and p38 activation at similar concentrations as JNK). Our results agreed with previous observations that the dominant-negative mutants of MEK1 and JNK1 blocked PEITC induced cell death in transfected 293 cells (16). However, it has been shown that curcumin, which inhibits JNK activation, suppressed the activation of JNK by PEITC and delayed but did not prevent the final onset of apoptosis in HL-60 cells (30). It has also been shown that PEITC induced apoptosis in p53-deficient PC-3 human prostate cancer cells is mediated by ERK but not JNK (21). Therefore, the role of JNK and other MAPKs in PEITC-induced apoptosis appears to be dependent on different cell lines.

In summary, in this report, we examined the molecular mechanism of PEITC-induced apoptosis in human HT-29 colorectal adenocarcinoma cell line. PEITC induces JNK pathway and leads to cytochrome c release. Caspase-9 and -3 are then subsequently activated, followed by nucleus condensation and DNA fragmentation, which are hallmarks of apoptotic cell death. Future in vivo studies using animal models and in patients would ascertain whether this pro-apoptotic effect of PEITC could potentially contribute to its overall chemopreventive effects against colonic carcinogenesis.

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**References**


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