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Effects of antioxidants and caspase-3 inhibitor on the phenylethyl isothiocyanate-induced apoptotic signaling pathways in human PLC/PRF/5 cells

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Abstract

Phenylethyl isothiocyanate (PEITC) is a well recognized potential chemopreventive compound against human cancers. In this study, the molecular mechanism of PEITC-induced apoptosis was examined with two antioxidants (*N*-acetyl-cysteine and vitamin E) and a caspase-3 inhibitor (z-DEVD-fmk). Results demonstrated that PEITC significantly induced human hepatoma PLC/PRF/5 (CD95-negative) cells undergoing apoptosis. Treatment with 0~10 μM PEITC-triggered cell apoptosis as revealed by the externalization of annexin V-targeted phosphatidylserine and the subsequent appearance of sub-G1 population. Results also displayed that PEITC-induced apoptosis involves the up-regulation of p53 and Bax protein, down-regulation of the XIAP, Bcl-2, Bcl-$_{XL}$ and Mcl-1 proteins, cleavage of Bid, and the release of cytochrome *c* and Smac/Diablo, which were accompanied by the activation of caspases -9, -3 and -8. PEITC-induced the generation of reactive oxygen species and the decrease of mitochondrial membrane potential ($\Delta\psi m$) in a time-dependent pattern. *N*-acetyl-cysteine and vitamin E at 100 μM, and z-DEVD-fmk at 50 μM markedly blocked PEITC-induced apoptosis, which was demonstrated by a decline in the reactive oxygen species generation and the release of the cytochrome *c* and Smac/Diablo from mitochondria to the cytosol. *N*-acetyl-cysteine, vitamin E and z-DEVD-fmk also prevented the PEITC in inducing the loss of $\Delta\psi m$. They also affected the activity of XIAP and Bax proteins. Taken together, these studies suggest that PEITC is an apoptotic inducer that acts on the mitochondria and the feedback amplification loop of caspase-8/Bid pathways in PLC/PRF/5 cells.

Keywords: Phenylethyl isothiocyanate; Apoptosis; Antioxidant; Caspase-3 inhibitor; PLC/PRF/5 cell

1. Introduction

Apoptosis or controlled cell death is a complex process, whose regulation requires the cell to integrate a variety of apoptotic signals. The common type of regulated cell death is characterized by nuclear and cytoplasmic condensation, DNA fragmentation, and alteration in cell membrane asymmetry. In the process of apoptosis, Smac/Diablo and/or Omi/HtrA2 are released from the mitochondria along with cytochrome *c*, which functions to promote caspase activity by eliminating the effect of apoptotic inhibitors, the inhibitor of apoptosis protein (IAP) family proteins (Chen et al., 2003; Martins et al., 2002; Wu et al., 2000). X-linked inhibitory of apoptosis protein (XIAP), cellular inhibitor of apoptotic protein-1 (cIAP-1) and -2, and survivin are proteins that exert anti-apoptotic effects on the processing and activities of the executioner caspases -3, -7 and -9 (Roy et al., 1997; Sasaki et al., 2000). Bax, Bad, Bid, Bak and Hrk promote cell death whereas Bcl-2, Bcl-$_{XL}$, Bcl-w, Mcl-1 and A-1 inhibit cell death (Borner, 2003; Harris and Thompson, 2000). The tumor suppressor of p53 can induce growth arrest and cell death via apoptosis in response to

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cellular stresses (Pyrzynska et al., 2002). The p53 protein is a potent regulator of apoptosis, most notably by mediating up-regulation and mitochondrial translocation of Bax (Karpinich et al., 2002).

Mitochondria have been noted to contribute to the apoptotic signaling via generation of reactive oxygen species, activation of caspases, loss of mitochondrial membrane potential ($\Delta\psi m$), and modulation of apoptotic inducing factors (Nakamura et al., 2002; Siraki et al., 2002). Reactive oxygen species have been reported to play a crucial role in the induction and progression of liver diseases. In all types of liver damage, whether acutely and/or chronically exposed to a toxic injury (alcohol, drugs, viruses etc), there is consistent evidence of enhanced production of free radicals and a significant decrease in the antioxidant defense (Loguercio and Federico, 2003). Antioxidants, both natural and synthetic, have been proposed and utilized as therapeutic agents in liver diseases. In vitro and in vivo studies have shown that hepatitis B virus and hepatitis C virus replications are counteracted by *N*-acetyl-cysteine, pyrrolidinedithiocarbamate and vitamin E (α-tocopherol) (Mahmood et al., 2003; Wang et al., 2003). *N*-acetyl-cysteine is a precursor of reduced glutathione that has the ability to quench hydroperoxides, hydroxyl radicals and superoxide anions ($O_2^{\bullet-}$) (Aruoma et al., 1989; Villagrasa et al., 1997). Vitamin E is a predominant lipophilic antioxidant, which is an efficient scavenger of alkoxyl, single oxygen and peroxyl radicals (Di Mascio et al., 1990; Ricciarelli et al., 2001). Antioxidants, as a quencher of reactive oxygen species, are well known to protect against drug-induced oxidative stress and apoptosis.

Dietary isothiocyanates such as phenylethyl isothiocyanate (PEITC), are present in large quantities in cruciferous vegetables including broccoli, cabbage, watercress, etc. (Fahey et al., 2001). Epidemiological studies have suggested that consumption of *Brassica* vegetables, can reduce risk of cancer in human populations (Block et al., 1992; Lampe and Peterson, 2002). The chemopreventive activity of isothiocyanates against chemically induced cancers was due to their ability to induce Phase II drug metabolizing enzymes such as glutathione *S*-transferase and quinine reductase, which detoxify the activated carcinogenic metabolites (Bogaards et al., 1990; Zheng et al., 1992). It has also been shown that certain isothiocyanates inhibit enzymes such as cytochrome *P*-450, which is involved in the bioactivation of carcinogens (Conaway et al., 1996). PEITC is effective in providing protection against chemically induced cancers in animal models (Hecht et al., 2002; Yang et al., 2002). Until now, the effects of antioxidants (i.e. *N*-acetyl-cysteine, vitamin E) and the caspase-3 inhibitor (z-DEVD-fmk) on the molecular pathways of PEITC-induced apoptosis have never been investigated.

In this study, we demonstrated that PEITC inhibited the expression of an anti-apoptotic protein (i.e. XIAP) and caused the release of cytochrome *c* and Smac/Diablo from mitochondria to the cytosol. PEITC activated caspases -9, -8 and -3, and enhanced the expression of apoptotic protease-activating factor-1 (Apaf-1) in human PLC/PRF/5 cells. Pretreatment with antioxidants and a caspase-3 inhibitor markedly blocked PEITC-induced apoptosis. *N*-acetyl-cysteine, vitamin E and z-DEVD-fmk significantly inhibited the generation of reactive oxygen species, the loss of $\Delta\psi m$, the activation of caspase-3, as well as the release of cytochrome *c* and Smac/Diablo from mitochondria to the cytosol.

2. Materials and methods

2.1. Chemicals

Dulbecco's modified Eagle's medium (DMEM), dimethyl sulfoxide (DMSO), penicillin, propidium iodide, ribonuclease A (RNase A), α-tocopherol acetate (vitamin E), *N*-acetyl-cysteine, trypan blue, streptomycin, rhodamine 123, caspase-3 substrate (Ac-DEVD-pNA), caspase-8 substrate (Ac-IETD-pNA), caspase-9 substrate (Ac-LEHD-pNA) and phenylethyl isothiocyanate (PEITC) with purity greater than 98% were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum was obtained from GIBCO BRL (Gaithersburg, MD, USA). 2′,7′-Dichlorodihydro-fluorescein (H_2DCF-DA) was bought from Molecular Probes (Eugene, OR, USA). Caspase-3 inhibitor (z-DEVD-fmk) was obtained from BD PharMingen (San Diego, CA, USA). All other chemicals used were of analytical grade reagents.

2.2. Cell culture and drug preparation

The PLC/PRF/5 cell line (ATCC CRL 8024; hepatitis B surface antigen, HBsAg [+]) was obtained from the American Type Culture Collection (Rockville, MD, USA). It is a human hepatoma cell line and is CD95-negative (Jiang et al., 1999; You et al., 2001). Cells were grown in 90% DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. They were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

The PEITC stock solution was prepared in DMSO at concentration of 10 mM and stored at − 20 °C until use. The concentrations used for the study were 1, 5 and 10 μM, which were freshly prepared for each experiment with a final DMSO concentration of 0.1%. Control was always treated with the same amount of DMSO (0.1% v/v) as used in the corresponding experiments.

2.3. Cell viability assay

Cells were seeded at a density of 5×10^5 cells per well onto 12-well plates. The concentrations of PEITC used in this study were 1, 5 and 10 μM. The control group was treated with 0.1% DMSO. After 6, 12 and 24 h of incubation, the number of viable cells was determined by staining cell populations with trypan blue. Cells in 6-well plates were pretreated for 1 h with 100 μM *N*-acetyl-cysteine or 100 μM vitamin E or 50 μM z-DEVD-fmk and then stimulated with 5 μM PEITC for 24 h and then harvested for assay. The number of unstained (viable) cells was counted using a hemocytometer.

2.4. Measurement of PEITC-induced apoptosis

Phosphatidylserine redistribution in a plasma membrane was measured by the binding of annexin V-fluorescein isothiocyanate (FITC) according to the manufacturer's protocol (Sigma Chemical Co, St. Louis, MO, USA). In brief, 1×10^6 cells were washed,

resuspended in HEPES buffer (10 mM HEPES at pH 7.4, 140 mM *N*-acetyl-cysteine and 5 mM $CaCl_2$) containing annexin V-FITC (1:50) and 1 μg/ml of propidium iodide for 15 min, and then analyzed by a flow cytometer.

To analyze the apoptotic cells with hypodiploid DNA content, both floating and adherent cells were collected after PEITC treatment. The cells in suspension were then fixed with 70% ice-cold methanol and then transferred to the freezer until use. After washing with phosphate buffered saline (PBS), cells were stained with 50 μg/ml propidium iodide in the presence of 25 μg/ml RNase A at 37 °C for 30 min. A minimum of 10,000 cells per sample was collected, and the DNA histograms were further analyzed by Multicycle software (Phoenix Flow Systems, San Diego, CA).

2.5. Measurement of mitochondrial membrane potential ($\Delta\psi m$) and intracellular reactive oxygen species

Cells were treated with 5 μM PEITC, 100 μM *N*-acetyl-cysteine, 100 μM vitamin E, 50 μM z-DEVD-fmk, 5 μM PEITC + 100 μM *N*-acetyl-cysteine, 5 μM PEITC + 100 μM vitamin E, 5 μM PEITC + 50 μM z-DEVD-fmk for 6, 12 and 24 h as described above. The control group received 0.1% DMSO. Cells were collected using a cell scraper and washed with PBS twice. Cells (5×10^5/ml) were then incubated with rhodamine-123 (5 mg/ml) at 37 °C for 30 min. After incubation, they were washed with PBS once and then analyzed by flow cytometry.

In the reactive oxygen species assay, 10 μM of H_2DCFDA was applied to the cells, which were then incubated for 30 min. A flow cytometer was used to detect the fluorescent dichlorofluorescein. The flow cytometer was equipped with an air-cooled argon 488 nm laser with a 525 nm band pass filter and a 550 nm dichroic mirror as detectors. At least 10,000 cells were collected for each group. To calculate the percentage of cells with damaged Δψm, and cells with fluorescent intensity of reactive oxygen species, data obtained from flow cytometry were analyzed using WinMDI 2.7 software (Scripps Research Institute, La Jolla, CA, USA).

2.6. Assay of caspases -3, -8 and -9

The enzymatic activity of caspases induced by PEITC was assayed according to the manufacturer's protocol. To evaluate the activity of caspases (-3, -8 and -9), cell lysates were prepared after their respective treatment with control (0.1% DMSO) and 5 μM PEITC for 0, 6, 12 and 24 h at 37 °C. Assays were performed by incubating 100 μg protein of cell lysate per sample in 100 μl of reaction buffer [1% NP-40, 20 mM Tris–HCl (pH 7.5), 137 mM *N*-acetyl-cysteine and 10% glycerol] containing 2 μl of caspase-3 substrate (Ac-DEVD-pNA) or caspase-8 substrate (Ac-IETD-pNA) or caspase-9 substrate (Ac-LEHD-pNA) in 96 well microtitre plates. Lysates were incubated at 37 °C for 4 h. The samples were measured with an ELISA reader at an absorbance of 405 nm (Anthos 2010, Austria).

2.7. Western blot analysis of cytohrome c, Smac/Diablo release and antibody-bound proteins

Untreated and drug-treated cells were harvested by centrifugation at 1000 ×*g* for 10 min at 4 °C. The cell pellets were washed once with ice-cold PBS and resuspended with 1 × volume of digitonin lysis buffer [75 mM *N*-acetyl-cysteine, 1 mM NaH_2PO_4, 8 mM Na_2HPO_4 and 190 μg/ml digitonin] containing 250 mM sucrose. The cells were homogenized and then centrifuged at 10,000 ×*g* for 10 min at 4 °C. The supernatants were further centrifuged at 10,000 ×*g* for 20 min. The resulting supernatants (cytosolic fraction) were collected, and the pellets (mitochondrial fraction) were resuspended in Triton lysis buffer [25 mM Tris–HCl (pH 8.0) and 0.1% Triton X-100], followed by incubating on ice for 30 min. The lysate was centrifuged at 15,000 ×*g* for 20 min at 4 °C. The supernatant collected was kept as a solubilized enriched mitochondrial fraction.

Cells were harvested and lysed in ice-cold buffer [10 mM Tris–HCl (pH 7.5), 0.1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanate and 120 mM sodium chloride] containing 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin and 1 μg/ml aprotonin (Sigma Chemical Co., St. Louis, MO, USA). Cell fractions were assayed for protein concentration using the Bio-Rad dye binding protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA).

A total of 50 μg/lane of the cytosolic and mitochondrial fractions, and lysate protein were used for Western blot analysis of cytochrome *c* (1:1000; BD PharMingen San Diego, CA, USA) and Smac/Diablo (1:1000; Alexis Biochemicals, Nottingham, UK), and with the specific primary antibodies, namely anti-XIAP (1:250), anti-Bax (1:250), anti-Bcl-2 (1:500), anti-Bcl-$_{XL}$ (1:500), anti-Mcl-1 (1:1000), anti-p53 (1:500), anti-CD95 (1:5000) and anti-caspase-3 (1:1000) antibodies (BD PharMingen San Diego, CA, USA), and the anti-ß-actin antibody (Sigma Chemical Co., St. Louis, MO, USA). In addition, the antibody of the positive control for mitochondrial protein was cytochrome *c* oxidase subunit IV (Cox IV; 1:1000; Molecular Probes, Eugene, OR, USA).

2.8. Statistical analysis

Data were presented as means ± standard deviations (S.D.) from three independent experiments. Results were evaluated by one way analysis of variance (ANOVA), followed by Duncan's multiple range tests using the Statistical Analysis System (SAS Institute, Cary, NC, USA). Control and treatment groups were compared among themselves using Student's *t*-test. Difference was considered significant when *P*-value was <0.05.

3. Results

3.1. Induction of apoptosis by PEITC

PEITC significantly inhibited the proliferation of PLC/PRF/5 cells in a dose- and time-dependent pattern (Table 1). After 24 h of

Table 1
Effect of phenylethyl isothiocyanate (PEITC) on PLC/PRF/5 cell viability

PEITC treatment (μM)	Cell viability (%)		
	6 h	12 h	24 h
Control (0.1% DMSO)	100.0 ± 0.01[a]	100.0 ± 0.01[a]	100.0 ± 0.02[a]
1	100.0 ± 0.02[a]	100.0 ± 0.05[a]	90.0 ± 1.00[b]
5	99.0 ± 0.01[a]	69.3 ± 1.60[b]	37.8 ± 0.50[c]
10	76.0 ± 0.80[b]	45.0 ± 1.25[c]	11.2 ± 0.70[d]

After PEITC treatment, cell numbers were estimated by trypan blue dye exclusion method. Values are means ± S.D. of three independent experiments. Values in the same column with different letters were significantly different at $P<0.05$ as analyzed by Duncan's multiple range tests.

Table 2
Effect of phenylethyl isothiocyanate (PEITC) on the incidence of PLC/PRF/5 cell apoptosis

PEITC treatment (μM)	% Apoptotic cells	
	Sub-G1 DNA	Annexin V
Control (0.1 % DMSO)	0.14±0.06[d]	0.06±0.01[d]
1	18.92±1.80[c]	12.37±0.09[c]
5	60.15±3.29[b]	61.90±3.25[b]
10	90.23±8.30[a]	88.22±7.00[a]

The DNA content (sub-G1 peak) and the binding of annexin V-FITC to the plasma membrane were analyzed by flow cytometry. Values are mean±S.D. of three independent experiments. Values in the same column with different letters were significantly different at $P<0.05$ as analyzed by Duncan's multiple range tests.

treatment, a trend of increasing cell growth inhibition was noted with increasing concentration of PEITC treatment.

To further confirm that PEITC causes apoptosis, PLC/PRF/5 cells were stained with annexin V-FITC and propidium iodide, and subsequently analyzed by flow cytometry. As indicated by FACS analysis, the percentage of apoptotic cells with hypo-diploid DNA content and the proportion of annexin V-staining cells were increased with increasing doses in the PEITC-treated cells (Table 2).

3.2. PEITC induces activation of caspases -3, -8 and -9

Compared to the control group, PEITC treatment caused a significant activation in caspases -3, -8 and -9 at 6, 12 and 24 h. After 24 h of PEITC treatment, the maximal activity was 502.20±6.25% for caspase-3 (Fig. 1A), 438.80±14.54% for caspase-8 (Fig. 1B) and 469.80±7.90% for caspase-9 (Fig. 1C).

3.3. Antioxidants and caspase-3 inhibitor prevent PEITC-induced cell death

To determine whether the PEITC induction of cell death was affected by the presence of 100 μM antioxidants (*N*-acetyl-cysteine and vitamin E) and 50 μM caspase-3 inhibitor (z-DEVD-fmk), PLC/PRF/5 cells were pre-incubated with *N*-acetyl-cysteine

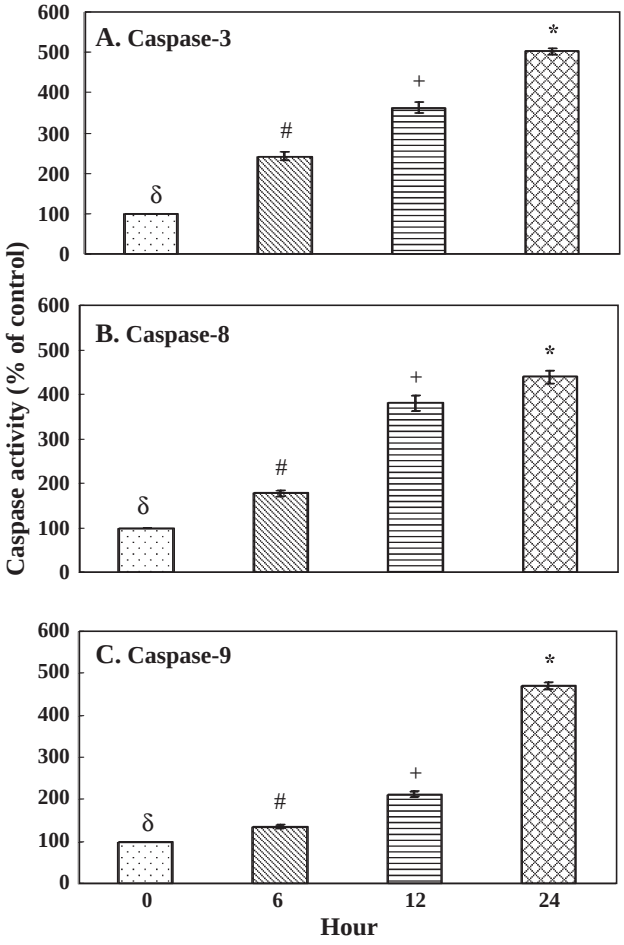

Fig. 1. Effects of PEITC on the activation of caspases. PLC/PRF/5 cells were untreated (control) or treated with 5 μM PEITC for the indicated time period. Cell lysates were then assayed for proteolytic cleavage of (A) caspase-3, (B) caspase-8 and (C) caspase-9 substrates. Data are mean±S.D. of three independent experiments. Values with different letters were significantly different at $P<0.05$ as analyzed by Duncan's multiple range tests.

or vitamin E or z-DEVD-fmk for 1 h, and then induced to undergo apoptosis by treatment with PEITC. As shown in Table 3, *N*-acetyl-cysteine, vitamin E and z-DEVD-fmk exhibited an inhibitory effect against PEITC-induced PLC/PRF/5 cell death.

3.4. Effects of antioxidants and caspase-3 inhibitor on mitochondrial membrane potential ($\Delta\psi m$) and reactive oxygen species generation in PEITC-treated cells

The induction of cell death is generally associated with, and probably mediated by, perturbations of the mitochondrial function, a manifestation of which is the dissipation of $\Delta\psi m$. The loss of relative fluorescent intensity percentage measured with rhodamine 123 dye reflects the collapse of $\Delta\psi m$. In Fig. 2, PLC/PRF/5 cells treated with PEITC at 5 μM significantly ($P<0.0001$) reduced the uptake of the fluorescent dye by 66.80±2.49% at 12 h and 47.90±1.25% at 24 h. The data also displayed a decline of $\Delta\psi m$ in PEITC treated cells in a time-dependent manner. *N*-acetyl-cysteine, vitamin E and z-DEVD-fmk treatment markedly prevented the loss of $\Delta\psi m$ in PEITC treated cells. These findings showed that treatment with PEITC together with *N*-acetyl-cysteine, vitamin E and z-DEVD-fmk significantly prevented the loss of $\Delta\psi m$.

After staining with H_2DCFDA (a fluorescent dye for peroxides, H_2O_2), results displayed a significant reactive oxygen species generation in 5 μM PEITC-treated cells as compared with the control (Fig. 3). We further investigated the involvement of reactive oxygen species by treating the cells with 100 μM *N*-acetyl-cysteine or 100 μM vitamin E or 50 μM z-DEVD-fmk before treatment with 5 μM PEITC for 6, 12, and 24 h. As shown in Fig. 3, treatment with 5 μM PEITC resulted in an increase in the mean fluorescent intensity of 131.67±3.43 (arbitrary unit, $P<0.01$) at 6 h, 168.92±6.82 (arbitrary unit, $P<0.0001$) at 12 h and 223.30±2.33 (arbitrary unit, $P<0.0001$) at 24 h. PEITC induces the formation of reactive oxygen species in a time-dependent pattern. Pretreatment with 100 μM *N*-acetyl-cysteine, 100 μM vitamin E and z-DEVD-fmk, followed by 5 μM PEITC treatment for 24 h showed a reduction in the fluorescent intensity to 85.67±0.66 (arbitary unit, $P<0.01$), 77.30±0.84 (arbitrary unit, $P<0.0001$) and 87.00±1.63 (arbitrary unit, $P<0.05$), respectively. The responses of cells treated with only 100 μM *N*-acetyl-cysteine, 100 μM vitamin E and z-DEVD-fmk cells for 24 h were 42.29±0.53 (arbitrary unit, $P<0.0001$), 13.19±0.64 (arbitrary unit, $P<0.0001$) and 0.23±1.25 (arbitrary unit, $P<0.05$), respectively. It is interesting to note that the inhibitory effect of vitamin E on the reactive oxygen species formation was superior to that of *N*-acetyl-cysteine and z-DEVD-fmk. These data indicated that reactive oxygen species generation was important for PEITC-induced apoptosis. Vitamin E exhibited the strongest reactive oxygen species scavenging activity in PEITC-treated cells.

Table 3
Effects of 100 μM antioxidants (*N*-acetyl-cysteine and vitamin E) and 50 μM caspase-3 inhibitor (z-DEVD-fmk) on the incidence of phenylethyl isothiocyanate (PEITC)-induced PLC/PRF/5 cell death

Treatment	% Cell death	
	Trypan blue	Annexin V
Control (0.1 % DMSO)	0.77±0.17	0.06±0.01
5 μM PEITC	69.33±0.94	61.90±3.25
N-acetyl-cysteine	4.00±0.20	4.53±0.40
N-acetyl-cysteine+5 μM PEITC	6.03±0.20	5.06±0.70
Vitamin E	4.70±0.50	2.23±0.33
Vitamin E+5 μM PEITC	5.50±0.10	4.78±0.90
z-DEVD-fmk	0.20±0.05	0.00±0.00
z-DEVD-fmk+5 μM PEITC	0.60±0.05	0.55±0.03

The binding of annexin V-FITC to the plasma membrane was analyzed by flow cytometry. The percentage of dead cells was calculated as the ratio between the number of trypan blue stained cells and the total cells. Values are mean±S.D. of three independent experiments.

3.5. PEITC induces cytochrome *c* and Smac/Diablo release in PLC/PRF/5 cells

To examine the release of cytochrome *c* and Smac/Diablo in 5 μM PEITC-treated cells at different time periods (0, 6, 12 and 24 h), Western blotting was used to analyze the mitochondrial and cytosolic fractions. Results showed that PEITC-mediated the release of cytochrome *c* and Smac/Diablo from mitochondria to the cytosol in the PEITC-treated groups (Fig. 4A). An increase in cytochrome *c* and Smac/Diablo within the cytosol was noted. The level of cytochrome *c* and Smac/Diablo was found to increase 12 h after PEITC treatment and reached the maximum at 24 h. Mitochondrial- and cytosolic-enriched fractions were assayed with antibodies to determine the presence of the mitochondrial protein (Cox IV). Cox IV protein was detected in the mitochondrial-enriched fraction, but not in the cytosolic-enriched fraction (data not shown). Together, the data demonstrated that PEITC-induced apoptosis is associated with the release of cytochrome *c* and Smac/Diablo from mitochondria to the cytosol.

3.6. PEITC treatment down-regulates the protein level of XIAP and up-regulates the levels of p53, Bid and Apaf-1 in PLC/PRF/5 cells

We evaluated the expression levels of p53, XIAP, Apaf-1 and Bid proteins in PLC/PRF/5 cells after exposure to 5 μM PEITC for 0, 6, 12 and 24 h. As shown in Fig. 4B, the result indicated that PEITC caused an increase in the p53 level. Our data also demonstrated that PEITC could induce cleavage of the pro-apoptotic protein (Bid) from 22 kDa to its truncated form t-Bid (15 kDa) after 24 h of PEITC treatment. Thus, it is quite likely that PEITC activated caspase-8, which led to the cleavage of Bid in a time-dependent manner (Figs. 1B and 4B). PLC/PRF/5 cells exposed to PEITC displayed an up-regulation of Apaf-1, and activation of caspase-3 and caspase-9, which consequently caused a down-regulation of XIAP protein (an inhibitor of apoptosis) in a time-dependent pattern (Figs. 1A, 1C and 4B). The expression of XIAP protein was found to decline gradually after 24 h of PEITC treatment (Fig. 4B).

3.7. Effects of CD95 and Bcl-2 family in PEITC-induced apoptosis

It is well documented that the CD95 (Apo-1/CD95) pathway is involved in apoptosis induced by certain anticancer drugs. However, in this study, the expression of CD95 (Apo-1/CD95) was not detected in PLC/PRF/5 cells (Fig. 4B).

PEITC-induced cell death was also examined by modulating the expression of Bcl-2 family members. After treating the PLC/PRF/5 cells with 0, 1 and 5 μM of PEITC for 24 h, results showed a marked down-regulation of Bcl-2, Bcl-$_{XL}$ and Mcl-1 expression and an up-regulation of the Bax protein in a dose-

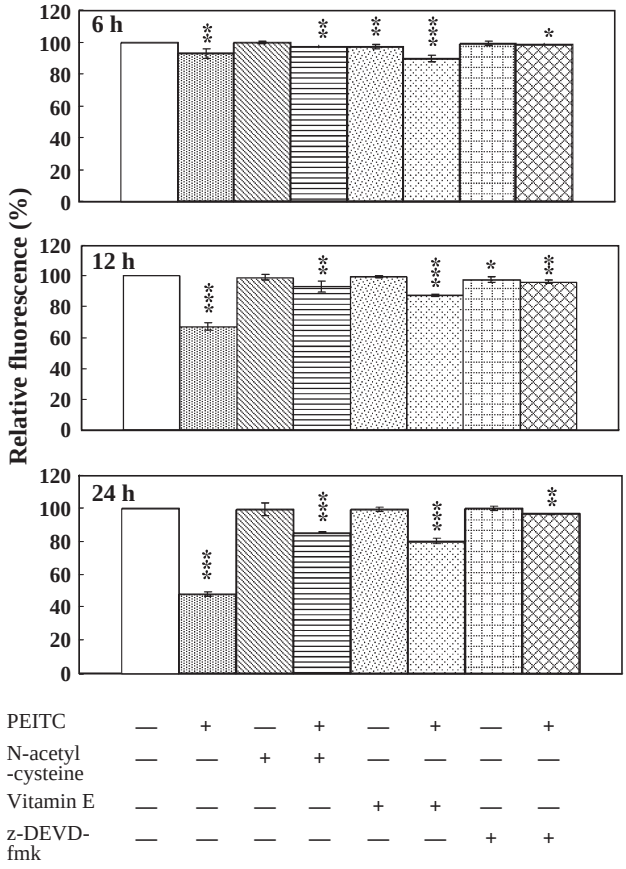

Fig. 2. Effects of PEITC on mitochondrial membrane potential (Δψm) loss in PLC/PRF/5 cells. To determine the effect of PEITC on mitochondria, cells were treated for 6, 12, and 24 h with 0.1% DMSO (control) or 5 μM PEITC following pretreatment for 1 h in the presence or absence of 100 μM *N*-acetyl-cysteine, 100 μM vitamin E and 50 μM caspase-3 inhibitor (z-DEVD-fmk), followed by staining with rhodamine 123. Change of Δψm was measured by flow cytometry. The results represent the relative fluorescence (%) of different treated groups. The asterisks (*$P<0.05$, **$P<0.01$ and ***$P<0.0001$) indicate significant differences between control and PEITC-treated cells as analyzed by Student's *t*-test.

dependent pattern (Fig. 5). These results suggest that the level of Bcl-2 family protein expression regulates PEITC-induced cell apoptosis.

Treatment with 5 μM PEITC for 0, 6, 12 and 24 h displayed an up-regulation of pro-apoptotic (Bax) protein in a time-dependent manner (data not shown). The expression of Bax protein in the 5 μM PEITC with or without *N*-acetyl-cysteine, vitamin E and z-DEVD-fmk treated cells at 6 h and 12 h appeared to be similar to the 24 h response (Fig. 6), where only the PEITC treated cells showed Bax protein expression. This suggests that treatment with 5 μM PEITC in the absence or presence of *N*-acetyl-cysteine, vitamin E and z-DEVD-fmk was not affected by time.

3.8. Effects of N-acetyl-cysteine, vitamin E and z-DEVD-fmk on caspase-3 activity, Bax and XIAP protein expression and release of cytochrome c and Smac/Diablo

To investigate the activity of caspase-3 in PEITC-induced apoptosis, *N*-acetyl-cysteine, vitamin E, and z-DEVD-fmk were used in the study. As observed in Fig. 6A, PEITC strongly stimulated caspase-3 activity (32 KDa), this activation was suppressed by pretreatment with 100 μM *N*-acetyl-cysteine or 100 μM vitamin E or 50 μM z-DEVD-fmk. Of note, PEITC-induced activation of caspase-3 was fully blocked in the presence of caspase-3 inhibitor (z-DEVD-fmk), but a lesser effect was noted in the antioxidant (*N*-acetyl-cysteine and vitamin E) pretreated cells. These data reveal that caspase-3 was activated in PEITC-induced apoptosis.

We determined whether the expression of Bax and XIAP proteins are affected by the presence of *N*-acetyl-cysteine, vitamin E or z-DEVD-fmk in PEITC-treated cells. PLC/PRF/5 cells treated with 100 μM *N*-acetyl-cysteine or 100 μM vitamin E or 50 μM z-DEVD-fmk resulted in a significant down-regulation of Bax protein and up-regulation of XIAP protein (Fig. 6A).

Pre-incubation with 100 μM *N*-acetyl-cysteine or 100 μM vitamin E or 50 μM z-DEVD-fmk inhibited the release of cytochrome *c* and Smac/Diablo from mitochondria to the cytosol in PEITC-treated cells (Fig. 6B). However, in treatments pre-incubated with *N*-acetyl-cysteine, vitamin E and z-DEVD-fmk for 1 h, followed by treating the cells with PEITC or 0.1% DMSO (control) for 24 h, cytochrome *c* and Smac/Diablo proteins were

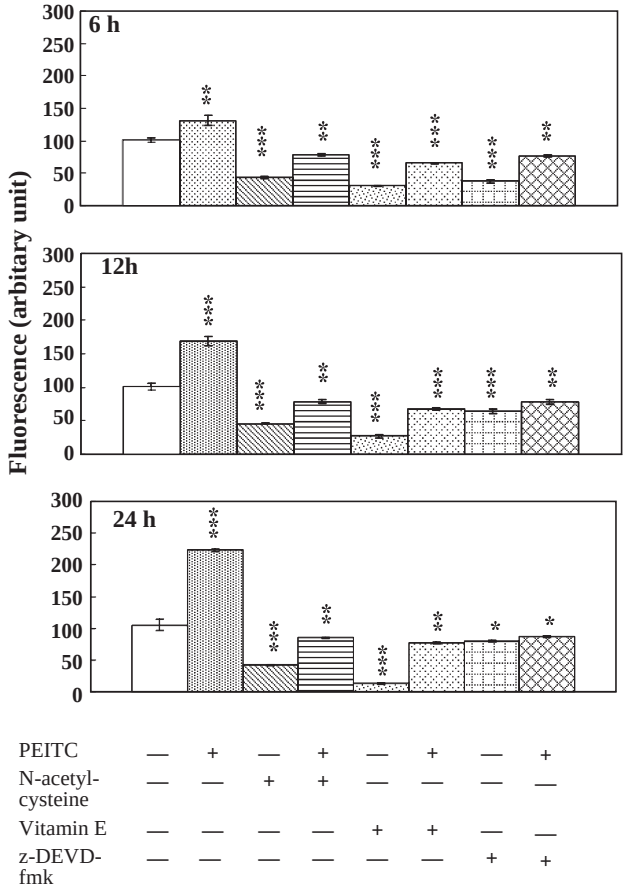

Fig. 3. Effects of PEITC on the reactive oxygen species generation in PLC/PRF/5 cells. To determine the intracellular content of peroxides, cells were treated for 6, 12, and 24 h with 0.1% DMSO (control) or 5 μM PEITC following pretreatment for 1 h in the presence or absence of 100 μM *N*-acetyl-cysteine, 100 μM vitamin E and 50 μM caspase-3 inhibitor (z-DEVD-fmk), followed by loading with H_2DCFDA. Change of reactive oxygen species generation was measured by flow cytometry. The results represent the mean fluorescence (arbitrary unit) of different treated groups. The asterisks (*$P<0.05$, **$P<0.01$ and ***$P<0.0001$) indicate significant differences between control and PEITC-treated cells as analyzed by Student's *t*-test.

found to remain in the mitochondrial-enriched fractions. Cox IV protein (as a positive control) was also detected in the mitochondrial-enriched fraction. These results suggest that PEITC-induced reactive oxygen species dependent apoptosis was associated with the release of cytochrome *c* and Smac/Diablo from the mitochondria, but the release of these death signal transduction proteins was suppressed by *N*-acetyl-cysteine, vitamin E and z-DEVD-fmk.

4. Discussion

Previous studies have revealed that PEITC is a potent inducer in the expression of enzymes implicated in the detoxification of a variety of chemical carcinogens (Bogaards et al., 1990; Conaway et al., 1996; Hecht et al., 2002; Hu et al., 2003; Xiao and Singh, 2002; Yang et al., 2002; Zheng et al., 1992). Triggering of apoptosis by cancer therapeutic agents involves simultaneous or subsequent activation of death receptor systems, perturbation of mitochondrial function and proteolytic processing of caspases. Thus, the cell death pathway may be entered at multiple sites, although the precise molecular mechanisms have not been characterized in detail for each particular drug and specific target cells. Here, we reported the PEITC-induced apoptosis in hepatitis B virus-hepatoma cells and its death signaling pathways. Our findings indicated that PEITC significantly inhibited cell proliferation and induced apoptosis in human PLC/PRF/5 cells. PEITC-induced PLC/PRF/5 cell apoptosis could be completely blocked by the caspase-3 inhibitor (z-DEVD-fmk) and antioxidants (*N*-acetyl-cysteine and vitamin E). The reactive oxygen species formation was noted to be an early apoptotic event in PEITC-treated cells, which was accompanied by a loss in mitochondrial transmembrane potential. *N*-acetyl-cysteine and vitamin E, antioxidants that act as reactive oxygen species scavengers, quenched PEITC-induced reactive oxygen species. The ability of *N*-acetyl-cysteine and vitamin E correlates with the prevention of mitochondrial dysfunc-

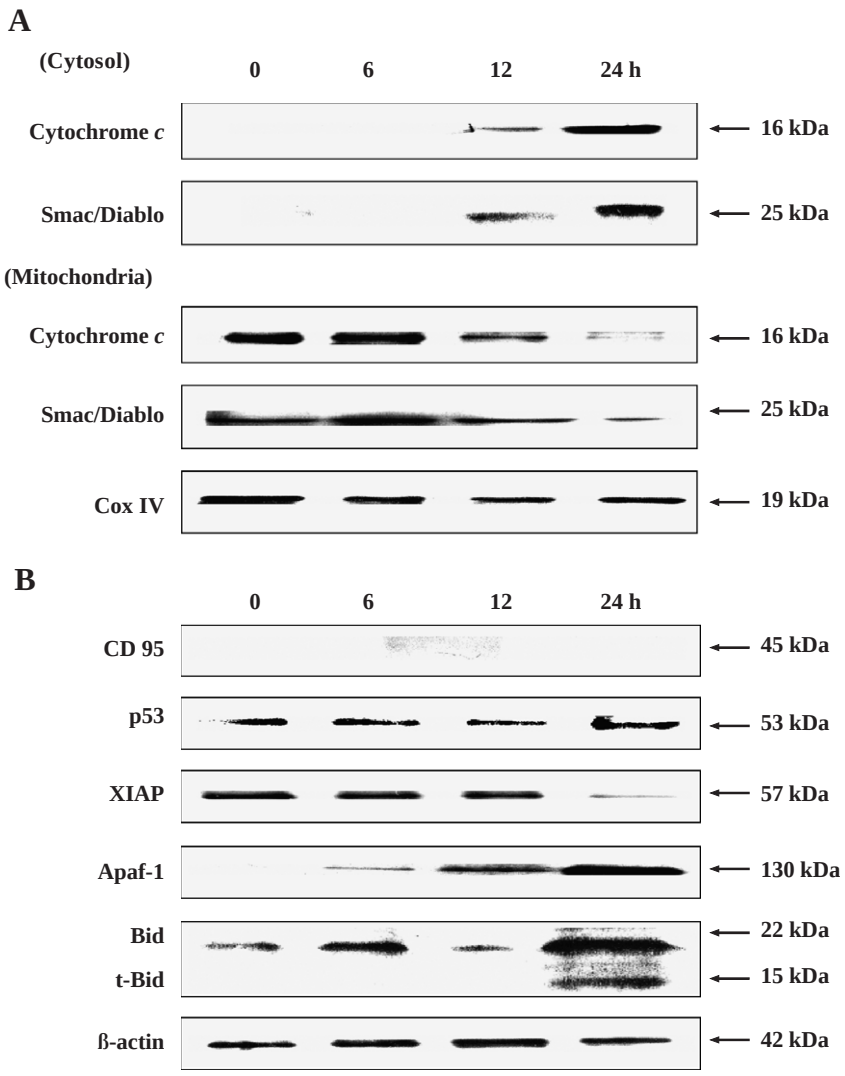

Fig. 4. Effects of PEITC on the release of apoptotic signal transduction factors (cytochrome *c* and Smac/Diablo), CD95, p53, XIAP, Apaf-1 and Bid in PLC/PRF/5 cells. (A) Effect of 5 μM PEITC on the expression of cytochrome *c* and Smac/Diablo proteins in cytosolic and mitochondrial fractions at 0, 6, 12, and 24 h. The cellular proteins were subjected to Western blot analysis with the specific antibody. (B) Effect of 5 μM PEITC on the expression of CD95, p53, XIAP, and Apaf-1 proteins, and Bid cleavage at 0, 6, 12, and 24 h. Mitochondrial protein Cox IV and cytosol β-actin were used as controls to verify the enrichment of mitochondrial and cytosolic fractions.

tions, and prohibits PEITC-induced apoptosis. Vitamin E was found to exert a stronger inhibitory effect than *N*-acetylcysteine and z-DEVD-fmk on the reactive oxygen species generation by PEITC. Hydrogen peroxide is an important member of reactive oxygen species and is generated predominantly by mitochondria (Gosslau and Rensing, 2002). Excessive production of hydrogen peroxide in mitochondria will damage lipid, proteins, and DNA, as well as leading to reactions that can cause cell death due to necrosis or apoptosis (Gosslau and Rensing, 2002; Sakura and Cederbaum, 1998).

Our results showed that treatment of PLC/PRF/5 cells with PEITC caused an up-regulation of p53 protein, a down-regulation of XIAP, and consequently caused the cleavage of Bid protein (22 kDa) to its truncated form (t-Bid; 15 kDa) and the release of cytochrome *c* and Smac/Diablo from mitochondria to the cytosol. Consistent with previous reports, CD95 (Apo-1/CD95) was undetectable in PLC/PRF/5 cells treated with chemotherapeutic drugs (Jiang et al., 1999; You et al., 2001). The attenuation of the levels and activities of IAP proteins sensitizes tumor cells to apoptosis as induced by anticancer agents (Chen et al., 2003; Sasaki et al., 2000). Higher levels of Apaf-1 were demonstrated to enhance apoptosis induced by chemotherapeutic agents such as etoposide and taxol through increasing the sequential cleavage and activities of caspase-9 and caspase-3 (Perkins et al., 1998, 2000). Our results indicated that overexpression of Apaf-1 could lead to an activation of caspase-9 and caspase-3, an increase in reactive oxygen species generation, a reduction in $\Delta\psi m$, and an accumulation of cytochrome *c* and Smac/Diablo in the cytosol during PEITC-induced apoptosis. Thus, it is possible that the

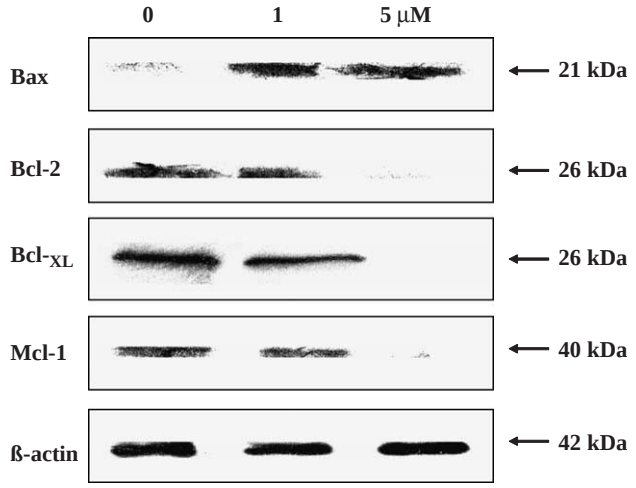

Fig. 5. Effects of PEITC on the release of Bcl-2 family proteins in PLC/PRF/5 cells. The expression of Bax, Bcl-2 and Bcl-$_{XL}$ and Mcl-1 proteins was measured at 24 h after treating the cells with 0~5 μM PEITC. β-actin was used as a positive control to verify the enrichment of the cytosolic fraction.

PEITC-induced cytochrome *c* release observed in PLC/PRF/5 cells was mediated through Bid cleavage and caspase-8 activation pathway.

PEITC-induced apoptosis also appeared to be through the up-regulation of the pro-apoptotic protein (Bax), and down-regulation of anti-apoptotic proteins (Bcl-2, Bcl-$_{XL}$ and Mcl-1). In the mitochondria, Bcl-2 and Bcl-$_{XL}$ promote cell survival by preserving the integrity of the external mitochondrial membrane, which prevents the release of cytochrome *c*, while Bax, Bid and Bak activity enhance the release of cytochrome *c* from mitochondria and hence induce cell death (Scorrano and Korsmeyer, 2003).

Certain chemotherapeutic agents such as arisostatins A and curcumin induced the release of cytochrome *c*, increased the production of reactive oxygen species, and activated caspase-3, but cell death could be prevented by preincubation with a general caspase inhibitor or *N*-acetyl-cysteine (Kim et al., 2003; Woo et al., 2003). In this study, *N*-acetyl-cysteine, vitamin E and z-DEVD-fmk

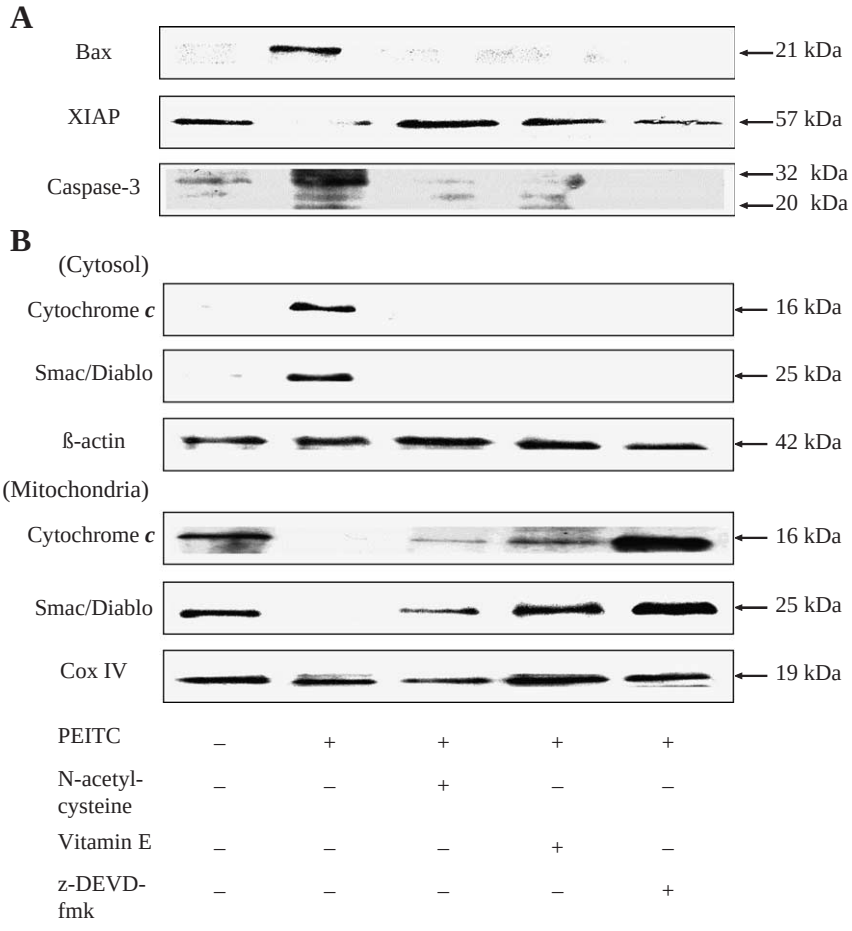

Fig. 6. Effects of PEITC in the presence or absence of *N*-acetyl-cysteine, vitamin E and caspase-3 inhibitor (z-DEVD-fmk) on apoptotic inhibitors, pro-apoptotic proteins, caspase-3 and death signal transduction factors (cytochrome *c* and Smac/Diablo). (A) After 24 h of treatment, cells were harvested and analyzed for Bax, XIAP proteins and caspase-3 activities. (B) The presence of cytochrome *c* and Smac/Diablo proteins in the cytosolic-and mitochondrial-enriched fractions. Mitochondrial protein Cox IV and cytosol β-actin were used as controls to verify the enrichment of mitochondrial and cytosolic fractions.

completely blocked the apoptotic signaling factors such as the release of cytochrome *c* and Smac/Diablo from mitochondria to the cytosol, which was found to remain in the mitochondrial-enriched fraction. Activation of caspase-3 activity, decreasing $\Delta\psi m$, stimulation of reactive oxygen species in the PEITC-induced apoptosis were all prevented by *N*-acetyl-cysteine, vitamin E and z-DEVD-fmk treatment. Thus, under our model system of study, the expression level of Bcl-2 family and XIAP proteins appeared to affect the response of PLC/PRF/5 cells to the PEITC treatment.

In summary, PEITC treatment significantly inhibited PLC/PRF/5 cell proliferation. PEITC-mediated apoptosis was found to associate with the activation of caspases -8, -9 and -3, cleavage of Bid, release of cytochrome *c* and Smac/Diablo, and increase in the level of Apaf-1. PEITC also up-regulated the p53 protein and pro-apoptotic protein (Bax), and down-regulated the anti-apoptotic protein (Bcl-2, Bcl-$_{XL}$ and Mcl-1). PEITC significantly enhanced apoptosis through the dropping of $\Delta\psi m$ and reactive oxygen species formation in a time-dependent pattern. *N*-acetyl-cysteine, vitamin E and z-DEVD-fmk markedly blocked PEITC-induced apoptosis and diminished caspase-3 activity through preventing the loss of $\Delta\psi m$, generation of reactive oxygen species, and release of cytochrome *c* and Smac/Diablo from mitochondria to the cytosol. They also affected the activity XIAP and Bax proteins. Taken together, antioxidants such as *N*-acetyl-cysteine and vitamin E are able to stabilize the intracellular redox status, reduce the caspase-3 activity, and inhibit the expression of apoptotic mitochondrial signal transduction factors, which consequently suppresses the hepatoma cell proliferation and induces cell apoptosis.

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