Mitochondria are the primary target in isothiocyanate-induced apoptosis in human bladder cancer cells

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Abstract

Many isothiocysanates (ITC) are promising cancer-preventive agents, and induction of apoptosis is one of their underlying mechanisms of action. We recently found that caspase-9 was preferentially activated over other initiator caspases in human bladder cancer UM-UC-3 cells. We report here that caspase-9 activation is the major step leading to ITC-induced apoptosis in this cell line. More importantly, our results show that caspase-9 activation by the ITCs may result primarily from mitochondrial damage. Four common naturally occurring ITCs were studied, including allyl ITC, benzyl ITC (BITC), phenethyl ITC (PEITC), and sulforaphane. BITC and PEITC showed more potent mitochondria-damaging ability than the other two ITCs, correlating well with their stronger apoptosis-inducing potentials. Furthermore, BITC and PEITC damaged both the outer and inner mitochondrial membranes. Use of isolated mitochondria allowed us to establish that ITCs, and more importantly their major intracellular derivatives (glutathione conjugates) at concentrations that are readily achievable in cells, damage mitochondria, leading to the collapse of mitochondrial *trans*-membrane potential and release of cytochrome *c*. The mitochondria-damaging potencies of the ITCs correlate well with their lipophilicities. Bcl-2 family members are known to influence the stability of mitochondrial membrane. Our results show that the ITCs caused phosphorylation of Bcl-2, induced mitochondrial translocation of Bak, and disrupted the association of Bcl-xl with both Bak and Bax in mitochondrial membrane, indicating that ITC-induced mitochondrial damage results at least in part from modulation of select Bcl-2 family members. [Mol Cancer Ther 2005;4(8):1250–9]

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Introduction

Many isothiocyanates (ITC), some of which are abundantly available from various cruciferous vegetables, are promising cancer-preventive agents (1–4). Among their cancer-preventive mechanisms that have been discovered to date is the induction of apoptosis. Indeed, many lines of evidence show that induction of apoptosis by ITCs is not cell specific (5–10). Moreover, there also is evidence showing that ITCs are significantly more toxic to cancer cells than to normal cells, although it is not fully understood to what extent induction of apoptosis is responsible for the differential cytotoxic effects (11–13). These compounds may be especially useful for the prevention of bladder cancer, because orally ingested ITCs are rapidly and almost exclusively disposed in the urine, and bladder cancers originate overwhelmingly from the epithelia that directly face the urine stored in the bladder (4, 14–20). Four common dietary ITCs, including allyl ITC (AITC), benzyl ITC (BITC), phenethyl ITC (PEITC), and sulforaphane, have been extensively studied for their cancer preventive activities. Our recent study in cultured human bladder cancer UM-UC-3 cells showed that BITC and PEITC were more potent than AITC and sulforaphane in inducing apoptosis and activating caspases, including caspase-3, caspase-8, and caspase-9 (21). Interestingly, both BITC and PEITC were markedly more effective in activating caspase-9 than caspase-8, suggesting that caspase-9-related apoptotic pathway might play a more important role in the induction of apoptosis by the ITCs in these cells. It is noteworthy, however, that the role of a specific caspase in ITC-induced apoptosis may be cell specific. For example, Xu and Thornalley reported that caspase-8 activation played the major role and caspase-3 activation played a minor role in PEITC-induced apoptosis in HL60 cells (7), whereas caspase-3 activation was critical in PEITC-induced apoptosis in human cervical cancer HeLa cells (22).

Activation of caspase-9 requires the release of cytochrome *c* from the mitochondrial intermembrane space, which often is a sign of compromised mitochondrial membrane integrity. Indeed, our previous study in human leukemia HL60 cells (10), as well as those of Nakamura et al. in rat liver RL34 cells (23), revealed that AITC and/or BITC could rapidly induce the loss of mitochondrial *trans*-membrane potential ($\Delta\Psi_m$). Nakamura et al. also reported that BITC induced cytochrome *c* release from isolated rat liver mitochondria (23). However, it is highly unlikely that the BITC concentration (200 μmol/L) used by these investigators can be achieved in cells, because ITCs, including BITC, are known to accumulate in cells mainly as dithiocarbamates, through conjugation with cellular thiols (glutathione in particular;

refs. 24, 25). Consequently, ITC-derived dithiocarbamates should be examined for their effect on mitochondria. It was also unclear as to what extent ITC-induced mitochondrial damage in human bladder cancer cells relies on the modulation of mitochondria sensitizing or desensitizing Bcl-2 family proteins, some of which are known to be modulated by ITCs (see Discussion).

The studies reported herein document the critical importance of caspase-9 activation in ITC-induced apoptosis in UM-UC-3 cells. Evidence is provided to show that mitochondria are damaged by ITCs and more importantly by their intracellular metabolites at concentrations that can be readily achieved in cells and that ITC-induced mitochondrial damage leads to cytochrome *c* release, caspase-9 activation, and apoptosis. The role of Bcl-2 family proteins, including Bcl-2, Bcl-xl, Bad, Bak, and Bax, as well as the mitochondrial permeability transition pore, in mediating ITC-induced mitochondrial damage was also examined. Our results suggest that the action of ITCs on mitochondria in UM-UC-3 cells involves Bcl-2 family proteins.

Materials and Methods

Materials

AITC, BITC, PEITC, and sulforaphane were purchased from LKT Laboratories (St. Paul, MN). Cyclosporin A and bongkrekic acid were purchased from A.G. Scientific (San Diego, CA) and EMD Biosciences (San Diego, CA), respectively. Caspase-9 inhibitor Z-LEHD-FMK was obtained from R&D Systems (Minneapolis, MN) and rhodamine 123 was purchased from Molecular Probes (Eugene, OR). All other chemicals were purchased from Sigma (St. Louis, MO).

Cell Culture

Human bladder cancer UM-UC-3 cells and their culture have been previously described (21). Normally, 1.5×10^6 cells were grown in a 10-cm dish with 10 mL McCoy's 5A medium containing L-glutamine and 10% fetal bovine serum for 48 hours and treated with an ITC at desired concentrations for 3 or 24 hours. Each ITC was dissolved in acetonitrile, the final concentration of which in the medium was 0.1% (v/v).

Western Blotting

Cells were treated with an ITC at 7.5, 15, and 30 μmol/L for 3 or 24 hours. The cells were then trypsinized, harvested by centrifugation ($500 \times g$ for 5 minutes at 4°C), and processed for preparation of whole cell lysates and cytosolic and mitochondrial fractions using published protocols with modifications (26). Briefly, for preparation of whole cell lysates, each cell pellet was washed twice with 10 mL ice-cold PBS, suspended in 200 μL of cell lysis buffer (Cell Signaling Technology, Beverly, MA) supplemented with 1 mmol/L phenylmethylsulfonyl fluoride, and sonicated by a Branson Model 450 sonicator. The lysates were centrifuged at $10,000 \times g$ for 5 minutes at 4°C, and the supernatant was used for analysis. For the preparation of mitochondrial and cytosolic fractions, cells were harvested and washed as above. The cell pellets were suspended in buffer A (pH 7.5) containing 20 mmol/L HEPES-KOH, 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, and a mixture of proteinase inhibitors (1 tablet of the complete mini inhibitor cocktail from Roche, (Indianapolis, IN) was added to 10 mL solution), which were incubated on ice for 15 minutes. After 15 strokes in a Kontes Dounce homogenizer, the homogenates were mixed with ~400 μL of 2.5 × buffer B (pH 7.5) containing 10 mmol/L HEPES-KOH, 210 mmol/L mannitol, 70 mmol/L sucrose, 0.1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, and the abovementioned proteinase inhibitor cocktail. After centrifugation at $1,000 \times g$ for 10 minutes at 4°C, the supernatant fraction was transferred to another tube and centrifuged at $17,000 \times g$ for 30 minutes at 4°C. The resulting supernatant fraction was collected as the cytosolic fraction, whereas the pellet containing the mitochondria was resuspended in buffer B. To prepare the medium fraction, cells were treated with an ITC as described above; the medium was then collected and centrifuged at $500 \times g$ for 5 minutes at 4°C; the supernatant was supplemented with the proteinase inhibitor cocktail and was concentrated using a centrifugal filtration device (Amicon, Bedford, MA). Protein content of each sample was measured using the bicinchoninic acid assay kit from Pierce (Rockford, IL). Each sample (40–50 g proteins) was resolved by SDS-PAGE (8–15%) and blotted to polyvinylidene difluoride membranes, which were probed by specific antibodies and visualized using an enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ). All antibodies were purchased from Cell Signaling Technology except anti-malate dehydrogenase (MDH) and anti-glyceraldehyde-3-phosphate dehydrogenase, which were obtained from Rockland (Gilbertsville, PA) and Chemicon (Temecula, CA), respectively.

Immunoprecipitation

Mitochondria were prepared as described above and used within 3 hours. The freshly isolated mitochondria were suspended in PBS at 1 mg protein equivalent/mL and incubated for 30 minutes at room temperature with 50 μmol/L BITC followed by centrifugation of the mixture at $17,000 \times g$ for 15 minutes at 4°C. The resultant pellet was suspended in 200 μL of cell lysis buffer (Cell Signaling Technology) supplemented with 1 mmol/L phenylmethylsulfonyl fluoride and the protease inhibitor cocktail described above and sonicated. Lysates were incubated overnight at 4°C with an anti-Bcl-xl antibody (Cell signaling Technology), which was then mixed with 20 μL of protein A-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) and incubated for 3 hours at 4°C. The immunoprecipitated complexes were washed five times with lysis buffer and subjected to SDS-PAGE followed by immunoblotting using anti-Bcl-xl, anti-Bak, and anti-Bax antibodies (Cell Signaling Technology).

Measurement of Integrity of Cellular and Mitochondrial Membranes

At the end of an ITC treatment, the cells were harvested and washed with buffer as described above. Each cell pellet was resuspended in fresh medium at 5×10^5 cells/mL.

Fluorescent propidium iodide (PI), a DNA-binding reagent, cannot penetrate a normal cell membrane but may stain cells with membrane damage. For the PI staining experiment, 1 mL cell suspension was mixed with 10 μg PI. After 5 minutes of incubation, each sample was immediately measured by flow cytometry for PI-stained cells. Rhodamine 123 was used to determine if $\Delta\Psi_m$ was lost. Rhodamine 123 is readily and selectively sequestered by normal mitochondria (cells showing strong fluorescence) but is removed from the latter when $\Delta\Psi_m$ is lost (cells showing greatly diminished fluorescence). Each above-described cell suspension (1 mL) was incubated with 10 μg rhodamine 123 for 30 minutes at 37°C; the cells were then washed twice with fresh medium, resuspended in an equal volume of fresh medium, and promptly measured by flow cytometry to determine fluorescence intensity of the rhodamine-treated cells. Ten thousand cells were examined in each sample, using a Becton Dickinson FACScan (BD Biosciences, San Jose, CA).

Measurement of $\Delta\Psi_m$ and Cytochrome *c* Release from Isolated Mitochondria

Measurement of $\Delta\Psi_m$ in isolated mitochondria was based on a previous report that the fluorescence of a rhodamine 123 solution is quenched upon addition of normal mitochondria that sequester the dye but recovers as the dye is leaked back out of the mitochondria when their $\Delta\Psi_m$ is lost (27). The mitochondria were prepared as described above, washed and resuspended in ice cold buffer B, and used within 3 hours. The protein concentration was measured using the bicinchoninic acid assay kit. To check $\Delta\Psi_m$, isolated mitochondria (0.3 mg protein equivalent/mL) were suspended in buffer C (pH 7.5) containing 150 mmol/L sucrose, 5 mmol/L $MgCl_2$, 5 mmol/L disodium succinate, 2.5 μmol/L rotenone, 5 mmol/L potassium phosphate, and 20 mmol/L HEPES-KOH (27) followed by incubation with 5 μmol/L rhodamine 123 for 5 minutes. A fluorescence reading of the solution was then promptly taken, and an ITC at a desired concentration was added to the solution. The fluorescence reading of the solution was taken again at 5, 10, 15, 20, 25, and 30 minutes after ITC addition. The experiment was carried out at room temperature, and the fluorescence of rhodamine 123 was measured at 538 nm (excitation, 485 nm). When cyclosporin A or bongkrekic acid was included in the experiment, mitochondria were pretreated with each compound for 10 minutes before ITC addition.

To measure cytochrome *c* release from mitochondria, freshly isolated mitochondria were suspended in PBS at 1 mg protein equivalent/mL and incubated for 30 minutes at room temperature with an ITC or a mixture of an ITC plus a 5-fold higher concentration of reduced glutathione (GSH). In the latter case, an ITC was mixed with GSH in PBS (pH 7.5) for 30 minutes at room temperature before addition of mitochondria. Based on spectroscopic changes previously described (28), all ITCs in the solution were found to be converted to the corresponding GSH conjugates (GS-ITC) before mitochondria treatment (results not shown). Thus, the mitochondria were exposed in effect to a GS-ITC at 0.5 mmol/L in the presence of 2 mmol/L GSH, mimicking the intracellular environment, where GSH concentration is in the millimolar range and ITCs are accumulated predominantly as GS-ITCs (24, 25). At the end of mitochondria treatment, each suspension was centrifuged at 17,000 × *g* for 15 minutes at 4°C, and the supernatant was examined for presence of cytochrome *c* by Western blot analysis.

Determination of Intracellular ITC Accumulation

The procedures involving cell exposure to an ITC, cell harvest, preparation of cell lysates, and measurement of total ITC content (free ITC plus its thiol conjugate) by the cyclocondensation assay were described previously (29). Intracellular fluid volume was determined using ^{14}C-inulin and ^3H_2O (Moravek Biochemicals, Brea, CA) following a previously described procedure (29).

Statistics

Results are expressed as means ± SD (at least three determinations). Data were analyzed by one-way ANOVA followed by Dunnett's *t* test for separate comparisons with the control group. Differences were considered significant at $P < 0.05$.

Results

ITC-Induced Apoptosis in UM-UC-3 Cells Was Mainly Mediated by Caspase-9

When UM-UC-3 cells were treated with either BITC or PEITC, caspase-9 was activated as early as 3 hours after the treatment started, although the activation was stronger at 24 hours (Fig. 1A). Caspase activation by BITC and PEITC was more significant at 15 than 7.5 μmol/L, but increasing the ITC concentration to 30 μmol/L did not lead to further caspase-9 activation, or in some cases even caused a decrease, which is in agreement with our previous results (21). Interestingly, as shown in Fig. 2C and 2D and described later, ITC treatment apparently causes the damage of cell surface membrane and the leakage of intracellular components into the extracellular space. Hence, it is likely that a majority of the cleaved caspases, especially cleaved caspase-3 (its molecular weight is relatively low: 17/19 kDa), leaked into the culture medium from cells treated with the highest ITC concentration. The activation of caspase-3 and the cleavage of poly(ADP-ribose) polymerase (PARP) follow a pattern similar to caspase-9 activation (Fig. 1A), in line with the fact that both caspase-3 and PARP are downstream of caspase-9. However, BITC- or PEITC-induced cleavage of PARP was substantially reduced in the presence of Z-LEHD-FMK (100 μmol/L), a caspase-9 specific inhibitor (Fig. 1B). Because our previous study showed that activation of caspases and cleavage of PARP closely correlate with the occurrence of apoptotic cells (terminal deoxynucleotidyl transferase–mediated nick end labeling–positive cells) in BITC- and PEITC-treated UM-UC-3 cells (21), the above results provide a clear indication that caspase-9 activation is a major step in mediating the induction of apoptosis in these cells by BITC and PEITC.

Mitochondria Were Damaged by ITCs

Because caspase-9 activation is caused by the release of cytochrome *c* to the cytoplasm from mitochondria, we next

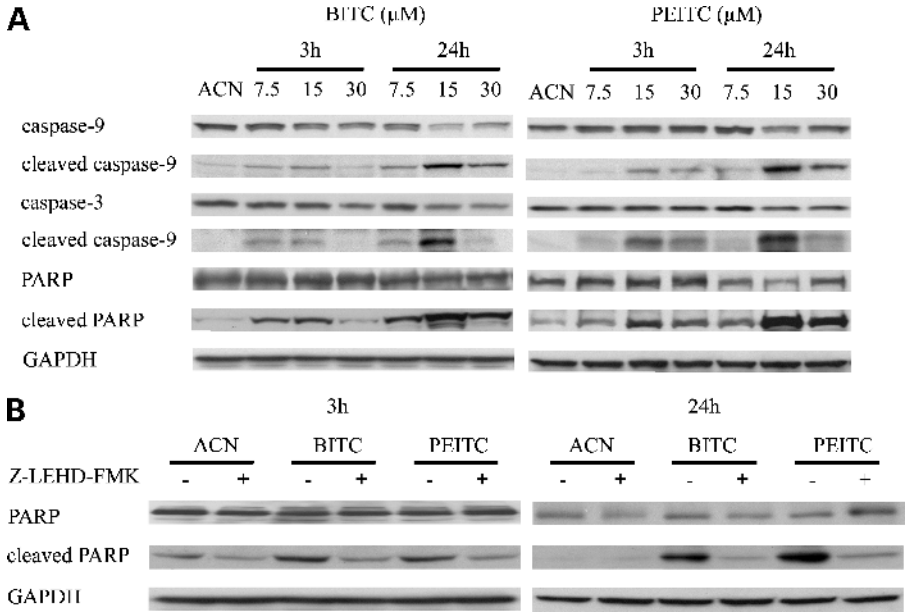

Figure 1. Effect of BITC and PEITC on caspase activation and PARP cleavage in UM-UC-3 cells. **A,** activation of caspases and cleavage of PARP. Cells were treated with BITC or PEITC at the specified concentrations for 3 or 24 h before they were harvested for analysis. ITCs were dissolved in acetonitrile (*ACN*). **B,** effects of Z-LEHD-FMK, a caspase-9-specific inhibitor, on ITC-induced PARP cleavage. Cells were pretreated with (+) or without (−) 100 μmol/L Z-LEHD-FMK for 30 min and were exposed to 15 μmol/L ITC or acetonitrile for 3 or 24 h in the presence of Z-LEHD-FMK. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used to confirm the equal loading of samples.

examined the effect of ITCs on mitochondria. When UM-UC-3 cells were treated with BITC or PEITC at 7.5, 15, or 30 μmol/L, both compounds rapidly caused a loss of $\Delta\Psi_m$ in a dose-dependent manner (Fig. 2A). At 24 hours of treatment, 30 μmol/L BITC and PEITC caused 60% and 70% of the cells to lose their $\Delta\Psi_m$, respectively. Even at 3 hours of treatment, BITC and PEITC at 30 μmol/L caused 54% and 35% of the cells to lose their $\Delta\Psi_m$, respectively, indicating that mitochondrial damage occurred rapidly. In fact, mitochondrial damage in these cells could be observed after an ITC treatment for only 30 minutes (results not shown). In contrast, mitochondrial damage caused by AITC and sulforaphane at 7.5 to 30 μmol/L was rather limited (Fig. 2A), which correlates with the negligible activation of caspase-3 and casapse-9 and cleavage of PARP in these cells (results not shown). However, the weak effects of AITC and sulforaphane were dose related because incubation of cells with each ITC at 100 μmol/L for 24 hours resulted in 43% and 94% of the cells to lose their $\Delta\Psi_m$, respectively. Clearly, the mitochondria-damaging potency varies with ITCs, and there is a correlation between loss of $\Delta\Psi_m$ and apoptosis caused by the ITCs.

We also examined whether ITC treatment would lead to cytochrome *c* release. It has been suggested that the collapse of $\Delta\Psi_m$ may not necessarily lead to cytochrome *c* release and apoptosis (30). However, both BITC and PEITC at 7.5 to 30 μmol/L caused the release of cytochrome *c* into the cytoplasm of UM-UC-3 cells in a manner that corresponds to the activation of caspases-9 and caspase-3 (Fig. 2B and Fig. 1A). More cytochrome *c* was detected in the cytoplasm when cells were treated with each ITC for 24 than for 3 hours. In addition, both ITCs at 15 μmol/L showed the strongest effect, which correlated well with the results of activation of the caspases. In line with the weak effect of both AITC and sulforaphane on $\Delta\Psi_m$, little cytochrome *c* was detected in the cytoplasm of cells treated with either ITC at concentrations up to 30 μmol/L. Interestingly, MDH (35 kDa) was also detected in the cytoplasm of cells treated with either BITC or PEITC (Fig. 2B). Cytochrome *c* is known to exist in the mitochondrial intermembrane space, whereas MDH is normally found in the mitochondrial matrix. Although MDH is not known to be involved in the induction of apoptosis, detection of both MDH and cytochrome *c* in the cytoplasm indicates that BITC and PEITC damaged both the outer and inner mitochondrial membranes.

Cytochrome *c* level in the cytoplasm dropped when the concentration of BITC or PEITC was increased from 15 to 30 μmol/L (Fig. 2B). We questioned whether this drop was a reflection of damage to the cell surface membrane, which may result in leaking cytochrome *c* to the extracellular medium. Indeed, cytochrome *c* was found in the medium after cell treatment with BITC or PEITC (Fig. 2C), and the amount detected in the culture media increased as the ITC concentration was increased. To further confirm cell membrane damage, we used PI, a fluorescent DNA-binding reagent, which cannot penetrate an intact cell membrane. UM-UC-3 cells treated with BITC or PEITC showed a dose-dependent increase in the number of cells stained by PI (Fig. 2D). The number of cells stained with PI increased 6- and 4-fold, respectively, after treatment with either BITC or PEITC at 30 μmol/L for 24 hours. Moreover, ITC-induced cell membrane damage occurred rapidly, for the number of cells stained with PI was similar between 3 and 24 hours.

Modulation of Bcl-2 Family Members by ITCs

Because mitochondrial membrane integrity is known to be influenced by Bcl-2 family proteins, we wondered if ITCs modulated these proteins in UM-UC-3 cells. Two groups of Bcl-2 family members were examined: the antiapoptotic (mitochondrial membrane stabilizing) proteins, including Bcl-2 and Bcl-xl, which are normally

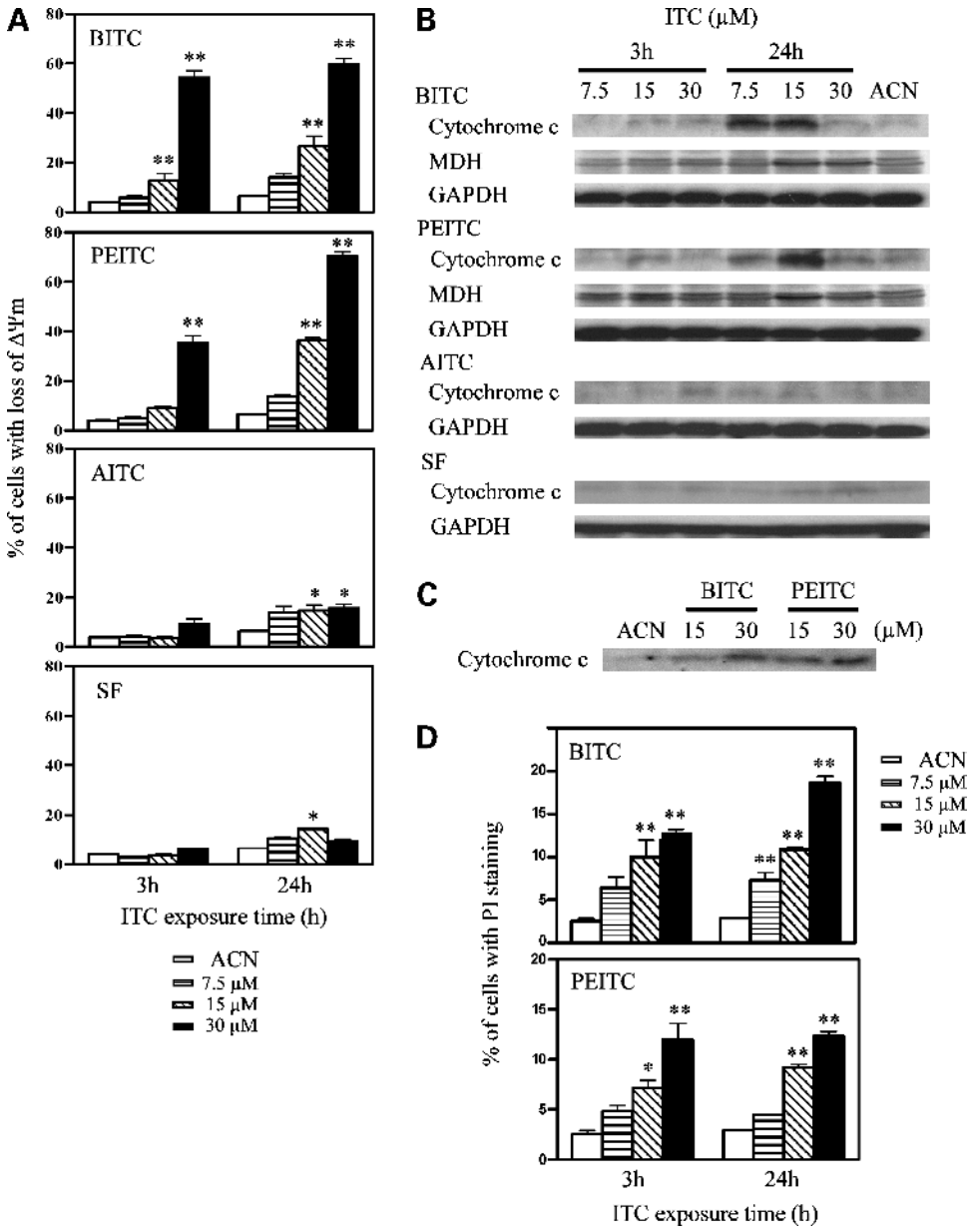

Figure 2. Effect of ITCs on the integrity of cellular and mitochondrial membranes in UM-UC-3 cells. Cells were exposed to each ITC at 7.5, 15, 30 μmol/L, or to acetonitrile (*ACN*) for 3 or 24 h before analysis. **A,** loss of $\Delta\Psi_m$, as determined by flow cytometry. *Columns,* means of three independent experiments; *bars,* SD. *, $P < 0.05$, versus control; **, $P < 0.01$, versus control. **B,** release of cytochrome c and MDH from mitochondria. The cytosolic fraction that was separated from ITC-treated cells was subjected to Western blot analysis for detection of cytochrome *c* and MDH. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the loading control. **C,** detection of cytochrome *c* in culture medium. Cells were treated with BITC or PEITC at 0 (acetonitrile), 15, or 30 μmol/L for 24 h. At the end of the treatment, medium was collected and concentrated for detection of cytochrome *c* by Western blot. **D,** damage of cell surface membrane. Cells treated with or without ITC were incubated with PI (10 μg/mL, 5 min) and flow cytometry was used to detect the cells with PI staining. *Columns,* means of three independent experiments; *bars,* SD. *, $P < 0.05$, versus control; **, $P < 0.01$, versus control.

located on the mitochondrial membrane and the proapoptotic (mitochondrial membrane destabilizing) proteins, including Bad, Bak, and Bax, which translocate to the mitochondria in response to apoptotic stimuli. When UM-UC-3 cells were treated with BITC or PEITC at 7.5, 15, or 30 μmol/L for 3 and 24 hours, the levels of Bcl-2, Bcl-xl, Bax, and Bak in whole cell lysates, as well as the levels of Bax and Bcl-2 in the mitochondria, did not change (Fig. 3A and B). Although total Bad levels seemed to increase in response to ITC treatment, its level in the mitochondria did not change, suggesting that Bad is unlikely to mediate ITC-induced mitochondrial damage. Phosphorylation of Bcl-2 is known to inactivate Bcl-2. However, increased phospho-Bcl-2 was detected only when cells were treated with each ITC at 7.5 μmol/L for 24 hours (Fig. 3A) but not at high ITC concentrations. Interestingly, whereas the total level of Bak in the cell did not change after ITC treatment, its level in the mitochondria was elevated by both ITCs, especially when the cells were treated by the compounds for 24 hours, indicating increased translocation of this protein to mitochondria, although it is unclear from where it is transferred. However, increase in mitochondrial Bak levels seem to reach a plateau at 15 μmol/L ITC and decline at a higher ITC concentration (Fig. 3B).

Because the mitochondria-destabilizing function of Bak and Bax are normally kept in check by association with Bcl-2 and Bcl-xl, we next asked if ITCs affected the association. Our immunoprecipitation experiment showed that treatment of isolated mitochondria with 50 μmol/L BITC (other ITCs not tested) for 30 minutes disrupted the association

of Bcl-xl with both Bak and Bax (Fig. 3C), although similar BITC treatment did not seem to affect the interaction of Bcl-2 with Bak and Bax (results not shown). The exact reason for BITC-caused disassociation of the proteins is unknown but it reveals for the first time a novel mechanism by which Bcl-2 family proteins are modulated by ITC.

ITCs Damaged Isolated Mitochondria

We next asked if ITCs would induce similar changes in isolated mitochondria. When freshly isolated mitochondria from UM-UC-3 cells were added to a rhodamine 123 solution, the fluorescence of rhodamine 123 dropped significantly due to its uptake and sequestration by the mitochondria (Fig. 4A). When the rhodamine 123–loaded mitochondria were treated with BITC and PEITC at 15, 50, or 100 μmol/L, there was a dose- and time-dependent collapse of $\Delta\Psi_m$ as shown by fluorescence increase in the solution (due to rhodamine 123 leaking out of the mitochondria; Fig. 4B). In contrast, AITC and sulforaphane seemed much weaker in causing $\Delta\Psi_m$ to collapse, which again is correlated with their weak stimulation of apoptosis in UM-UC-3 cells (21). Moreover, the different potencies of each of the four ITCs in collapsing $\Delta\Psi_m$ of isolated mitochondria is also closely correlated with their effects on $\Delta\Psi_m$ in intact cells (Fig. 2A). The opening of mitochondrial permeability transition pore is believed to result in the collapse of $\Delta\Psi_m$ in response to various stimuli (31). Two types of mitochondrial permeability transition pore inhibitors, cyclosporin A (a ligand of cyclophilin D) and bongkrekic acid (a ligand of adenine nucleotide translocase) were used to explore whether the ITCs affected $\Delta\Psi_m$ by modulating the mitochondrial permeability transition pore. Both compounds were previously shown to inhibit mitochondrial permeability transition pore opening (32–34). When mitochondria were preincubated with the inhibitor for 10 minutes and then exposed to each ITC in the presence of the inhibitor, cyclosporin A at 10 μmol/L and bongkrekic acid at 50 μmol/L had no effect on BITC-induced loss of $\Delta\Psi_m$ (Fig. 4C). Addition of bongkrekic acid at 50 μmol/L seemed to enhance PEITC-induced loss of $\Delta\Psi_m$, presumably due to drug toxicity. Although addition of cyclosporin A at 10 μmol/L seems to slightly protect the mitochondria against PEITC-induced damage, as reflected by a slight drop of fluorescence (Fig. 4C), this may not be a true protection, because the decrease was not statistically significant. In fact, when UM-UC-3 cells were pretreated with cyclosporin A at concentrations ranging from 2.5 to 50 μmol/L, neither the loss of $\Delta\Psi_m$ nor PARP cleavage, which were induced by BITC and PEITC, was attenuated (results not shown). Because the concentrations of cyclosporin A and bongkrekic acid

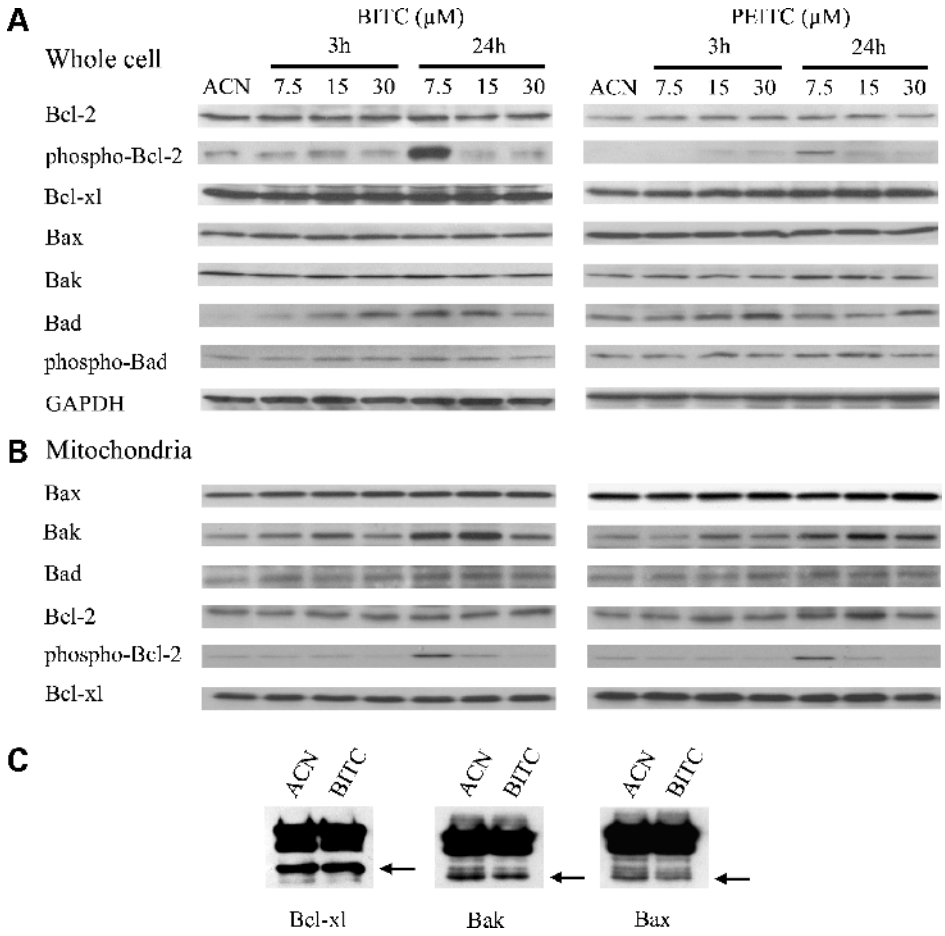

Figure 3. **A** and **B**, Western blot analysis of selected Bcl-2 family members in UM-UC-3 cells treated by BITC and PEITC. Cells were exposed to an ITC at 0 (acetonitrile, *ACN*), 7.5, 15, or 30 μmol/L for 3 or 24 h. Whole cell lysates and the mitochondrial fraction were analyzed. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the loading control. **C**, mitochondria were isolated from UM-UC-3 cells and treated with 50 μmol/L BITC for 30 min. The lysates were immunoprecipitated with anti-Bcl-xl antibody, and the immunoprecipitates were immunoblotted with anti-Bcl-xl, anti-Bak, and anti-Bax antibodies. *Arrow,* specific bands recognized by each antibody, and the top nonspecific IgG bands indicate an equal sample loading.

used in the above experiments are apparently sufficient to inhibit cyclophilin D or adenine nucleotide translocase (35–39), our findings suggest that it is unlikely that these two mitochondrial permeability transition pore components are the targets of ITCs.

Exposure of mitochondria isolated from UM-UC-3 cells to BITC or PEITC (50 μmol/L, 30 minutes) also induced the release of cytochrome *c* (Fig. 4D). However, does this type of experiment really address what happens in cells? It is now well established that ITCs, including all four ITCs used in the current study, are accumulated 100- to 200-fold in cells within 1 to 3 hours (24, 25). Indeed, as shown in Fig. 5B, both BITC and PEITC were dramatically accumulated in UM-UC-3 cells. For example, total intracellular BITC and PEITC accumulation levels were 1.04 and 0.66 mmol/L, respectively, when cells were exposed to each ITC at 7.5 μmol/L for only 1 hour (Fig. 5B). Previous studies have shown that ITCs are accumulated in cells predominantly as GS-ITC through conjugation with GSH and the concentrations of free ITCs are relatively low in cells (24). It was therefore important to examine whether GS-ITCs at intracellularly achievable concentrations could cause cytochrome *c* release from mitochondria. Thus, each ITC at 0.5 mmol/L was preincubated with 2.5 mmol/L GSH at pH 7.5 for 30 minutes, then mitochondria were added and incubated for another 30 minutes. Based on spectroscopic changes of these solutions as mentioned before, it was found that all ITC molecules were converted to the corresponding GS-ITCs before mitochondria were added (results not shown); thus, the mitochondria were actually treated with 0.5 mmol/L GS-ITC in the presence of 2 mmol/L GSH, which mimics the intracellular environment. As shown in Fig. 4E, cytochrome *c* was released from the mitochondria treated with GS-BITC or GS-PEITC, whereas GS-AITC and GS-SF under similar

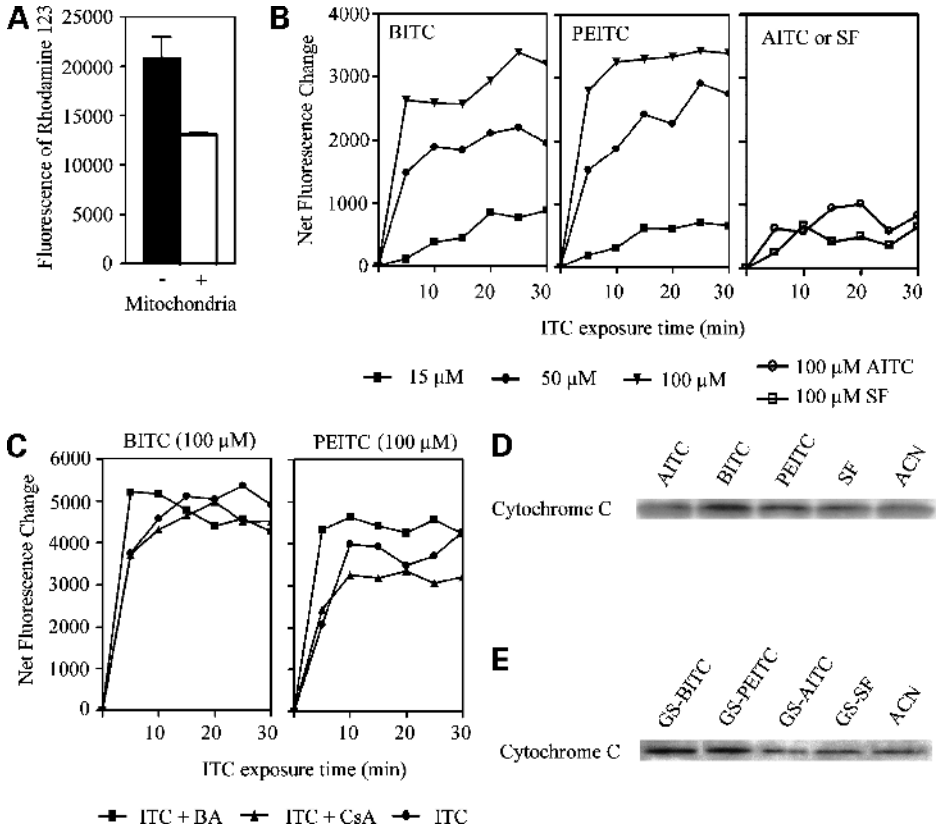

Figure 4. The effect of ITCs on isolated mitochondria. Mitochondria were freshly prepared from UM-UC-3 cells. **A,** quenching of rhodamine 123 fluorescence by mitochondria. The fluorescence of the incubation buffer containing 5 μmol/L rhodamine 123 was measured before (*dark column*) and after addition of mitochondria (0.3 mg protein equivalent/mL, *open column*). **B,** ITC-induced loss of $\Delta\Psi_m$ in isolated mitochondria. The rhodamine 123 – loaded mitochondria were then incubated with an ITC at the specified concentrations for 30 min. The fluorescence of rhodamine 123 in the solution was measured every 5 min. The increase of fluorescence intensity in the solution indicates the loss of $\Delta\Psi_m$. Each value is a mean of three measurements (SD ≤8% of the mean), and the presented values are adjusted by subtraction from control values. **C,** effect of mitochondrial permeability transition pore inhibitors on ITC-induced loss of $\Delta\Psi_m$. Mitochondria were pretreated with or without 50 μmol/L bongkrekic acid (*BA*) or 10 μmol/L cyclosporin A (*CsA*) for 10 min followed by exposure to 100 μmol/L BITC or PEITC in the presence of the inhibitor for 30 min. The fluorescence intensity of rhodamine 123 in the solutions was measured every 5 min. Each value is a mean of three measurements (SD ≤4% of the mean). Again, the presented values are adjusted by subtraction from control values. **D,** release of cytochrome *c* from isolated mitochondria after ITC treatment. Mitochondria were exposed to an ITC at 50 μmol/L for 30 min. The incubation buffer was then separated from the mitochondria and subjected to Western blot analysis. **E,** effect of GS-ITCs in the presence of excess amount of GSH on the release of cytochrome *c* from isolated mitochondria. Mitochondria were incubated with 0.5 mmol/L of an GS-ITC in the presence of 2 mmol/L GSH for 30 min. Each GS-ITC conjugate was prepared *in situ* just before the addition of mitochondria. The incubation buffer was then separated from the mitochondria by centrifugation and analyzed by Western blot for detection of cytochrome *c*.

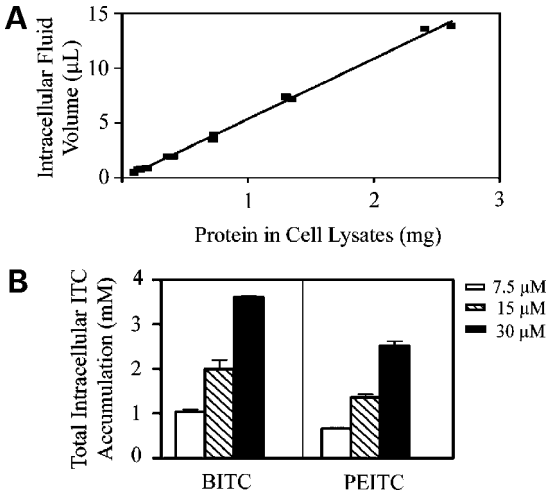

Figure 5. Total intracellular accumulation of ITCs in UM-UC-3 cells. **A,** standard curve relating the protein content in cell lysates to the intracellular fluid volume. The cell volume was determined as described before (27), using $^{3}H2O$ with ^{14}C-inulin. **B,** total intracellular ITC accumulation levels. Cells were exposed to an ITC at the specified concentrations for 1 h. Total intracellular amounts of ITCs were measured by the cyclocondensation assay (27) and calculated based on cell volume, using the standard curve shown above. *Columns,* means of three measurements; *bars,* ±SD. The cyclocondenation assay measures the total amount of both free ITC and its thiol conjugate (dithiocarbamate), including GS-ITC.

conditions were ineffective. Furthermore, the rank order of the potencies of these compounds in damaging mitochondria *in vitro* matches the rank order of the potencies of the free ITCs in causing mitochondrial damage in treated UM-UC-3 cells. These results clearly indicate that exposure of cells to BITC and PEITC at 7.5 to 30 μmol/L directly damage mitochondria.

Discussion

The four dietary ITCs showed very different apoptosis-inducing abilities in UM-UC-3 cells, providing a good model to study the targets of ITCs in the induction of apoptosis. Our current and previous results (21) show that BITC and PEITC are more potent inducers of apoptosis than AITC and sulforaphane. The ITCs induce apoptosis in UM-UC-3 cells primarily through the caspase-9-mediated pathway because in the presence of Z-LEHD-FMK, a specific caspase-9 inhibitor, PARP cleavage induced by the ITCs is largely abolished. The activation of caspase-9 apparently resulted from mitochondrial damage caused by these compounds. BITC and PEITC damaged both outer and inner mitochondrial membranes as shown by the release into cytoplasm of cytochrome *c* from the mitochondrial intermembrane space and MDH from the mitochondrial matrix (Fig. 2). Indeed, the abilities of the four ITCs in activating caspase-9 are closely correlated with their abilities in causing loss of $\Delta\Psi_m$ and mitochondrial cytochrome *c* release (compare the results in Fig. 1 with those in Fig. 2A and B). Similar effects of both BITC and PEITC on mitochondria were seen in the isolated mitochondria. Both ITCs at 15 to 100 μmol/L caused a dose- and time-dependent loss of $\Delta\Psi_m$ and cytochrome *c* release (Fig. 4). AITC and sulforaphane were much weaker in damaging the isolated mitochondria, correlating well with the results obtained from the whole cell experiments. The experimental system using isolated mitochondria also allowed us to show that the main intracellular metabolites of ITCs (GS-ITCs) caused mitochondrial damage, as cytochrome *c* was released after treatment of mitochondria for 30 minutes with 500 μmol/L GS-BITC or GS-PEITC in the presence of 2 mmol/L GSH (Fig. 4E). The treatment condition mimics the intracellular environment after cells are exposed to the ITCs.

Although Bcl-2 family proteins are widely known to affect the mitochondrial membrane integrity, neither BITC nor PEITC have any detectable effect on the expression of Bcl-2, Bcl-xl, Bak, and Bax in UM-UC-3 cells (Fig. 3). Both compounds elevated whole cell Bad levels but did not change its content in the mitochondria. However, BITC and PEITC markedly stimulated phosphorylation of Bcl-2, although the change occurred only when the cells were treated with each compound at 7.5 μmol/L for 24 hours (Fig. 3) but not at high ITC concentrations (15 and 30 μmol/L), which caused more damage to the mitochondria (Fig. 2A). Moreover, although total Bak levels in UM-UC-3 cells were not altered after ITC treatment, both BITC and PEITC apparently stimulated Bak translocation to mitochondria (Fig. 3). Choi and Singh recently reported that Bak as well as Bax play critical roles in apoptosis induction by sulforaphane in SV40-transformed mouse embryonic fibroblasts (40). However, increased translocation of Bak occurred when cells were treated with 7.5 to 15 μmol/L BITC or PEITC, and further increase in ITC concentration (30 μmol/L) resulted in a decrease in mitochondrial Bak levels although more cells suffered loss of $\Delta\Psi_m$ (compare results in Fig. 3B with those in Fig. 2A). These results show that the effect of ITCs on Bcl-2 and Bak is highly dependent on the concentrations of the compounds. It is noteworthy that published data show that the effect of ITCs on Bcl-2 family proteins varies with cell lines. Xiao et al. reported that AITC, which markedly induced apoptosis in human prostate cancer LNCaP cells and PC-3 cells, did not affect the expression of proapoptotic Bax and Bid, but decreased the expression of both Bcl-2 and Bcl-xl in these cells (12). However, both AITC and PEITC significantly activated Bid in HL60 cells (41), and BITC significantly induced the expression of Bax and phosphorylation of Bcl-2 in human T-cell leukemia Jurkat cells (42). Rose et al. also recently reported that PEITC induced conformational change and mitochondrial translocation of Bax in human hepatoma HepG2 cells and suggested that loss of $\Delta\Psi_m$ resulted from Bax-dependent membrane pore formation (43). Importantly, our results also show that ITC can cause the disassociation of Bcl-xl with Bak and Bax in the mitochondria, which likely frees the latter from inhibition by Bcl-xl, thus revealing a novel mechanism by which an ITC causes mitochondrial damage.

The mitochondrial permeability transition pore opening is known to lead to loss of $\Delta\Psi_m$, and subsequently, osmotic

swelling of the mitochondrial matrix, causing the rupture of the outer membrane and the release of cytochrome *c* (44, 45). The mitochondrial permeability transition pore is a preexisting channel at the contact site between the inner and outer mitochondrial membrane, primarily consisting of three components: voltage-dependent anion channel in the outer mitochondrial membrane, adenine nucleotide translocase in the inner mitochondrial membrane, and cyclophilin D in the matrix. Cyclosporin A and bongkrekic acid are known to bind to cyclophilin D and adenine nucleotide translocase, respectively, and inhibit the mitochondrial permeability transition pore opening. However, both cyclosporin A and bongkrekic acid in our hands did not inhibit the BITC- and PEITC-induced loss of $\Delta\Psi_m$, suggesting that the ITCs likely do not modulate mitochondrial permeability transition pore, which is in line with what was reported by Rose et al. (43).

Several synthetic ITCs, including *p*-bromophenyl ITC, 4,4′-diisothiocyanatebiphenyl, and β-naphthylmethyl ITC, were shown to act on isolated liver mitochondria as uncouplers with stimulation of ATPase activity (46). However, disruption of $\Delta\Psi_m$ by a mitochondrial uncoupler, such as carbonyl cynide *m*-chlorophenylhydrazone, was not followed by cytochrome *c* release and cell death, and additional triggers were required for induction of apoptosis (47, 48). It is not known if the ITCs in the current report also act as uncouplers. However, even if they do, this function alone may not be sufficient to rapidly induce mitochondrial damage. Exposure of cells to ITCs including BITC and PEITC also results in a rapid and dose-dependent increase of cellular reactive oxygen species levels in several cell lines (23, 49–51), including UM-UC-3 cells.[1] Mitochondria are the major site of reactive oxygen species production, which may result from disruption of the respiratory chain (52–54). Although mitochondrial respiration was suppressed by several ITCs including BITC (23, 46, 55), it remains to be determined to what extent reactive oxygen species production mediates ITC-induced damage of mitochondrial membrane.

Interestingly, the potency of the four ITCs in damaging mitochondria (causing the loss of $\Delta\Psi_m$, see Fig. 2A) seems to correlate with their lipophilicity (log*P* values), which was previously determined to be 3.2 (PEITC), 3.0 (BITC), 2.2 (AITC), and 0.3 (sulforaphane), respectively (25). The two aromatic ITCs (BITC and PEITC) are rather hydrophobic and potent mitochondria-damaging agents, whereas AITC, and sulforaphane in particular, are more water-soluble and show lower mitochondria-damaging potential. Certainly, higher lipid solubility facilitates the delivery of the compound to the membrane. In fact, Miko and Chance reported that the uncoupling activities of some ITCs positively correlated with their log*P* values (46). The importance of lipophilicity is also reflected by the damaging of the cell surface membrane. Both BITC and PEITC at 7.5 to 30 μmol/L caused dose-dependent damage to cell membrane (Fig. 2D), whereas AITC and sulforaphane showed much weaker effects at the same concentration range (results not shown).

In summary, the differential effects of the four dietary ITCs on apoptosis of UM-UC-3 cells are associated with their damaging effects on the mitochondria. Treatment of UM-UC-3 cells with BITC and PEITC at low micromolar concentrations caused the damage of both outer and inner mitochondrial membranes, leading to the release of cytochrome *c* into the cytoplasm, caspase-9 activation, and induction of apoptosis. Mitochondrial damage was caused by the main intracellular metabolites of ITCs (their glutathione conjugates). Increased phosphorylation of Bcl-2, mitochondrial translocation of Bak, and disruption of the association of Bcl-xl with both Bak and Bax in the mitochondria occur following ITC treatment, all of which likely contribute to mitochondrial damage and induction of apoptosis.

Acknowledgments

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