Dietary isothiocyanate mediated apoptosis of human cancer cells is associated with Bcl-xL phosphorylation

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Abstract. Benzylisothiocyanate (BITC), a major phase II enzyme inducer in the organic solvent of papaya fruit, has been shown to induce apoptosis specifically in cancer cells. The exposure of pancreatic, prostate as well as leukemic cells to this dietary isothiocyanate resulted in significant extent of apoptosis as evident from PARP cleavage, chromatin condensation or profound attenuation of procaspase-3 level. We also investigated whether BITC induces apoptosis by converging two major pathways: the death receptor mediated extrinsic and the mitochondrial intrinsic pathway. The exogenous expression of dominant-negative caspase-8 or dominant-negative caspase-9 can attenuate BITC-mediated cell death of prostate cancer cells. In parallel with this observation, BITC can activate both procaspase-8 and -9 in pancreatic and prostate cancer cells. Furthermore, flow cytometry analysis demonstrated the enrichment of sub-G_0-G_1 phase population with G_2-M arrest in BITC challenged pancreatic cancer cells. In order to comprehend the molecular mechanism underlying the relationship between BITC-mediated cell cycle arrest and apoptosis we report here for the first time that the anti-apoptotic protein Bcl-xL was phosphorylated by BITC treatment. Subsequent investigation using Jun kinase inhibitor exhibits the involvement of Jun kinase in BITC triggered Bcl-xL phosphorylation and apoptosis.

Introduction

The abundant epidemiological studies as well as experimental animal studies noticeably validate that high intake of cruciferous vegetables protects against tumorigenesis (1-10). Thus, cruciferous vegetables, a rich source of glucosinolates, have been of great interest for potential use in the chemoprevention of cancer. The glucosinolates are known to be degraded into isothiocyanates by enzymatic action of plant-specific myrosinase or intestinal flora in the body. It is emergent that considerable portion of the chemopreventive effects of isothiocyanates may be associated with the inhibition of the metabolic activation of carcinogens by cytochrome P450s (Phase I), tied with robust induction of Phase II detoxifying and cellular defensive enzymes (9). Besides, apoptosis and cell cycle perturbations seem to be yet another possible chemopreventive mechanisms extracted by isothiocyanates (ITC), especially with respect to the effects on neoplastic cells (8). Previous studies report that naturally occurring ITCs can exert chemopreventive effect against tumors induced by chemical carcinogen in experimental animals (6,7). High intake of cruciferous vegetables, including broccoli and cauliflower, may be associated with reduced risk of aggressive prostate cancer, particularly extraprostatic disease (10).

Previously multiple pathways involved in apoptosis induction by ITCs were suggested (11-14). Interestingly, benzylisothiocyanate (BITC) induced apoptosis was reported to be linked with G_2-M arrest and Bcl-2 phosphorylation in Jurkat T lymphoma cells. In this report (15), the authors demonstrated that the p38 MAPK pathway could be operative in cell cycle arrest induced by BITC, whereas JNK pathway plays a major role in apoptosis but not in the cell cycle regulation. The Bcl-2 family comprises of two counteracting groups of proteins: the pro-apoptotic and anti-apoptotic (16-18). Among the anti-apoptotic members, Bcl-2, Bcl-xL or Mcl-1 is phosphorylated by microtubule disarraying agents such as Taxol, nocodazole or 2-Methoxyestradiol (19-42). In most cases, phosphorylation of the Bcl-2 family members leads to the loss of their biological function. The emerging concept yielded from these studies is that phosphorylation induced inactivation of Bcl-2 protein on Ser 70 residue inside the unstructured 'loop region' (LR) during mitosis might work as a checkpoint to permit apoptosis (28,29). The LR of both Bcl-2 and its close homologue Bcl-xL can negatively regulate their functions as evident by enhanced anti-death activity of LR deficient or phosphorylation-defective mutants (21-25). Of note, the endogenous phosphorylation of Bcl-2 without treatment of any trigger can be detected in M phase of normally cycling cells (21). Another interesting observation demonstrates the ability of phosphorylated Bcl-2 to regulate Ca^{2+} homeostasis and apoptosis (43). In line with our previous

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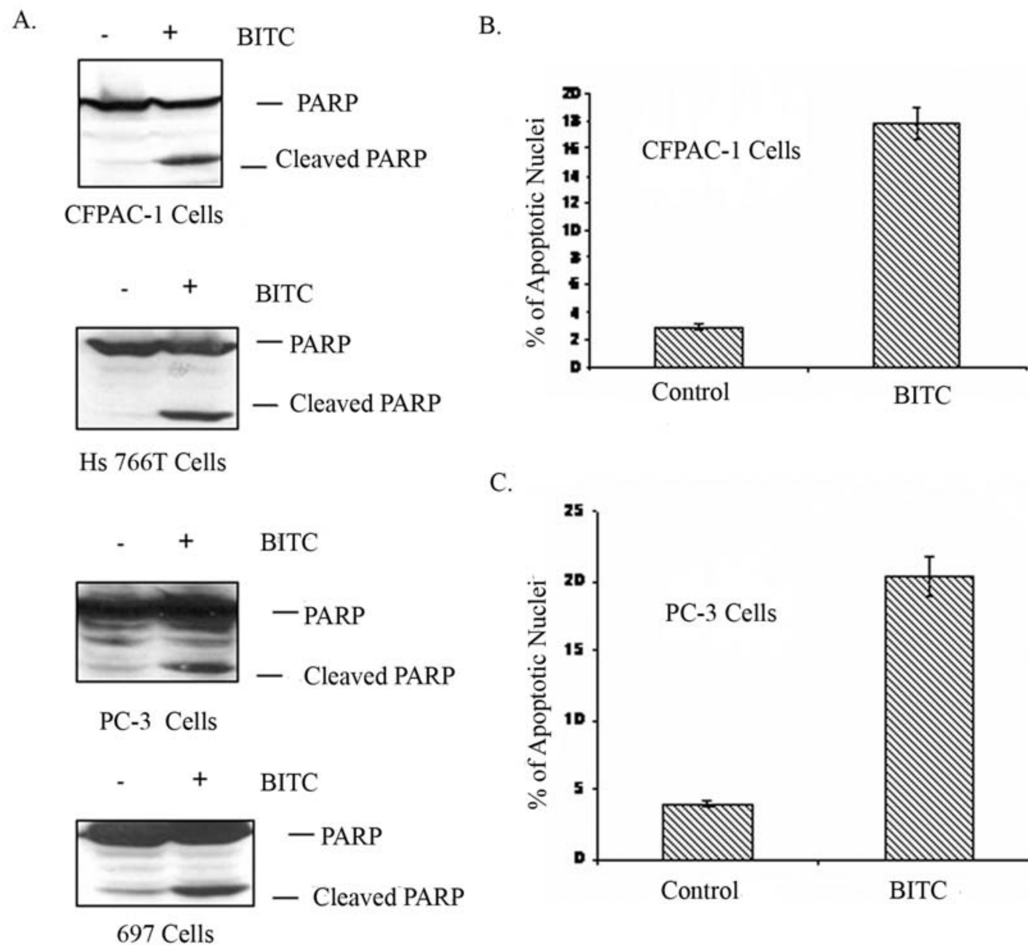


Figure 1. BITC induces apoptosis in a panel of human cancer cells. Pancreatic cancer (CFPAC-1, Hs 766T), prostate adenocarcinoma (PC-3) or Pre-B leukemia (697) cells were exposed to BITC for 24 h. A, PARP degradation. Western blotting of vehicle control (DMSO) and BITC-treated cell lysate using monoclonal antibody against PARP. B and C, Apoptotic cell death visualized by DAPI staining. Percentage of fragmented nuclei indicated in B and C was determined by scoring ~500 cells from randomly chosen field. For PC-3 and 697 cells, 5 μmol/l BITC was used while CFPAC-1 and Hs 766T cells were treated with 2.5 and 7.5 μmol/l BITC respectively (A-C).

finding (19), Bassik *et al* (43) reported that phospho-Bcl-2 binds less BH3 domain only BIM or multidomain BAX pro-apoptotic protein. Precisely, phosphorylation of Bcl-2 at mitosis would increase calcium in endoplasmic reticulum (ER) and could account for the increased G_2/M susceptibility to apoptosis (43,44). Further mechanistic studies demonstrate that PP2A phosphatase can regulate Bcl-2 phosphorylation and proteasome mediated degradation of phospho-Bcl-2 (44).

We have investigated the effect of BITC on the post-translational modification (phosphorylation) of Bcl-xL protein and apoptosis in a panel of human cancer cells. In the current report, we for the first time document that chemopreventive agent BITC can invoke death advantage with concomitant phosphorylation of Bcl-xL on serine 62 residue.

Materials and methods

Cell lines. Human pancreatic cancer cells (CFPAC-1, Hs 766T), human lymphoid cells 697 (pre-B-cell line harboring a t(1;19) chromosomal translocation) and Jurkat T lymphoma cells were grown in RPMI supplemented with 10% fetal bovine serum (FBS) and 50 μg/ml gentamycin. Hormone-independent human prostate carcinoma cells (PC-3 or DU145) were cultured in MEM with 10% FBS and 50 μg/ml gentamycin.

Apoptosis assay. Cells ($5x10^6$) were seeded (in triplicate plates) in the growth medium and, the next day, were treated with BITC in the presence or absence of Jun kinase inhibitor (EMD Biosciences, San Diego, CA), for designated time periods. Dominant-negative cDNA constructs encoding caspase-8 and -9 (45) in mammalian expression vectors were transiently transfected (21,45) prior to BITC exposure. Subsequently, cells were either processed for chromatin condensation analysis by DNA binding dye, 4',6'-diamidino-2-phenylindole (DAPI) fluorescence (23-26,45) or PARP cleavage analysis (23-25,45) by immunoblotting with monoclonal antibody against PARP (BD Biosciences, La Jolla, CA). For DAPI staining, cells were washed, fixed, and permeabilized followed by mounting in a fluid containing 2 μg/ml DAPI (Vector Laboratories, Burlingame, CA). A Nikon Eclipse E600 Fluorescence microscope was used to visualize nuclear stain (23-26,45).

Immunoblotting. Cells at $5x10^6$ per 100-mm dish were seeded and treated 1 day later with specified concentrations of BITC. Cells were preincubated for 2-6 h with 10 μmol/l JNK inhibitor II (EMD Biosciences) followed by 24-h exposure of 5 μmol/l of BITC. Following treatment, total cellular proteins were extracted (23-26,45). After normalization for total protein

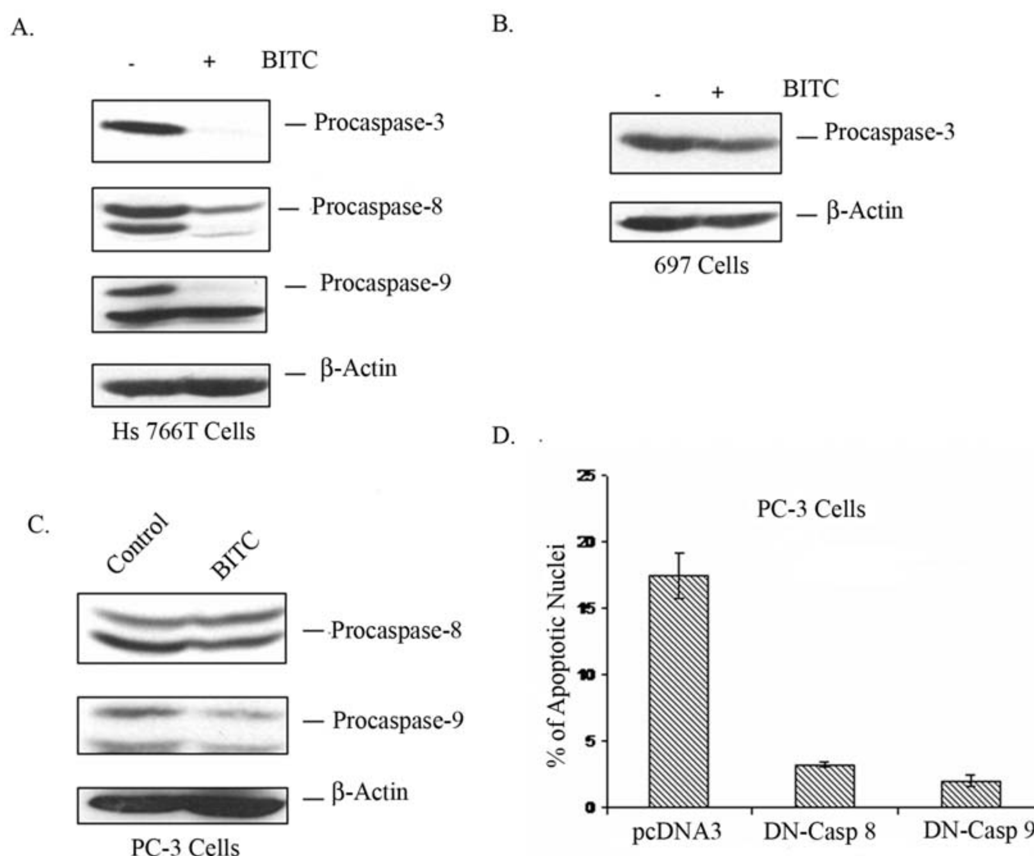


Figure 2. BITC-mediated apoptosis requires activation of procaspase-8, -9 and -3. A, Diminished procaspase-3 level following BITC exposure. Total protein extract from control and 24 h BITC-treated Hs 766T/697 cells were subjected to immunoblot analysis with caspase-3 antibody. B and C, BITC induced activation of procaspase-8 and -9. Hs 766T and PC-3 cells were challenged with BITC for 24 h and subsequently cellular extract was immunoblotted with procaspase-8 and -9 antibodies. D, Attenuation of BITC induced apoptosis by dominant negative constructs of caspase-8 and -9. Cells were transfected with either vector (pcDNA3) or the indicated constructs by calcium phosphate co-precipitation method. Twenty-four hours post transfection, cell culture media was replaced with the media containing 5 μmol/l BITC. Following 24 h of BITC treatment, cells were harvested and stained with DAPI for scoring apoptotic nuclei as described above in Fig. 1.

content, the resulting lysate was subjected to SDS-PAGE and blotted into nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ). Membranes were probed with the following antibodies: phospho-Bcl-xL (46), phospho-Bcl-2 (23), procaspase-8 and -9 (Santa Cruz Biotechnology, Santa Cruz, CA), procaspase-3 and PARP (BD Biosciences). Immunodetection was accomplished by enhanced chemiluminescence method (Amersham Biosciences). Immunoblotting with ß-actin antibody (Sigma, St. Louis, MO) was performed for protein loading control.

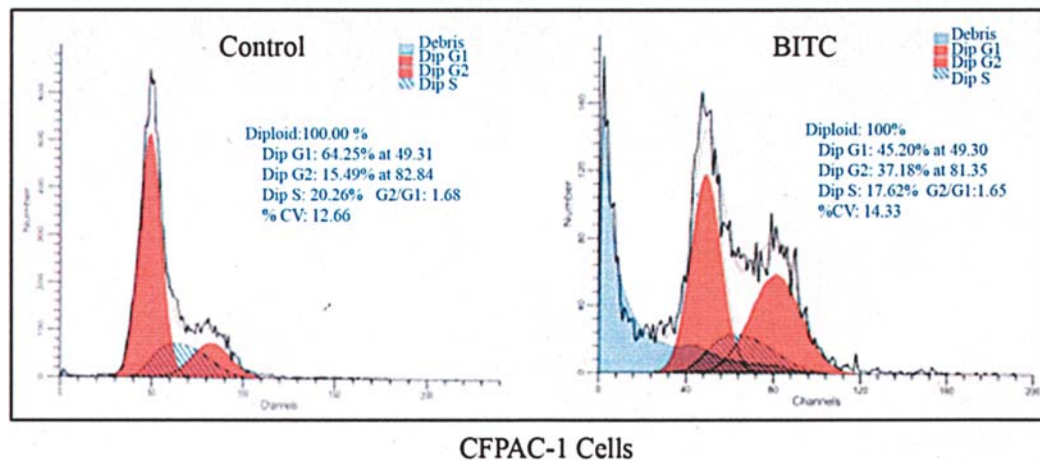
Cell sorting. Control or BITC-treated pancreatic cancer cells were sorted by using a fluorescence activated cell sorter at the Case Comprehensive Cancer Center's core facility. Cells were stained with Hochest 33342 (Sigma) at a concentration of 15 μg/ml for 1 h at 37˚C. In order to increase the resolution of DNA distribution, 3,3'-dipentyloxacarbocyanine iodide (Molecular Probes) was added at a concentration of 0.2 μg/ml simultaneously with Hochest 33342 (20,21).

Results

Chemopreventive agent BITC and apoptosis of cancer cells. Initially, the potential apoptosis inducing effect of BITC was investigated in different actively proliferating human cancer cells that exhibit aggressive clinical behavior. The caspase family of cysteine proteases plays a key role in mediating apoptosis through proteolysis of specific target. Among the targets are Poly-(ADP-ribose) polymerase (PARP) and nuclear lamins. PARP is a 116-kDa nuclear protein that is specifically cleaved by caspase-3 into a signature 85-kDa fragment (45). In response to BITC treatment, pancreatic carcinoma, prostate carcinoma as well as pre-B leukemic cells showed elevated level of 85-kDa fragmented PARP, which is considered to be the hallmark of apoptosis (Fig. 1A). Since PARP is activated in the presence of nicked or damaged DNA, we stained the control and BITC-treated pancreatic (CFPAC-1)/prostate (PC-3) cancer cells with 4,6-diamidino-2-phenylindole, DAPI (26,42,45). Both CFPAC-1 and PC-3 cells, which underwent enhanced PARP cleavage, also exhibited significant increase in apoptotic nuclei in response to BITC treatment compared to vehicle control (Fig. 1B and C).

BITC-mediated apoptosis requires activation of both initiator and executioner caspases. As mentioned previously, the activation of caspases results in cleavage and inactivation of key cellular proteins. Subsequently, we examined whether BITC-induced cell death was mediated by caspases. Exposure of pancreatic cancer cells Hs766T or pre-B leukemic cells 697 to BITC resulted in the disappearance of procaspase-3 (Fig. 2A

A.



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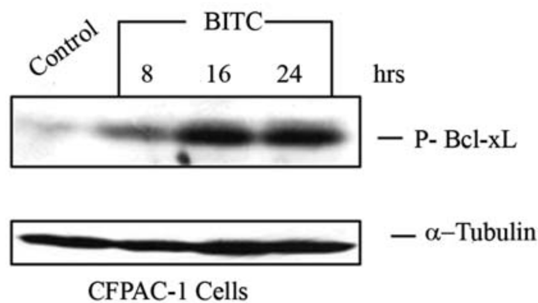

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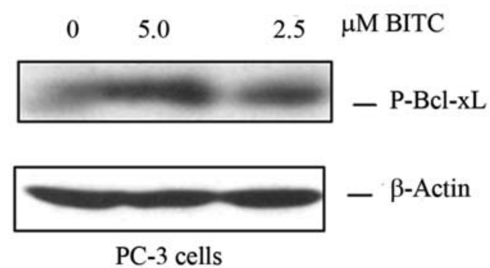

Figure 3. BITC induces Bcl-xL phosphorylation with simultaneous cell cycle arrest. A, Cell cycle distribution of BITC-treated CFPAC-1 cells. Approximately 2.5-fold greater population of cells are at G_2 phase in BITC-treated in comparison to control. B and C, Western blotting with phospho-specific Bcl-xL antibody (46). B, Appearance of phospho-Bcl-xL in CFPAC-1 cells as early as 8-h treatment of 2.5 μmol/l BITC. C, Status of phospho-Bcl-xL in prostate cancer cells PC-3-treated with 0-5 μmol/l BITC for 24 h. Immunoblots of α-tubulin and ß-actin were presented as internal controls.

and B). Next, we examined whether activation of both initiator and executioner caspases are necessary for BITC-mediated cell death of prostate and pancreatic cancer cells. Fig. 2A and C demonstrate the activation of both pro-caspase-8 and -9 in BITC challenged prostate carcinoma cells PC-3 as well as metastatic pancreatic carcinoma cells Hs 766T. The activity was assessed by the disappearance of the inactive forms procaspase-8 and -9 on Western blotting. The two species of procaspases are previously reported isoforms (45). BITC treatment caused a decrease in the levels of both procaspase-9 and -8. We also determined apoptosis in PC-3 cells transfected with pcDNA3 vector, DN-caspase-8 and -9 respectively. In contrast to empty vector, both dominant negative constructs inhibited BITC induced apoptosis (Fig. 2D). These results pointed toward involvement of both caspase-8 and -9 pathways in execution of BITC-induced apoptosis.

BITC exposure causes cell cycle arrest and Bcl-xL phosphorylation. The growth inhibition of cancer cells by many dietary agents, including ITCs and garlic-derived organosulfides has been observed to be associated with block of cell cycle progression and apoptosis (8,15). Accordingly, we assessed the effect of BITC on cell cycle distribution, by flow cytometry following staining with Hoechst 33342 (20,21). Representative histograms for cell cycle distribution in CFPAC-1 cells following a 24-h exposure to DMSO (control) or 5 μM BITC are presented in Fig. 3. As shown in Fig. 3A, treatment of pancreatic cancer cells CFPAC-1 to 5 μM BITC for 24 h resulted in statistically significant enrichment of sub-G_0-G_1 fraction, an indicator of DNA fragmentation. Furthermore, BITC caused ~2.4-fold increase in G_2-M population compared to DMSO-treated control.

In the present study, we initiated the investigations on understanding the regulatory mechanisms of cell death elicited by BITC in prostate adenocarcinoma cells, PC-3 or pancreatic adenocarcinoma cells, CFPAC-1. The oncoprotein Bcl-xL, like Bcl-2, is phosphorylated at mitotic phase of the cell cycle (23,38,46). BITC is also known to arrest cells at mitotic phase of cell cycle (15). As shown in Fig. 3B and C, the ability of BITC at pharmacological concentration (2.5-5 μM) to induce Bcl-xL phosphorylation is quite evident in both PC-3 and CFPAC-1 cells. Bcl-xL phosphorylation was determined by Western blotting using phospho-Bcl-xL specific antiserum (46). The ability of phospho-Bcl-xL (Ser 62) specific antibody to detect enhanced Bcl-xL in BITC exposed cancer cells suggests Ser 62 of Bcl-xL to be the phosphorylation target of BITC. Thus our studies indicate that BITC induced cancer cell death is mediated by activation of caspases and is accompanied by simultaneous phosphorylation of anti-apoptotic protein Bcl-xL.

JNK pathway is responsible for BITC triggered Bcl-xL phosphorylation and cell death. The phosphorylation site(s)

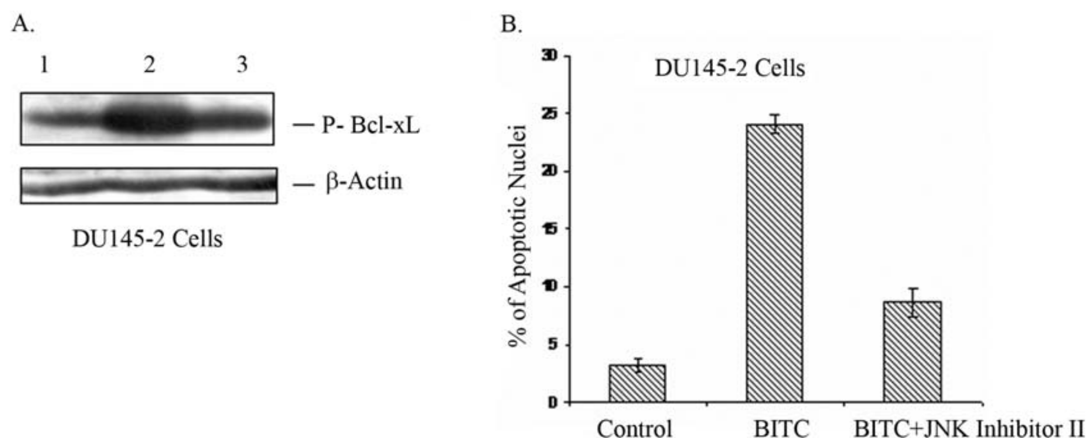

Figure 4. JNK inhibitor can diminish BITC induced Bcl-xL phosphorylation as well as apoptosis. A, DU145-2 cells (genetically engineered to overexpress wild-type Bcl-xL) were pretreated for 8 h (23) with JNK inhibitor II (10 μM) followed by treatment with 1 μM BITC for 24 h. Lane 1, control; lane 2, 1 μmol/l BITC for 24 h; lane 3, BITC and JNK inhibitor II. For analyzing Bcl-xL phosphorylation (A), phosphorylation site-specific Bcl-xL antibody (46) was used. Under identical conditions, apoptotic nuclei were visualized by DAPI staining (B). Approximately 500 cells were scored in each category. Results are mean ± SD of three independent experiments.

for Bcl-2/Bcl-xL conforms to the consensus motif for substrates of MAP kinase and JNK/SAPK (22,46). The pretreatment of JNK inhibitor II can successfully abrogate the phosphoforms of Bcl2/Bcl-xL in a concentration-dependent manner (22,23,46). When DU145-2 cells (prostate cancer DU145 cells genetically engineered to overexpress wild-type Bcl-xL; 23) were pretreated with 10 μM of JNK inhibitor II, BITC induced Bcl-xL phosphorylation was significantly decreased (Fig. 4A, lane 3). This inhibitor is known to effectively block the accumulation of phospho-Jun in response to cytokines (47). Subsequently, we examined whether blocking of Jun kinase activity can prevent BITC initiated cell death in DU145-2 cells. As shown in Fig. 4B, co-incubation with 10 μM JNK inhibitor II rendered the cells more resistant to BITC-triggered apoptosis. To summarize, our results indicate that Jun kinase mediated Bcl-xL phosphorylation on Serine 62 residue inside 'loop region' of Bcl-xL is critical for rendering death advantage against BITC.

Discussion

The epidemiological studies demonstrate an inverse correlation between consumption of fruits and vegetables with the development of various human malignancies. A score of preclinical investigations link isothiocyanates to exerting anti-cancer effect (1-9). The studies presented herein describe the ability of BITC to induce apoptosis in human cancer cells by phosphorylation of anti-apoptotic protein Bcl-xL. Among the two major apoptotic pathways the mitochondrial 'intrinsic' and transmembrane 'extrinsic', the latter comprises of activation of death receptors (DR) such as Fas, TNF receptor 1, DR4 or DR5 (45,48). Upon activation by interacting with their respective ligands such as FasL, TRAIL (45,48), a signal transduction cascade ensues by the recruitment of DR associated molecules such as FADD. Subsequent activation of initiator caspases such as caspase-8 can lead to cleavage of a proapoptotic protein Bid. Truncated Bid (t-Bid) can translocate to mitochondria and can orchestrate mitochondrial events that can result in biochemical as well as morphological alterations implicated with programmed cell death. Since our preliminary studies demonstrate the involvement of both caspase-8 and -9, it is very likely that both death receptors and mitochondrial machinery are responsible for BITC triggered apoptosis of prostate cancer cells. Future investigations will be directed towards establishing cross-talk between extrinsic and intrinsic pathway to execute BITC-mediated cell death of cancer cells as observed in the cases of other chemopreventive agents such as EGCG (49-51). Interestingly, epigallocatechin-3-gallate (EGCG), the major polyphenolic constituent of green tea, sensitizes TRAIL-resistant LNCaP cells to TRAIL-mediated apoptosis through modulation of intrinsic and extrinsic apoptotic pathways. When combined with EGCG, Apo2L/TRAIL exhibited enhanced apoptotic activity in LNCaP cells (50).

The investigation on the dissection of the molecular mechanism of the apoptosis-inducing effect of BITC is important because BITC is quickly and continuously accumulated into cells, and the intracellular concentration of BITC increased up to 300 μM (52). The studies depicted here have potential clinical significance. The anti-apoptotic proteins Bcl-2/Bcl-xL play an important role in tumor cell survival. The overexpression of Bcl-2/Bcl-xL in chemo-resistant tumors is well known (16,53). Given the synergistic involvement of phospho-Bcl-2/Bcl-xL and death receptors in BITC-mediated demise of cancer cells should be helpful to identify biomarkers for future prevention trial. The results yielded from our investigation significantly contribute towards the understanding a blueprint of signaling network involving BITC and apoptosis in human cancer.

Acknowledgments

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