Involvement of the Mitochondrial Death Pathway in Chemopreventive Benzyl Isothiocyanate-induced Apoptosis*

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In the present study, we studied the molecular mechanism underlying cell death induced by a cancer chemoprotective compound benzyl isothiocyanate (BITC). The cytotoxic effect of BITC was examined in rat liver epithelial RL34 cells. Apoptosis was induced when the cells were treated with 20 μM BITC, characterized by the appearance of phosphatidylserine on the outer surface of the plasma membrane and caspase-3 activation, whereas no caspase activation and propidium iodide incorporation into cell were detected with 50 μM BITC that induced necrosis. The mitochondrial death pathway was suggested to be involved in BITC-induced apoptosis because the treatment of cells with BITC-induced caspase-9-dependent apoptosis and mitochondrial transmembrane potential (ΔΨm) alteration. We demonstrated here for the first time that BITC directly modifies mitochondrial functions, including inhibition of respiration, mitochondrial swelling, and release of cytochrome c. Moreover, glutathione depletion by diethyl maleate significantly accelerated BITC-triggered apoptosis, suggesting the involvement of a redox-dependent mechanism. This was also implicated by the observations that intracellular accumulation of reactive oxygen species, including superoxide (O2·−) and hydroperoxides (HPOs), was indeed detected in the cells treated with BITC and that the intracellular HPO level was significantly attenuated by pretreatment with N-acetylcysteine. The treatment with a pharmacological scavenger of O2·−, Tiron, also diminished the HPO formation by ~80%, suggesting that most of the HPOs were H2O2 derived from the dismutation of O2. These results suggest that BITC induces apoptosis through a mitochondrial redox-sensitive mechanism.

A number of studies support that certain food phytochemicals protect against cancer. An important group of compounds that have this property are organosulfur compounds including isothiocyanates (ITCs). ITCs are compounds that occur as glucosinolates in a variety of cruciferous vegetables such as *Brassica* species. Many ITCs are effective chemoprotective agents against chemical carcinogenesis in experimental animals (1–5). Previous studies have suggested that ITCs inhibit phase I enzymes that are required for the bioactivation of carcinogens and increase carcinogen excretion or detoxification by the phase II enzyme including glutathione (GSH) S-transferase in animal tissues (6–8). In addition, treatment of ITCs induces a stress signaling pathway-, caspase-3,- and/or p53-dependent apoptosis, which may be one possible mechanism to achieve the anticarcinogenic function (9–13). On the other hand, we have recently reported the induction of intracellular reactive oxygen species (ROS) by benzyl-ITC (BITC) (14), one of the well-known ITC derivatives having a potency for phase II enzyme induction (15). Therefore, it has been suggested that oxidative stress may be involved in the ITCs-induced apoptosis (10).

Apoptosis plays important roles in developmental processes, maintenance of homeostasis, and elimination of seriously damaged cells. The aberrant regulation of apoptosis has been observed in many disorders including cancer. The caspases are a novel class of at least 10 cysteine proteases, known to be a general mechanism in the induction of apoptosis. Especially, caspase-3 (CPP32) plays a crucial role in the process of apoptosis (16). Numerous therapeutic and preventive agents eliminate cancer cells by inducing apoptotic cell death through the caspase-3 pathway (17). Involvement of mitochondria has been implicated in various types of stimuli-induced cell death upstream of caspase activation. Activators of apoptosis, such as caspase-9, cytochrome c, and apoptosis-inducing factor, are all found in mitochondria (18, 19). Mitochondria also contain proteins that regulate apoptosis, such as members of the Bcl-2 family, namely Bcl-2, Bcl-xL, Bax, and Bad, which can prevent and accelerate apoptosis. ROS are recognized as mediators of the apoptotic signaling pathway. Mitochondria are also known to be a major physiological source of ROS, which are generated during mitochondrial respiration and have been implicated as mediators of apoptotic signaling pathway (20).

In the present study, we studied the regulatory mechanism of cell death elicited by BITC. Here we examined the cell death

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§ The abbreviations used are: ITC, isothiocyanate; ROS, reactive oxygen species; BITC, benzyl isothiocyanate; GSH, glutathione; H2DCF-DA, 2′,7′-dichlorofluorescein diacetate; DHE, dihydroethidium; FITC, fluorescein isothiocyanate; PI, propidium iodide; DEVD-pNA, Ac-Asp-Glu-Val-Asp-p-nitroanilide; YVAD-fmk, Z-Tyr-Val-Ala-Asp-fluoromethyl ketone; DEVD-fmk, Z-Asp-Glu-Val-Asp-fluoromethyl ketone; LEHD-fmk, Z-Leu-Glu-His-Asp-fluoromethyl ketone; ΔΨm, mitochondrial transmembrane potential; Rho123, rhodamine green; HPO, hydroperoxide; DCF, dichlorofluorescein; JNK, c-Jun NH2-terminal kinase; PT, permeability transition; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; DEM, diethyl maleate; PBS, phosphate-buffered saline; NAC, N-acetylcysteine; TNF-α, tumor necrosis factor-α.
Redox-regulated Apoptosis Induced by Benzyl Isothiocyanate

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pattern induced by BITC and found that the mitochondrial death pathway might be involved in BITC-induced apoptosis. We demonstrated for the first time that BITC directly modifies mitochondrial function including mitochondrial swelling and inhibition of respiration. We also indicate the important roles of intracellular defensive factors, including GSH, in the electrophile-induced activation of caspase-3 protease and apoptosis and discuss the redox-sensitive mechanism.

EXPERIMENTAL PROCEDURES

Materials—BITC was obtained from Nacalai Tesque, Inc., Kyoto, Japan. Tiron™ was purchased from Sigma. 2,7'-Dichlorofluorescein diacetate (H2DCF-DA) and dihydroethidium (DHE) was obtained from Molecular Probes, Inc., Leiden, The Netherlands. All other chemicals were purchased from Wako Pure Chemical Industries, Osaka, Japan. Protein concentration was measured using the BCA protein assay reagent from Pierce.

Cell Culture—RL34 cells were obtained from the Health Science Research Resources Bank, Osaka, Japan (21). The cells were grown as monolayer cultures in Dulbecco’s modified Eagle’s medium supplemented with 5% heat-inactivated fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), l-glutamine (0.5 mg/ml), pyruvic acid (0.11 mg/ml), and 0.37% NaHCO3 at 37 °C in an atmosphere of 95% air and 5% CO2. Cells post-confluency were exposed to the test compounds (resolved in 0.1% MeSO) in the medium containing 5% fetal bovine serum.

Cytotoxicity—Phosphatidylserine redistribution in a plasma membrane was measured by the binding of annexin V-fluorescein isothiocyanate (FITC) according to the protocol outlined by the manufacturers in the Annexin V-FITC Apoptosis Detection Kit (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan). Cells were also stained with 100 μg/ml propidium iodide (PI), before being analyzed with a Flow cytometer.

Apoptotic cell death was also evaluated by fluorescence microscopy after staining with Hoechst 33258 (Wako Pure Chemical Co., Osaka, Japan). Cells were collected and fixed for 20 min in 3.7% (w/v) paraformaldehyde in PBS. After being washed with PBS, cells were stained with 1.6 μM Hoechst 33258 for 10 min. Samples were observed with a fluorescence microscope (Nikon, Tokyo, Japan), equipped with a ×40 objective (488 nm excitation and 518 nm emission).

Caspase Activity Determination—The measurement of caspase-3 specific activity was done by the cleavage of Ac-Asp-Glu-Val-Asp-pNA (21) according to the protocol outlined by the manufacturers in a Caspase-3 Colorimetric Protease Assay Kit (Medical & Biological Laboratories Co., Ltd.). Caspase-3 like activity was monitored in the cytosol of RL34 cells that had been incubated with BITC at 20 μM for 4 h. Inhibitory experiments were performed by the pretreatment of caspase isozyme specific inhibitors including Z-Tyr-Val-Ala-Asp-fluoromethyl ketone (ZVAD-fmk), Z-Asp-Glu-Val-Asp-fluoromethyl ketone (DEVD-fmk), and Z-Leu-Glu-His-Asp-fluoromethyl ketone (LEHD-fmk) (Medical & Biological Laboratories Co., Ltd.) at 50 μM or Tiron at 1 mM for 1 h before BITC stimulation.

Assessment of Mitochondrial ∆Ψm—Changes in the inner mitochondrial transmembrane potential (∆Ψm) were determined by incubating the BITC-treated cells in 10 μM rhodamine green (Rho123) for 30 min and scoring immediately by flow cytometry.

GSH Assay—Measurement of GSH was performed spectrophotometrically using a commercial kit (BIOXYTECH™ GSH-400™ Assay; OXIS International, Inc., Portland, OR) as previously reported (14, 22). Cells were lysed and extracted with 5% metaphosphoric acid solution containing 5 mM EDTA. After centrifugation (10,000 × g, 20 min), 50 μL of 12 mM chromogenic reagent in 0.2 M HCl was then added to the resulting supernatant (300 μL) and mixed thoroughly. After 50 μL of 7.5 mM NaOH was added and mixed, the mixture was incubated at 25°C for 10 min, and then absorbance was determined spectrophotometrically at 400 nm.

Mitochondrial Preparation, Measurement of Respiration, and Mitochondrial Sculling Assay—Rat liver mitochondria were prepared by the method of Schneider (23) using 0.25 M sucrose solution containing 0.5 mM EDTA and 10 mM Tris-HCl (pH 7.4). The respiratory of mitochondria was measured using a Galvani-type oxygen electrode (Eijima Electronic Mfg. Co. Ltd.). The reaction medium was essentially composed of 0.15 M KCl, 5 mM MgCl2, 1 mM EDTA and 20 mM Tris-HCl in a final volume of 2 ml (pH 7.4). Reaction was initiated after saturating O2 in the reaction medium at 30 °C and was carried out at the same temperature. State 3 respiration (ADP-driven respiration), state 4 respiration (respiration without ADP), RC index (ratio of state 3 to state 4 respiration), and P/O ratio (ratio of added ADP to the amount of oxygen consumed during state 3 respiration) were calculated from an oxynograph according to the method of Chance and Williams (24). Inhibitory effect of BITC on state 3 respiration was expressed by the relative ratio of oxygen consumption rate of BITC-treated mitochondria and that of a control; inhibition (%) = [(control) – (BITC)]/(control) × 100. Mitochondrial swelling was photometrically measured following the absorbance decrease at 520 nm as previously reported (25).

The reaction medium essentially contained 0.15 mM KCl, 1 mM EGTA, 20 mM Tris-HCl, and 0.3 mg of mitochondrial protein in a final volume of 2 ml (pH 7.4), and the reaction was carried out in a glass cuvette mounted on the recording-spectrophotometer (Uvisec 610C, JASCO, Japan).

Cytochrome c Determination—Mitochondria (0.3 mg of mitochondrial protein), resuspended in the reaction medium (0.15 mM KCl, 20 mM Tris-HCl, and 1 mM EGTA, pH 7.4), were stimulated by BITC (100 μM) and then incubated for 15 min at room temperature with magnetic stirring. The mitochondrial suspension was centrifuged at 12,000 rpm for 20 min, and the supernatant solution was supplied for the detection of cytochrome c release. Cytochrome c reduced by sodium dithionite was detected by measuring the visible absorption at 550 nm. The final result was obtained by subtracting the absorbance of a reference tube containing mitochondria without BITC stimulation.

Intracellular ROS Determination—Intracellular ROS were detected by H2DCF-DA and DHE as an intracellular fluorescence probe (14, 15). H2DCF-DA is oxidized by H2O2 and lipid hydroperoxide (HPO) to yield fluorescent dichlorofluorescein (DCF). DCF is oxidized by O2 to yield fluorescent ethidium. Briefly, the cells under confluency were treated with BITC at various concentrations for 15 min at 37°C. After washing with PBS twice, H2DCF-DA (50 μM) or DHE (50 μM) was applied to the cells, which were incubated for 15 min. A flow cytometer (CytoACE 150, JASCO, Tokyo, Japan) was used to detect DCF and ethidium.

Protein Quantitation—Concentration of proteins was determined with the BCA protein assay reagent (Pierce) with bovine serum albumin as the standard.

RESULTS

Induction of Apoptosis and Necrosis by BITC in a Dose-depended Manner—We characterized the cytotoxic response of RL34 cells to BITC. Although distinctive apoptotic changes were detectable by 3 h (data not shown), maximal induction was observed at 8 h after treatment. Flow cytometric analysis of the cells treated with 20 μM BITC using annexin V-FITC conjugate showed that phosphatidylserine, normally confined to the inner leaflet of the plasma (26), appeared on the outer surface of 35% of the cells, characterized by annexin (+) and PI (−) staining pattern (Fig. 1B). The exclusion of PI showed that the cells maintained their plasma membrane integrity at this stage; however, higher concentrations of BITC (50 μM) led to the appearance of necrotic cells (Fig. 1C; annexin (−), PI (+)). On the other hand, the cells treated with BITC (20 μM) for 24 h exhibited severe membrane damage and were stained using both annexin V-FITC conjugate and PI (Fig. 1D; annexin (+), PI (+)). The apoptotic cell death by BITC at 20 μM was also confirmed by nuclear morphology using a fluorescent DNA-binding agent, Hoechst 33258 (Fig. 2). Thus, the present flow cytometric study clearly demonstrated a dose-dependent change in cell death pattern induced by BITC. The narrow threshold of BITC to exhibit apoptosis was consistent with the results previously reported (9).

Involvement of the Mitochondrial Death Pathway in BITC-induced Apoptosis—Caspases are believed to play a central role in mediating various apoptotic responses. Caspases are activated in a sequential cascade of cleavages from their inactive forms (27-29). As shown in Fig. 3A, caspase-3 activity in the BITC-treated cells was detectable using a DEVD-related mCherry conjugated to the chromophore pNA. At 20 μM BITC, there was a 3-fold increase in the rate of DEVD-pNA cleavage compared with control cells. Cleavage was inhibited by treatment with an inhibitor of caspase-3-like proteases, DEVD-fmk (Fig. 2A), indicating that it was specific for caspase-3 or caspase-3-related activities. The question remains as to how caspase-3 or...
caspase-3-relating proteases are activated by BITC. In a variety of cell deaths, a caspase-1/ICE-like protease was found to activate CPP32-like proteases (27, 28). In fact, ICE can directly activate CPP32 \textit{in vitro} (30). In contrast to the large peak of DEVD specific activity, little caspase-1 specific activity (data not shown) and no inhibitory effect of a caspase-1 inhibitor YVAD-fmk on caspase-3 activity were observed (Fig. 3A). These results suggested that a caspase-1/ICE-like protease might be ruled out as the cause of the caspase-3-dependent apoptosis induced by BITC. On the other hand, c-Jun NH$_2$-terminal kinase (JNK) activation is involved in ITC-induced caspase-3 activity and apoptosis (10). ITC-induced JNK activation could be inhibited by overexpression of Bcl-2 and exogenous antioxidants (10). This led us to the hypothesis that the mitochondrial death pathway may be involved in BITC-induced apoptosis. 

In \textit{vitro} studies have previously identified Apaf1, cytochrome c, and caspase-9 as participants in a complex important for caspase-3 activation (31). Therefore, we examined the participation of caspase-9 in apoptosis induced by the treatment of cells with BITC. As shown in Fig. 3B, an inhibitor of caspase-9, LEHD-fmk had a significant inhibitory effect on BITC-induced caspase-3 activity. As shown in Fig. 3B, inhibitors of caspase-3 and 9 also completely protected BITC-induced apoptosis.

There is increasing evidence that an altered mitochondrial function is linked to apoptosis and a decrease in $\Delta V_{m}$, mediated by a polyprotein channel called the permeability transition (PT) pore including the voltage-dependent anion channel (32), is associated with mitochondrial dysfunction. It has been sug-
As shown in Fig. 4, the addition of BITC for 30 min substantially induced a loss of $\Delta \Psi_m$ in a dose-dependent manner. The mitochondrial uncoupler, carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), used as a positive control for mitochondrial uncoupler, carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), used as a positive control for $\Delta \Psi_m$ disruption, also induced a significant change in $\Delta \Psi_m$.

BITC Directly Modifies Mitochondrial Functions and Induces Cytochrome c Release—Following a diverse array of apoptotic stimuli, cytochrome c is released from the mitochondria to the cytosol where it participates in activating the caspase cascade leading to cell death. Vander Heiden et al. (35) have recently provided evidence that, during apoptosis, there is a disruption of the mitochondrial membrane, which may be responsible for cytochrome c release. One way this could happen would be through induction of swelling of the membrane until the membrane breaks, as was suggested (35). Cell-free systems have been extremely valuable for the analysis of apoptosis mechanisms, including modification of mitochondrial respiration, mitochondrial depolarization, and cytochrome c release (34, 36). We therefore asked whether BITC is able to induce cytochrome c release in vitro and whether this directly or indirectly links to another function of the mitochondria. As shown in Fig. 5A, we found an absorption peak at about 550 nm in the supernatant from mitochondria, consistent with the known typical absorption peak of reduced cytochrome c. Control mitochondria did not release cytochrome c during a 15-min incubation (data not shown). Next, we examined mitochondrial swelling as a function of light scattering. As shown in Fig. 5B, BITC induced rapid swelling of the mitochondria in vitro in a dose-dependent manner. Because one of the best indicators of the mitochondrial PT is swelling of the organelle, our results provide evidence that BITC may induce cytochrome c release in vivo through a morphological and functional alteration of the mitochondrial inner membrane.

Because of the relevance of mitochondrial function to BITC-induced apoptosis, we planned to analyze the effects of BITC on the respiratory parameters of isolated mitochondria. BITC suppressed the ADP-derived state 3 respiration with complex I-linked substrates, whereas it produced only a slight alteration of the respiration with complex II-linked substrates (Fig. 6A). As shown in Fig. 6B, the representative oxygraph of the respiration with complex I-linked substrates demonstrated that state 4 respiration was not significantly changed by the concentrations of BITC tested. It should be noted that, although BITC significantly attenuated the RC index (ratio of state 3 to state 4 respiration) due to suppression of state 3 respiration, the P/O ratio (ratio of added ADP to the amount of oxygen consumed during state 3 respiration) was invariant. Addition of the uncoupler dinitrophenol restored BITC-inhibited respiration to control the state 3 rate, and BITC did not alter the succinate oxidase and NADH oxidase of submitochondrial particles (data not shown), indicating that BITC is able to suppress the energy transduction system producing ATP, whereas complex I or complex II through complex IV of the respiratory electron transport chain is not a target of BITC. The potential remaining targets of BITC-mediated inhibition of state 3 respiration include P$_i$ transport, substrate transport, the adenine nucleotide translocation, or citric acid cycle enzymes. Respiratory impairment by a thiol-alkylating agent in hepatic mitochondria has been reported to occur in part by interrupting P$_i$ transport (37). As the preincubation period of BITC with mitochondria was increased, stronger inhibitory effects on ADP-derived state 3 respiration were observed (data not shown). These results suggested that respiratory modulation by BITC in mitochondria may be responsible for mitochondrial swelling and/or cytochrome c release.
Redox-regulated Apoptosis Induced by Benzyl Isothiocyanate

Involvement of Redox Alteration in BITC-induced Apoptosis—We have recently shown that the pretreatment with diethyl maleate (DEM), which blocks the intracellular thiol groups including mitochondrial GSH, produced intracellular peroxides and enhanced the BITC-induced oxidative stress (14). The level of intracellular GSH fell to ~35% of the control level after treatment of the cells with 1 mM DEM (Fig. 7A). Buthionine sulfoximine, a specific inhibitor of cytosolic GSH synthesis, also significantly reduced the intracellular GSH level (38) but did not enhance apoptotic responses (data not shown). Therefore, we examined the effects of GSH depletion by DEM pretreatment on BITC-induced apoptosis. As shown in Fig. 7B, optimal apoptosis induction was observed in normal cells at 20 μM BITC, followed by a decline in activity at higher concentrations. Optimal caspase activation also occurred at 20 μM BITC, whereas above 50 μM, caspase-3 activity dropped below the background activity seen in control cells (Fig. 7C). The effective concentration of BITC required to activate a caspase-3-like protease closely paralleled that for apoptosis, also supporting the assumption of involvement of a caspase-3 (like) pathway. The cells treated with 1 mM DEM alone for 1 h did not show any significant cytotoxic effect or caspase activation (Fig. 7, B and C). GSH-depleted cells required lower concentrations of BITC (5–10 μM) to induce apoptosis than normal cells (Fig. 7B). In GSH-depleted cells, a caspase-3-like protease was also activated in cells cultured with 5–10 μM BITC; however, the enzymatic activity could not be detected in cells with more than 50 μM BITC (Fig. 7C). Thus, the threshold of BITC required to exhibit apoptosis was significantly decreased in the DEM-treated cells, suggesting that intracellular GSH may play a negatively regulating role in BITC-induced cell death.

ROS are regarded as important regulators of apoptosis. Pro-oxidants and redox cycling agents including H2O2 (39) and semiquinones (40) can induce apoptosis. Other apoptotic stimuli such as TNF-α (41) and ceramide (42) can stimulate ROS production. Studies with TNF-α and ceramide have shown that these agents activated ROS generation from the mitochondria (42). In addition, it has been reported that protection from apoptosis was associated with the overexpression of mitochondrial glutathione peroxidase (43). Thus, ROS, and the resulting cellular redox change, can be part of the signal transduction pathway during apoptosis. Therefore, we determined the intracellular levels of superoxide and HPO using specific fluorescent indicators. Rapid and significant increases in the levels of intracellular O2− and HPOs, including hydrogen and lipid peroxides, were observed after exposure of the cells to BITC (Fig. 8A). This induction of ROS production is time- and dose-dependent. Although a receptor-mediated apoptosis inducer, including TNF-α, induces ROS production after a couple of hours after treatment with cells (44), the intracellular ROS accumulation was observed within 15 min of exposure to BITC.

Based on the fact that O2− is rapidly dismutated to H2O2, a...
possible involvement of H$_2$O$_2$ in the BITC-induced intracellular HPO production was assessed with the cells pretreated with a cell-permeable scavengers. Flow cytometric analysis revealed that the $O_2^-$ scavenger, Tiron, and a substrate of GSH synthesis, N-acetylcysteine (NAC), substantially attenuated HPO accumulation by $\sim$80 and 60%, respectively, while BITC-induced HPO accumulation was observed regardless of the presence of ONOO$^-$ scavenger, uric acid, or an hydroxyl radical scavenger, mannitol (Fig. 8B). These results suggested that excessive intracellular H$_2$O$_2$, comprising most of the intracellular HPOs and being derived from $O_2^-$, was possibly produced from the mitochondria.

**DISCUSSION**

Diverse types of cancer chemopreventive agents, including naturally occurring and pharmaceutical compounds, have been studied for efficacy in vitro and in vivo. Among the most extensively investigated are the ITCs, which occur naturally in a variety of cruciferous vegetables. BITC is an ITC that has been isolated from papaya fruit as the major phase II enzyme inducer present in organic solvent extracts of this fruit (15). Our isolated rat mitochondria (Figs. 5 and 6). Following a diverse array of apoptotic stimuli, cytochrome c is released from the mitochondria to the cytosol where it participates in activating the caspase cascade leading to cell death. The results in the present study thus provide evidence that cytochrome c release, through a morphological and functional alteration of the mitochondrial membrane, may be potentially involved in BITC-induced apoptotic responses.

Zhang and Talalay (38) have quantified the intracellular accumulation of ITCs measured by a probe specifically reacted with ITCs. When murine hepatoma cells were exposed to BITC for 30 min, the intracellular concentration of BITC was increased up to 300 $\mu$M (38). Therefore, it is very likely that BITC at a relatively higher concentration ($\sim$300 $\mu$M) is localized around the mitochondria. This speculation was also supported by the present findings that treatment with BITC induced a rapid $\Delta V_m$ alteration accompanied by ROS generation. The change in redox potential regulated by thiol (-SH/disulfide (-SS-) exchange or chemical modification of thiols may also at least in part serve as a regulator of BITC-induced apoptosis, because thiol antioxidants such as GSH, forming dithiocarbamates (R-NH-C=S)-SR by means of reaction with BITC, attenuate the intracellular active BITC level and several biological responses (14, 38). In the present study, the pretreatment with DEM, which also blocks intracellular thiol groups including mitochondrial GSH (43, 45), enhanced BITC-triggered apoptosis (Fig. 7B). The optimal concentrations of BITC required to enhance caspase activity were also lowered by DEM treatment (Fig. 7C). We more recently also observed that the pretreatment with NAC prevented apoptotic cell death by ITCs. A thiol-alkylating agent such as an $\alpha,\beta$-unsaturated aldehyde has been reported to induce respiratory impairment in rat brain mitochondria (36). Respiratory modulation in rat liver mitochondria by BITC is also likely the result of covalent at-

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2 Y. Nakamura, A. Yoshihiro, H. Ohigashi, and K. Uchida, unpublished data.

3 Y. Nakamura, N. Miyoshi, T. Osawa, and K. Uchida, unpublished data.
apoptosis occurs through a mitochondrial damage-dependent pathway. It should be noted that higher concentrations of BITC produce a large amount of ROS, resulting in severe oxidative damage to caspase activity and/or other cellular components. These oxidative phenomena may lead to necrotic cell death and thus damage to the surrounding cells. Although ITCs are promising and effective candidates as anticancer drugs, some ITCs showed enhancement of carcinogenicity or lack of chemopreventive effects in rat liver and kidney especially dosed at the post-initiation phase (52, 53). It is very likely that the intracellular ROS generation plays an important role in unfavorable side effects of BITC on carcinogenesis. Because the cancer preventive or promoting potential, threshold, and target organ have to be distinguished in detail, further mechanistic studies on intracellular oxidative stress and the following events induced by ITCs are necessary.

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Redox-regulated Apoptosis Induced by Benzyl Isothiocyanate

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