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1,4-Butanediyl-bismethanethiosulfonate (BMTS) Induces Apoptosis Through Reactive Oxygen Species-Mediated Mechanism

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ABSTRACT

Although methane sulfonate compounds are widely used for the protein modification for their selectivity of thiol groups in proteins, their intracellular signaling events have not yet been clearly documented. This study demonstrated the methane sulfonate chemical 1,4-butanediyl-bismethanethiosulfonate (BMTS)-induced cascades of signals that ultimately led to apoptosis of Jurkat cells. BMTS induced apoptosis through fragmentation of DNA, activation of caspase-9 and caspase-3, and downregulation of Bcl-2 protein with reduction of mitochondrial membrane potential. Moreover, BMTS intensely and transiently induced intracellular reactive oxygen species (ROS) production and ROS produced by BMTS was mediated through mitochondria. We also found that a reducing agent dithiothreitol (DTT) and an anti-oxidant *N*-acetyl cysteine (NAC) inhibited BMTS-mediated caspase-9 and -3 activation, ROS production and induction of Annexin V/propidium iodide double positive cells, suggesting the involvement of ROS in the apoptosis process. Therefore, this study further extends our understanding on the basic mechanism of redox-linked apoptosis induced by sulfhydryl-reactive chemicals. J. Cell. Biochem. 108: 1059–1065, 2009.

KEY WORDS: BMTS; APOPTOSIS; ROS

There are many sulfhydryl reactive chemicals, drugs, metal ions, and physical agents that are involved in the development of many disorders and diseases in human and animal model [Bagenstose et al., 1998; Asano et al., 2000; Mazumder et al., 2005; Luchese et al., 2007; Singh et al., 2007]. Paradoxically some sulfhydryl reacting chemicals such as arsenic is being used for treating acute promyelocytic leukemia for its strong ability to induce apoptosis [Soignet et al., 1998]. Due to their multi-dimensional effects on the development of human pathogenesis and on the treatment of diseases, sulfhydryl chemicals have created renewed attentions. Recent studies have revealed that sulfhydryl-reactive chemicals triggered signals to determine cell death or survival through modification of molecules by the oxidation of two free cysteine thiol (SH) groups on proteins to form disulfide bond [Akhand et al., 1998; Hossain et al., 2000, 2003]. Such modification triggered activation of protein tyrosine kinases followed by either activation of downstream signals for apoptosis or proliferation. Sulfhydryl reacting chemicals activate the multiple intracellular signaling pathways depending upon the duration of stimulation, concentration of chemicals and types of cells. Sulfhydryl-reactive chemicals may regulate the expression and amplification of genes, and determine cell death or survival involving the modulation of

Abbreviations used: AIF, apoptosis inducing factor; BMTS, 1,4-butanediyl-bismethanethiosulfonate; DTT, dithiothreitol; HE, Hydroethidine; NAC, *N*-acetyl cysteine; ROS, reactive oxygen species.

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cell surface molecules and or the redox status of the cells. We previously found that arsenic, a potent sulfhydryl-reactive chemical, and a notorious environmental pollutant, produced reactive oxygen species (ROS) through the aggregation of cell surface molecules in a redox-linked manner that played a pivotal role for generating and transmitting intracellular signals [Hossain et al., 2000, 2003].

Many sulfhydryl reactive chemicals have long been used for the chemical modification of peptides and proteins [Kenyon and Bruice, 1977; Akhand et al., 2002] and for the study of redox-linked signaling events [Nakashima et al., 1994; Akhand et al., 1998]. Methane thiosulfonate chemicals are best known for their high selectivity for sulfhydryl groups and have been successfully used for structural and functional elucidation of a number of receptor proteins, including neuronal acetylcholine receptor [Akabas et al., 1992; Ramirez-Latorre et al., 1996], G-protein-linked dopamine receptor [Javitch et al., 1995] and NMDA glutamate receptor [Kuner et al., 1996]. In our previous study, we reported that 1,4-butanediyl-bismethanethiosulfonate (BMTS) induced sulfhydryl-linked aggregation of receptor-type protein tyrosine kinase, RET, that caused autophosphorylation and activation of RET kinase [Akhand et al., 2002]. In spite of the wide uses of methane sulfonate chemicals for protein modification, their potentiality for changing cellular oxidative and biochemical properties has remained largely unexplored. In this study, we for the first time showed that BMTS as a thiol reacting methane sulfonate chemical, induces ROS-mediated apoptosis in human leukemia Jurkat cells through mitochondrial damage.

MATERIALS AND METHODS

CELLS, REAGENTS, AND ANTIBODIES

Human T cell leukemia Jurkat cells were obtained from Japanese Cancer Research Resource Bank (Tokyo, Japan). For the experiment, 2×10^6 Jurkat cells were seeded in 24-well plates with 1 ml RPMI-1640 medium without fetal bovine serum (FBS) in the presence or absence of BMTS (Toronto Research Chemical, Inc., Ontario, Canada) at 37°C before analysis. Murine NIH3T3 cells were obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS. Hydroethidine (HE); 3,3′-dihexyloxacarbocyanin iodide ($DiOC_6$); dithiothreitol (DTT); *N*-acetyl cysteine (NAC); sodium arsenite; anti-β actin monoclonal antibodies were purchased from Sigma (St. Louis, MO). Anti-cleaved caspase-9, anti-caspase-3, anti-AIF, and anti-GAPDH were purchased from Cell Signaling (Beverly, MA). Anti-full-length caspase-9 antibody was purchased from Calbiochem and anti-Bcl-2 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Dihydrorhodamine 123 (DHR) was purchased from Cayman Chemical Co. (Ann Arbor, MI).

IMMUNOBLOTTING AND IMMUNOPRECIPITATION

SDS–PAGE and immunoblotting were performed as described previously [Nakashima et al., 1991]. For cytosolic fractions, cells were homogenized in buffer (20 mM HEPES–KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium-EGTA and 1 mM DTT, 250 mM sucrose and protease inhibitor cocktail). Supernatant was collected by pelleting at 17,000*g* for 20 min at 4°C. Lysates were immunoprecipitated overnight at 4°C with AIF antibodies, followed by 2-h incubation at 4°C with Protein A sepharose beads and washed three times in lysis buffer. Immune complexes were boiled in 2× sample buffer. Proteins thus obtained were subjected to SDS–PAGE. The proteins were then transferred to a PVDF membrane. Subsequently, the membrane was stained with different antibodies and then with anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase. The protein bands were visualized by the chemiluminescence reagent. Expression level of protein was analyzed using quantification software, CS Analyzer 2.0 (ATTO Corp., Tokyo, Japan).

CYTOFLUOROMETRIC AND MICROSCOPIC ANALYSIS OF ROS PRODUCTION

Generation of ROS was measured as described previously [Rothe and Valet, 1990; Zamzami et al., 1995] by incubating the cells with 2 μM HE for 15 min at 37°C followed by flow cytometric analysis. Mitochondrial ROS was also measured by incubation the cells with 10 μM dihydrorhodamine 123 (DHR) for 30 min at 37°C. Cytofluorometric analysis was performed by FACSCanto (Becton Dickinson, Mountain View, CA) using FL1 or FL2 signal detector for detection of DHR or HE oxidation, respectively. Images of cellular DHR fluorescence were acquired to visualize mitochondrial ROS production using a FluoView 1000 (Olympus, Tokyo, Japan) confocal microscope using a 60× objective with excitation at 488 nm and emission at 510 nm.

CYTOFLUOROMETRIC ANALYSIS OF MITOCHONDRIAL TRANSMEMBRANE POTENTIAL ($\Delta\psi$)

To evaluate mitochondrial transmembrane potential ($\Delta\psi$) cells were incubated for 15 min at 37°C in PBS containing 40 nM $DiOC_6$ [Zamzami et al., 1995]. Thereafter, cells were kept on ice until cytofluorometric analysis within 60 min. Analysis was performed by flow cytometry (excitation, 488 nm, emission, 525 nm) using FACSCanto.

ANALYSIS OF DNA FRAGMENTATION BY ELECTROPHORESIS

DNA fragmentation was detected as described previously [Herrmann et al., 1994; Dohi et al., 1996]. In brief, cells were pelleted by centrifugation. One hundred microliters of lysis buffer containing 10 mM Tris–HCl (pH 7.4), 10 mM EDTA and 0.5% Triton X-100 was added to the pellet and the preparation was left at 4°C for 15 min. After centrifugation at 20,000*g* for 20 min at 4°C, the supernatant was incubated at 37°C with 0.8 mg/ml of RNase A for 1 h followed by additional incubation for 1 h with 0.8 mg/ml of proteinase K at 55°C. Fragments of DNA were then precipitated with 20 μl of 5 M NaCl and 120 μl of 2-propanol and left overnight at −20°C. Following centrifugation for 20 min, DNA fragmentation was analyzed by using 1.4% agarose gel electrophoresis at 50 V for 1.5 h.

ANALYSIS OF APOPTOSIS BY ANNEXIN V AND PI STAINING

Annexin V-FITC apoptosis detection kit (BioVision, Mountain View, CA) was used according to the manufacturer's instructions. In brief, 1×10^5 NIH 3T3 cells were trypsinized and resuspended in 1× binding buffer. The cells were stained with Annexin V-FITC and

propidium iodide (PI), and then analyzed with a FACSCanto using FITC (FL1) and PI (FL2) signal detector.

RESULTS

DECREASE OF MITOCHONDRIAL MEMBRANE POTENTIAL BY A THIOL REACTIVE BMTS

We first investigated whether exposure of Jurkat cells to sulfhydryl group reactive chemical BMTS (Fig. 1A) could generate signals for inducing apoptosis. Exposure of cells to 10 μM of BMTS reduced the mitochondrial membrane potential which has been measured by staining the cells with fluorochrome $DiOC_6$ and analyzed by flow cytometry (Fig. 1B). Incorporation of $DiOC_6$ was reduced in BMTS-treated cells, which actually indicated the reduction of mitochondrial membrane potential. A substantial reduction in membrane potential (more than 41%) was observed at 2 h and it was almost 100% at 4 h of BMTS treatment suggesting that reduction of membrane potential by BMTS was an early event.

ANALYSIS OF APOPTOSIS BY BMTS

We next examined the effect of BMTS on the expression of Bcl-2 protein which is known as a negative regulator of cell death. As shown in Figure 2A (upper panel), 10 μM BMTS caused a gradual decrease in Bcl-2 expression with increasing time. It has been known that many oxidative chemicals induce apoptosis through the reduction of mitochondrial membrane potential, regulation of Bcl-2 family proteins, release of apoptosis inducing factor (AIF) and cytochrome *c* from the mitochondria, activation of caspase cascades and fragmentation of DNA [Hossain et al., 2000; Banerjee et al., 2008]. To characterize further the BMTS-induced apoptotic signal, we next performed time- and dose-dependent experiment to see whether BMTS could induce caspase activation. Caspase-9 is a member of caspase family proteins which is known to be degraded into smaller fragments upon activation. By using antibodies which could recognize 46 kDS full length and 35 kDa degraded fragment of caspase-9, we found that BMTS increased caspase-9 degradation in time- and dose-dependent manner (Fig. 2A, middle panel and B). Highest degradation was observed by 10 μM of BMTS within 8–16 h. Apoptosis induction was further confirmed by the detection of increased caspase-3 cleavage (Fig. 2A, lower panel). We also found that BMTS released AIF from the mitochondria to cytosol, suggesting that apoptosis induced by BMTS was involved in mitochondrial damage and activation of caspases (Fig. 2C). Agarose gel electrophoresis of fragmented DNA showed that BMTS induced fragmentation of DNA in a dose-dependent manner (Fig. 2D). Taken together, these data demonstrate that BMTS induced apoptosis via mitochondrial membrane depolarization and activation of caspases and fragmentation of DNA in Jurkat cells.

TRANSIENT PRODUCTION OF REACTIVE OXYGEN SPECIES IN CELLS BY BMTS

Previously we and other groups found that ROS production induced by oxidative stress acts as a second messenger for transducing intracellular signals [Kamata and Hirata, 1999; Hossain et al., 2000, 2003]. We later investigated whether BMTS could induce ROS production. BMTS-treated and -untreated cells were labeled with HE and analyzed by flow cytometry. HE is known to be oxidized by superoxide to ethidium, which emits red fluorescence. As shown in Figure 3A, the percentage of ethidium-containing cells clearly increased in 10 μM of BMTS-treated cells within 10 min compared to untreated cells. Maximum increase was observed at 1 h and then

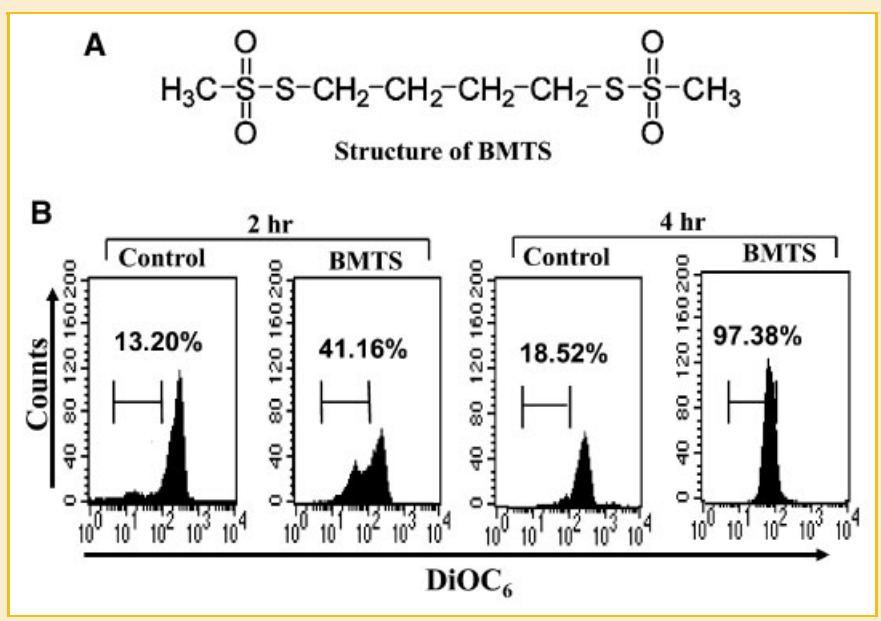

Fig. 1. Loss of mitochondrial membrane potential by 1,4-butanediyl-bismethanethiosulfonate (BMTS). A: Basic structure of BMTS. B: Assessment of mitochondrial transmembrane potential ($\Delta\psi$). Jurkat cells were treated with or without 10 μM of BMTS for indicated time at 37°C followed by staining with $DiOC_6$ (40 nM) and subjected to cytofluorometric analysis. Representative results of three experiments with consistent results are shown.

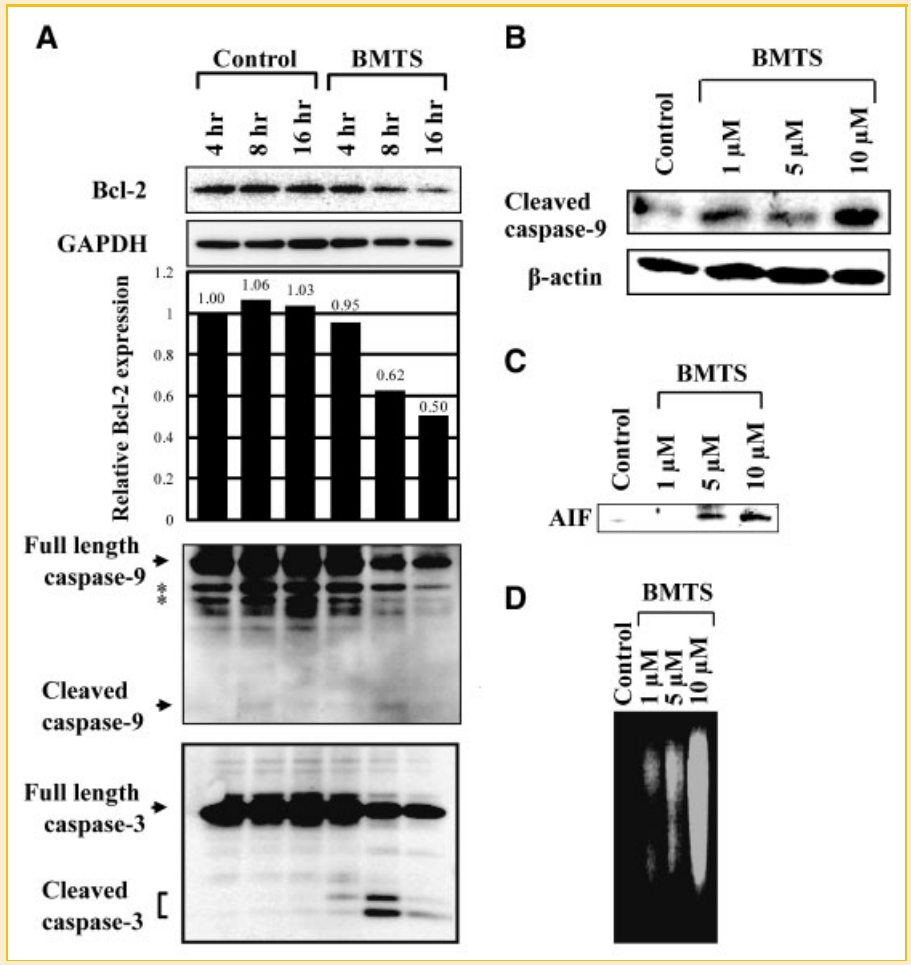

Fig. 2. Induction of apoptosis by BMTS. Jurkat cells were incubated with or without 10 μM of BMTS at 37°C for indicated time (A) or incubated with or without indicated concentrations for 16 h (B). Immunoblot assay was performed using anti-Bcl-2 or anti-caspase-9 and anti-cleaved caspase-9, or anti-caspase-3 antibodies which recognizes both full length and cleaved forms. Bar graph represents the relative Bcl-2 expression levels normalized to GAPDH as compared with control (4 h, DMSO), calculated by densitometric analysis of immunoblot. Asterisks indicate non-specific bands. The membranes were then stripped and reprobed with anti-GAPDH or β-actin antibody as internal controls (upper panel of A and B). Representative results of three experiments with consistent results are shown. C: Release of AIF into the cytosol by BMTS. Jurkat cells were incubated with or without BMTS at indicated concentrations for 16 h. Cytosolic fractions were separated and subjected to immunoprecipitation followed by immunoblotting with anti-AIF antibody. D: BMTS induces DNA fragmentation. Cells were incubated at 37°C with or without the indicated concentration of BMTS for 16 h. Fragmented DNAs were analyzed by agarose gel electrophoresis. Representative results of three experiments with consistent results are shown.

gradually decreased. These results suggested that BMTS, like other sulfhydryl-reactive chemicals, also promoted the production of ROS. Next, we explored how BMTS induced ROS in the cells. It is proposed that a small percentage (1–2%) of the electrons passing through the mitochondrial electron transport chain leak out and combine with molecular oxygen to form superoxide [Halliwell and Gutteridge, 1990; Davies, 1995]. As described above, BMTS decreased mitochondrial membrane potential, indicating that oxidative damage occurred in mitochondria. Therefore we hypothesized that BMTS promoted the production of mitochondrial ROS. To address this possibility, we used dihydrorhodamine 123 (DHR), an uncharged, non-fluorescent agent that can be converted by oxidation to the fluorescent laser dye rhodamine 123 which is used to detect mitochondrial reactive oxygen including super oxide anion. As shown in Figure 3B, some bright and condensed fluorescence spot, indicating oxidized DHR in mitochondria, was observed in BMTS-treated Jurkat cells by confocal microscopic analysis. Quantitative analysis by flow cytometry further revealed that BMTS induced DHR oxidation in a time-dependent manner (Fig. 3C). We also examined the possibility of the involvement of NADPH oxidase in BMTS-induced ROS production. The NADPH oxidase inhibitor diphenyleneiodonium (DPI) did not inhibit BMTS-induced ROS (data not shown) production, indicating that this enzyme might not be involved in the ROS production. Collectively, these results suggested that BMTS-induced mitochondria-mediated ROS production.

REDOX-LINKED MECHANISM OF APOPTOSIS BY BMTS

Interestingly, antioxidant NAC and reducing agent DTT clearly inhibited the BMTS-induced caspase-9 degradation as well as caspase-3 (Fig. 4A). Furthermore, pretreatment of NAC and DTT

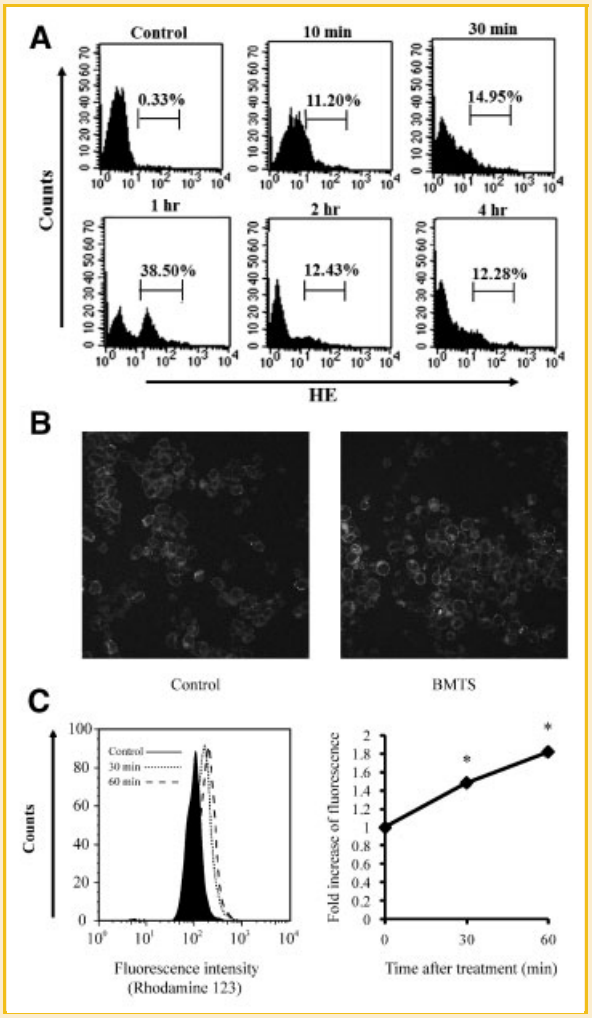

Fig. 3. Induction of intracellular ROS in Jurkat cells by BMTS. A: Jurkat cells were incubated with or without 10 μM BMTS at 37°C for indicated time. Cells were then labeled with HE and subjected to cytofluorometric analysis. Representative results of three experiments with consistent results are shown. B: DHR was used to visualize mitochondrial ROS. Representative confocal images showing the increase in DHR oxidation in Jurkat cells 30 min after exposure to 10 μM BMTS. C: Jurkat cells pre-incubated with DHR were then incubated with 10 μM BMTS for indicated time followed by flow cytometric analysis. The mean values ± SD determined in three independent experiments were obtained to calculate fold increase of fluorescence intensity in a time-dependent manner. $^{*}P < 0.005$ (Student's *t*-test) compared with the control (0 min, DMSO) value.

almost completely abrogated the ROS production as demonstrated by the drop of ethidium-containing cells to control level (Fig. 4B), suggesting the clear involvement of redox-linked mechanism in BMTS-mediated apoptosis. To ask whether BMTS had same effect on other cell line, we used murine fibroblast NIH3T3 cells. BMTS induced apoptosis as shown by increased Annexin V and propidium iodide positive cells, and consistent with Figure 4A,B, both NAC and DTT significantly inhibited apoptosis (Fig. 4C). These results indicate that ROS production by BMTS is a common phenomenon for the induction apoptosis in a variety of cells.

DISCUSSION

In this study we demonstrated that BMTS, a sulfhydryl reactive chemical induced apoptosis through the reduction of mitochondrial membrane potential, downregulation of Bcl-2 protein with the concomitant release of AIF, activation of caspases and finally fragmentation of DNA. We also found that BMTS-induced mitochondrial ROS production for the induction of apoptosis.

The methane sulfonate compounds are highly selective for sulfhydryl group. It can cross-link many signaling molecules through the binding of sulfhydryl group of protein [Akhand et al., 2002]. Usually sulfhydryl reactive chemicals such as mercury and arsenic induce apoptosis through the activation of caspases and fragmentation of DNA. Many studies are converging on a central hypothesis that, in many cell death cascades, the mitochondria and the nucleus play key roles in conducting cell death programs [Zamzami et al., 2000; Wang et al., 2002; Arnoult et al., 2003]. Decreased mitochondrial membrane potential promotes the release of mitochondrial death factors which ultimately lead to the DNA fragmentation and cell death [Joza et al., 2001; Li et al., 2001; Arnoult et al., 2003]. Decrease mitochondrial membrane potential is therefore an early event of apoptosis. In this study we also found that BMTS induced the reduction of mitochondrial membrane potential with in short time of BMTS challenge (Fig. 1B). This mitochondrial membrane potential reduction was followed by the reduction of Bcl-2 protein expression and AIF release (Fig. 2A,C). Oxidative elements especially sulfhydryl group modifying agents transduce the intracellular signals through the production of ROS which act as second messenger for determining the fate of cells. Like other oxidative element, in this study we also found that BMTS-induced ROS production (Fig. 3A) ultimately led to the cell death as the reducing agent DTT and antioxidant NAC almost completely abrogated BMTS-induced caspase activation (Fig. 4A) as well as apoptosis (Fig. 4C). DTT and NAC also inhibited the BMTS-induced ROS production. We also investigated site or sources of ROS production by BMTS. Previously it has been reported that mitochondria and the Nox family of membrane NADPH oxidase have emerged as major sources of ROS induction [Fleury et al., 2002; Lambeth, 2004]. Intriguingly, in this study we found that BMTS-induced ROS production through mitochondria (Fig. 3B,C) as BMTS induced DHR oxidation in a time-dependent manner. Pretreatment of cells with DPI, an inhibitor NAPDH oxidase, could not inhibit the ROS production at all (data not shown) which ruled out the possibility of the involvement of NADPH oxidase in this process. Although the precise mechanism of action of BMTS on mitochondria remains elusive, these results clearly indicated that ROS produced in the mitochondria acted as messenger for inducing BMTS-mediated cell death. This result is different from the other sulfhydryl group chemicals such as arsenic in which previously we found that arsenic-induced ROS production was a cell surface event [Hossain et al., 2000, 2003]. The possible explanation of this difference is that BMTS contains a long hydrocarbon backbone in its structure which can more rapidly and efficiently penetrate BMTS inside the cells through the hydrophobic portion of cell membrane compared to other oxidative chemicals such as arsenic. We suppose that after entering the cells BMTS can directly target the mitochondria for ROS

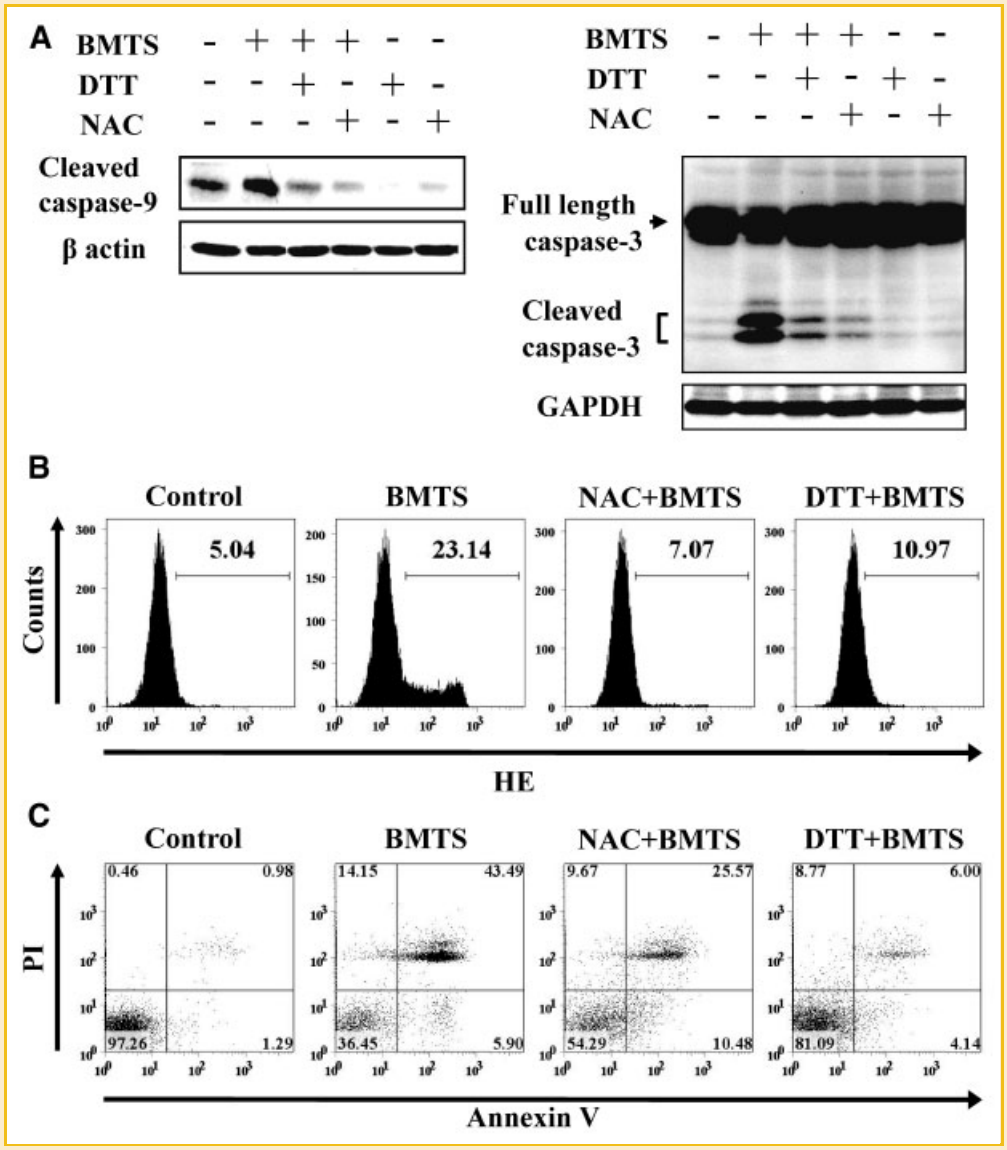

Fig. 4. BMTS induces apoptosis through a redox-linked mechanism. A: NAC and DTT inhibit BMTS-induced degradation of caspase-9 and caspase-3. Jurkat cells were incubated with or without 10 μM BMTS at 37°C for 16 h. In some groups DTT (500 μM) or NAC (10 mM) were added 1 h before the addition of BMTS. The cells were then lysed and subjected to immunoblot assay with anti-cleaved caspase-9 (left panel) or anti-caspase-3 (right panel) antibody. The membranes were then stripped and reprobed with anti-β-actin or anti-GAPDH antibody. B; NAC and DTT inhibit BMTS-induced ROS production. Jurkat cells were incubated with or without 10 μM BMTS at 37°C for 1 h. In some groups NAC or DTT were added 1 h before the addition of BMTS. Cells were then labeled with HE and subjected to cytofluorometric analysis. Representative results of three experiments with consistent results are shown. C: NAC and DTT inhibit BMTS-induced apoptosis in NIH3T3 cells. Annexin V and PI staining were measured as indicated in the Materials and Methods Section. NIH3T3 cells were pretreated with or without NAC or DTT for 1 h before addition of 10 μM BMTS at 37°C for 1 h. Viable cells were Annexin V negative/PI negative (lower left quadrant), early apoptotic cells were Annexin V positive/PI negative (lower right quadrant), and necrotic or late apoptotic cells were Annexin V positive/PI positive (upper right quadrant). Representative results of three experiments with consistent results are shown.

production for the induction of apoptosis. In many cases, intracellular signaling events induced by a chemical/stress are not always common phenomenon for all cell types such as arsenic induces apoptosis in leukemia cells more efficiently compared to the solid tumor cell lines. We found that BMTS induced ROS production and apoptosis in murine NIH3T3 cells as it did in human Jurkat cell lines. From the results of two cell lines tested in this study it can be assumed that BMTS-induced ROS production and apoptosis are common phenomena irrespective of cell types.

Taken together, our results demonstrate for the first time that BMTS induces apoptosis through redox-linked mechanism in which mitochondria play a pivotal role for the production of ROS which further extends and diversifies our understanding on the basic intracellular mechanism of sulfhydryl-reactive chemicals.

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