

Dithiothreitol Enhances Arsenic Trioxide-Induced Apoptosis in NB4 Cells

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ABSTRACT

Recently, arsenic trioxide (As_2O_3) was reported to induce clinical remission in patients with acute promyelocytic leukemia. Modulation of protein phosphorylation by binding to the vicinal thiols has been suggested as a possible mechanism. We found that phenylarsine oxide, a strong vicinal thiol-binding agent, neither induced nuclear fragmentation or DNA laddering nor increased caspase activity in NB4 cells; however, As_2O_3 and a weak thiol-binding agent, dimethylarsinic acid, did increase activity. Dithiothreitol (DTT) effectively suppressed the phenylarsine oxide-inhibited cellular reductive capacity, but unex-

pectedly, enhanced As_2O_3 -induced apoptosis in NB4 cells. As_2O_3 -induced and As_2O_3 -plus-DTT-induced apoptosis in NB4 cells was modulated by oxidant modifiers, but not by nitric oxide synthase inhibitors. These results demonstrate that DTT, a dithiol agent and known antidote for trivalent inorganic arsenic, enhances the toxicity of As_2O_3 , thereby opening a new research direction for the mechanisms of arsenic toxicity and perhaps also helping in the development of new therapeutic strategies for treating leukemias.

Recently, the inorganic arsenical arsenic trioxide (As_2O_3) was reported to induce complete remission in a high proportion of patients with refractory acute promyelocytic leukemia (APL) (Shen et al., 1997; Huang et al., 1998; Soignet et al., 1998). Subsequently, As_2O_3 was shown to induce apoptosis, down-regulate *bcl-2* gene expression, and modulate the PML/RAR α fusion protein in NB4 cells, an APL cell line (Chen et al., 1996). Because PML/RAR α , the oncogenic fusion protein, is linked to leukemogenesis and clinical sensitivity to all-trans-retinoic acid (Miller et al., 1992), it was hoped that As_2O_3 might target this oncogenic fusion protein and destroy leukemic cells specifically but leave normal cells unharmed. However, it was shown very recently that arsenic can inhibit cell growth and induce apoptosis independent of oncogenic fusion protein (Wang et al., 1998). In addition to As_2O_3 , sodium arsenite (Ma et al., 1998) and melarsoprol, an organic arsenical (Wang et al., 1998), have also been shown to induce apoptosis in NB4 cells. Moreover, melarsoprol and As_2O_3 can also inhibit growth and induce apoptosis in several chronic B-cell and myeloid leukemia cell lines (Konig et al., 1997; Wang et al., 1998). Therefore, the sunny side of this story is that arsenical compounds may be more broadly used for treatment of leukemias other than APL and continuous research along these lines may lead to a new pathway for killing leukemic cells.

The arsenite-induced apoptosis has been shown to be triggered by reactive oxygen species (Wang et al., 1996; Watson et al., 1996; Chen et al., 1998). Moreover, arsenite has been shown to induce DNA strand breaks, stimulate poly(ADP-ribosylation) and induce micronuclei. These seem to be caused by the generation of nitric oxide and superoxide (Gurr et al., 1998; Lynn et al., 1998). Because severe DNA damage may result in apoptosis, it is also possible that arsenic compounds may trigger apoptosis by increasing cellular nitric oxide and superoxide levels. However, a more popular hypothesis comes from the well established fact that trivalent inorganic arsenicals have high affinity to vicinal thiols. Trivalent inorganic arsenicals are sulfhydryl-complexing agents that exert many of their acute toxic effects by inhibiting the pyruvate dehydrogenase multienzyme complex and, consequently, the mitochondria-based citric acid cycle (Aposhian and Aposhian, 1989). Arsenite has also been shown to complex with the lipoic acid of pyruvate dehydrogenase (Aposhian and Aposhian, 1989) and with three closely spaced cysteines on the rat glucocorticoid receptor (Chakraborti et al., 1992). The binding affinity of arsenite was shown to be inversely related to the distance between the two thiol groups (Delnomdedieu et al., 1993). The active sites of many phosphatases contain adjacent sulfhydryl residues (Cavigelli et al., 1996). Many phosphotyrosine phosphatases behave as vicinal thiol proteins and require dithiothreitol (DTT) for activity measurements in vitro. They are inhibited

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ABBREVIATIONS: APL, acute promyelocytic leukemia; DTT, dithiothreitol; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; pNA, *p*-nitroanilide.

by oxidation and by phenylarsine oxide, a vicinal thiol-specific reagent (Gitler et al., 1997). Therefore, As_2O_3 may modulate protein phosphorylation to induce apoptosis (Chen et al., 1997).

Initially, we conducted some experiments to test the hypothesis that binding to the vicinal thiols and modulating protein phosphorylation are involved in As_2O_3 -induced apoptosis in NB4 cells. During these experiments, we found that phenylarsine oxide, a strong vicinal thiol-binding agent and an inhibitor of protein phosphatase, did not induce apoptosis; unexpectedly, DTT, a dithiol compound that was supposed to compromise the activity of As_2O_3 , enhanced As_2O_3 -induced apoptosis in NB4 cells. In this article, we have gathered data from nuclear fragmentation, DNA laddering, and caspase activity to verify this unexpected result.

Materials and Methods

Cells and Chemicals. NB4 cells (kindly provided by Dr. C. Y. Liu of Veterans Hospital, Taipei, ROC) were cultured in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 0.03% glutamate. Cultures were incubated at 37°C in water-saturated atmosphere containing 5% CO_2 .

Acetic acid, As_2O_3 , ethanol, β -mercaptoethanol, and trichloroacetic acid were purchased from Merck (Darmstadt, Germany); phenylarsine oxide was purchased from Aldrich Chem. Co. (Milwaukee, WI); DTT was purchased from Bio-Rad (Hercules, CA); N_ω -nitro-L-arginine methyl ester, *S*-methyl-L-thiocitrulline chloride, and Sybr Green were purchased from Molecular Probes Co. (Eugene, OR); diethyldithiocarbamic acid, dimercaptosuccinic acid, dimercaptopropionol, sulforhodamine B, dimethylarsinic acid, mercaptosuccinic acid, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), paraquat, sodium nitrosopruesside, and sodium selenite were purchased from Sigma Co. (St. Louis, MO); glutathione was purchased from Boehringer Mannheim (Mannheim, Germany); and sodium pyruvate was purchased from Calbiochem (San Diego, CA).

Flow Cytometric Determination of subG1 Cells. The procedure described previously (Wang et al., 1996) was followed. Briefly, 1×10^6 cells were treated with chemicals for 24 h, washed with PBS, fixed with 70% ethanol, and stored at -20°C overnight. The cells were then collected by centrifugation and extracted with 40 μl of phosphate-citric acid buffer at room temperature for 1 h. The extracted cells were stained with a propidium iodide solution and the fluorescence intensities of 10,000 cells were measured by an EPICS XL-MCL flow cytometer (Coulter, Miami Lake, FL) with excitation at 488 nm and emission at 620 nm.

Determination of Nucleus-Fragmented Cells. Cells treated with chemicals for 24 h were fixed at -20°C in 70% ethanol overnight, collected by centrifugation, and resuspended in an ethanol-acetate mixture (3:1, v/v). Cell suspensions were dropped onto a glass slide and air-dried. The slides were stained with 40 \times diluted Sybr Green solution. The slides were examined under an epifluorescence microscope with a 365-nm excitation filter, a 400-nm dichroic mirror, and a 435-nm barrier filter. For each treatment, the nuclear integrity of 500 cells was examined.

Determination of Cellular Reductive Capacity. Cellular reduction of the tetrazolium salt MTT was carried out according to the procedure of Mosmann (1983) with some modification. Briefly, 6×10^4 NB4 cells were treated with chemicals for 4 or 0.5 h, as indicated. They were then collected by centrifugation, washed twice with PBS, and resuspended in 0.5 ml of medium. An aliquot of 125 μl of 2 mg/ml MTT was added, and the cells were reincubated at 37°C for 4 h. After washing with PBS, 800 μl of acidified isopropanol (0.04 N HCl in isopropanol) was added to dissolve the dark blue crystals. After centrifugation, the spectrophotometrical absorbance at 570 nm of

supernatant was read in a Hitachi (Tokyo, Japan) U-2000 spectrophotometer.

Determination of Cell Growth Inhibition. Cell-growth inhibition was determined by estimating the cell density at the end of a 48-h chemical treatment. The cell density was estimated by sulforhodamine B staining (Skehan et al., 1990). NB4 cells grown in suspension were harvested by centrifugation and fixed with 1 ml of 10% trichloroacetic acid (in PBS) at 4°C for 1 h. The cell suspension was poured onto a 0.45- μm GN-6 filter paper (Gelman Sciences, MI) connected to a suction pump. The filter membranes were washed three times with distilled water, stained with 0.4% sulforhodamine B for 30 min, and washed twice with 1% acetic acid. After air-drying, the membranes were washed with 10% Tris solution, pH 10.5, to dissolve the protein-bound sulforhodamine B. The attached cultures of human umbilical vein endothelial cells were washed twice with PBS, and fixed at 4°C for 1 h with 1 ml of 10% trichloroacetic acid. Dishes were washed three times with distilled water and stained with 1 ml of 0.4% sulforhodamine B for 30 min. Dishes were washed twice with 1% acetic acid, air-dried, and the protein-bound sulforhodamine B was dissolved by 1 ml of 10% Tris solution, pH 10.5. The absorbance was determined at 565 nm.

DNA Laddering Analysis. Cells (1×10^6) treated with chemicals for 24 h were fixed with 70% ethanol and stored at -20°C overnight. They were collected by centrifugation and extracted with 40 μl of phosphate-citric acid buffer at room temperature for 1 h. The extracted phosphate-citric acid buffer solution was vacuum-dried, and the powder was resuspended with 3 μl of 0.25% Nonidet P-40 and 3 μl of 1 mg/ml RNase, and was then incubated at 37°C for 30 min. Three microliters of 1 mg/ml proteinase K was added to the solution and incubated at 37°C for another 30 min. The mixture, together with 2 μl of 6 \times loading buffer, was loaded on 1.5% agarose gel containing 0.5 mg/ml ethidium bromide, and electrophoresed at 25 V. The DNA ladder was recorded with a Gel-DC 1000 image analyzer (Bio-Rad).

Caspase Activity Analysis. The caspase activity was measured with the ApoAlert CPP32/caspase-3 assay kit (Clontech, Palo Alto, CA). This assay used a synthetic tetrapeptide, Asp-Glu-Val-Asp (DEVD), labeled with a colorimetric molecule, *p*-nitroanilide (pNA), as substrate, and the protease activity was assayed by detection of the free pNA cleaved from the substrate (Gurtu et al., 1997). DEVD-pNA can be digested by caspase-3 and caspase-7 (Thornberry et al., 1997). Briefly, 1×10^6 NB4 cells were treated with chemicals for 18 h. After removing medium by centrifugation, the cells were resuspended in 50 μl of chilled cell-lysis buffer (provided in the kit) and incubated on ice for 10 min. The cell lysates were then collected by centrifugation at 12,000 rpm for 3 min at 4°C. DEVDase activities were tested by adding 50 μl of 2 \times reaction buffer (provided in the kit) and 5 μl of 1 mM conjugated substrate, DEVD-pNA (50 μM final concentration), and then incubated at 37°C for 1 h. Absorbance of these reaction mixtures were read in a Hitachi spectrophotometer model U-2000 at 405 nm.

Statistical Analysis. Results are expressed as mean and S.E.M. Statistical analyses were performed with Student's two-tailed paired *t* test and ANOVA when more than two treatments were compared. Values of *p* < .05 were considered statistically significant.

Results

As_2O_3 Induced Apoptosis in NB4 Cells But Phenylarsine Oxide Did Not. Because nuclear fragmentation is a hallmark of apoptosis, we examined the integrity of nuclei of NB4 cells treated with 2 μM As_2O_3 for various times. The results show that the proportion of cells with fragmented nuclei increased with the interval of exposure to As_2O_3 (Fig. 1A). With a 24-h As_2O_3 treatment, a concentration-dependent increase of nucleus-fragmented cells was observed (Fig. 1B). The apoptotic cells can be separated from normal cells by

their lower DNA content (subG1 cell population) after phosphate-citric acid buffer extraction. Flow cytometric analysis of DNA content showed that 2 μM As_2O_3 lowered cellular DNA content, and the percentage of cells with subG1 DNA content increased with increasing drug treatment time (Fig. 1C). With a 24-h As_2O_3 treatment, a concentration-dependent increase of subG1 cells was also observed (data not shown). These results are consistent with the reports that As_2O_3 effectively induces apoptosis in NB4 cells (Chen et al., 1996; Gianni et al., 1998; Shao et al., 1998). We then used phenylarsine oxide and dimethylarsinic acid to test the hypothesis that As_2O_3 may bind vicinal thiols and modulate protein phosphorylation to cause apoptosis. Phenylarsine oxide, a widely used protein tyrosine phosphatase inhibitor, is a trivalent arsenical compound that can react with two thiol groups of closely spaced protein cysteinyl residues to form stable dithioarsine rings. The complex cannot be reversed by monothiol, but in the presence of dithiols, such as 2,3-dimercaptopropanol or 1,4-dithiolthreitol, the binding is competitively reversed (Liao et al., 1991). Conversely, dimethylarsinic acid is a methylated pentavalent arsenical that has been shown to be a fairly weak enzyme inhibitor; it does not bind to proteins (Healy et al., 1997). Contrary to the dithiol-binding hypothesis, the results show that a 24-h treatment with phenylarsine oxide in the concentration range from 1 to 20 μM did not induce many nucleus-fragmented cells (Fig. 1D), but a 24-h treatment with dimethylarsinic acid in the con-

centration range from 0.25 to 5.00 mM induced nucleus-fragmented cells up to almost 100% (Fig. 1E).

We examined the inhibitory effects of these arsenical compounds on the cellular reductive capacity. Decreased cellular uptake of phenylarsine oxide may account for the lack of nuclear fragmentation. The reduction of the tetrazolium salt by actively growing cells to produce a blue formazan product has been shown to use NADH, NADPH, and succinate as substrates (Berridge and Tan, 1993). The results indicate that a 4-h treatment with phenylarsine oxide in concentrations >0.25 μM effectively decreased the cellular reductive capacity in NB4 cells. In contrast, there was no apparent decrease of cellular reductive capacity after a 4-h treatment with $\text{As}_2\text{O}_3 \leq 4$ μM or with dimethylarsinic acid ≤ 4 mM (Fig. 2A). These results suggest that reduced cellular uptake does not account for the lack of nuclear fragmentation of phenylarsine oxide. Moreover, DTT suppressed the inhibitory effect of phenylarsine oxide on cellular reductive capacity (Fig. 2B). Therefore, in the concentration range tested, phenylarsine oxide was effective in decreasing the cellular reductive capacity, but was ineffective in inducing apoptosis in NB4 cells. On the other hand, As_2O_3 and dimethylarsinic acid were effective in inducing apoptosis but not effective in inhibiting the cellular reductive capacity.

DTT Enhanced As_2O_3 -Induced Apoptosis. We reasoned that if binding to vicinal thiols is the key mechanism, then dithiol compounds would reduce As_2O_3 -induced apoptosis in NB4 cells. Therefore, the effect of DTT on As_2O_3 -induced apoptosis was examined. In an initial experiment, we observed that a 24-h treatment with DTT alone at a concentration >50 μM induced an appreciable amount of nucleus-fragmented cells (data not shown). Thus, lower concentrations of DTT were tested. Results presented in Fig. 3A show that DTT at concentrations below 25 μM did not induce nucleus-fragmented cells; however, instead of the expected

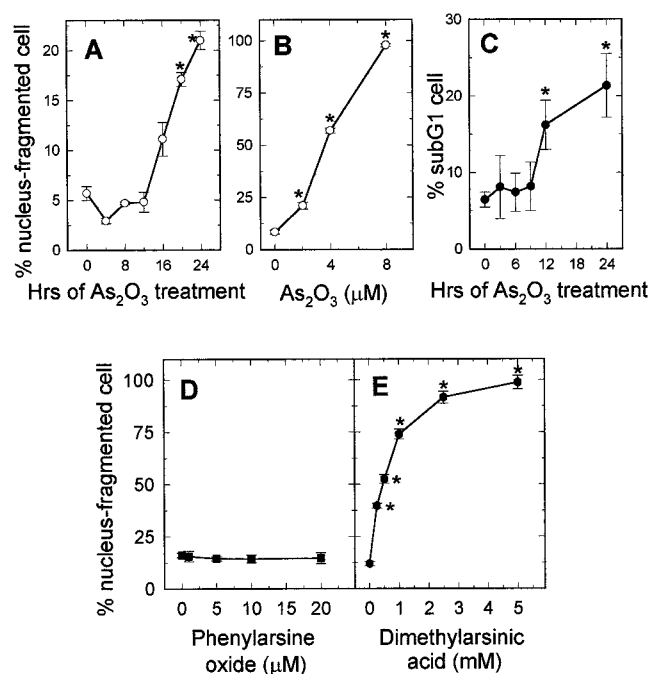


Fig. 1. Induction of apoptosis in NB4 cells by treatment with As_2O_3 , phenylarsine oxide, and dimethylarsinic acid. A, cells were treated with 2.0 μM As_2O_3 for various lengths of time. They were fixed, stained with Sybr Green, and observed under a fluorescence microscope as described in *Materials and Methods*. B, cells were treated with various concentrations of As_2O_3 for 24 h. C, cells were treated with 2.0 μM As_2O_3 for various lengths of time. After fixation and extraction, the DNA content of each cell was analyzed with a flow cytometer as described in *Materials and Methods*. D and E, NB4 cells were treated for 24 h with various concentrations of phenylarsine oxide or dimethylarsinic acid. The cells were fixed, stained with Sybr Green, and examined for nuclear integrity. The results are from three independent experiments. * $p < .05$ in Student's t test by comparing the values from treated with untreated cells.

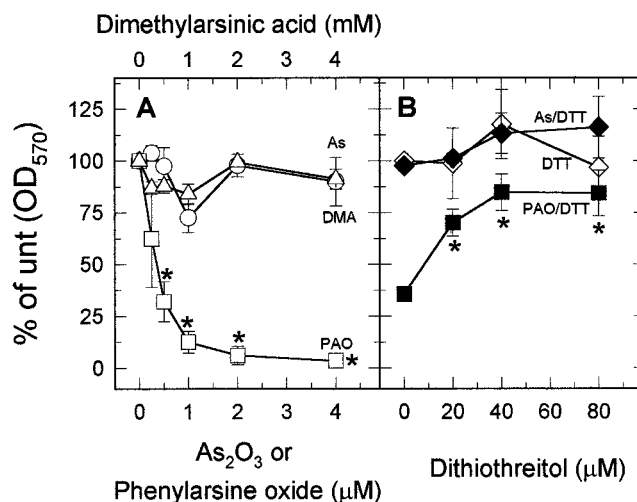


Fig. 2. Effects of treatment with As_2O_3 , phenylarsine oxide, dimethylarsinic acid, and DTT on cellular reductive capacity. A, NB4 cells were treated for 4 h with various concentrations of As_2O_3 (○), phenylarsine oxide (□), dimercaptosuccinic acid (Δ), or DTT (◇), or with 3.0 μM As_2O_3 plus various concentrations of DTT (◇). B, cells were treated for 0.5 h with 3.0 μM phenylarsine oxide plus various concentrations of DTT (◇). After treatment, the cells were subjected to MTT staining assay as described in *Materials and Methods*. Results are from three experiments. * $p < .05$ in Student's t test by comparing the values from treated versus untreated cells (A) or the values from phenylarsine oxide with DTT versus PAO without DTT (B).

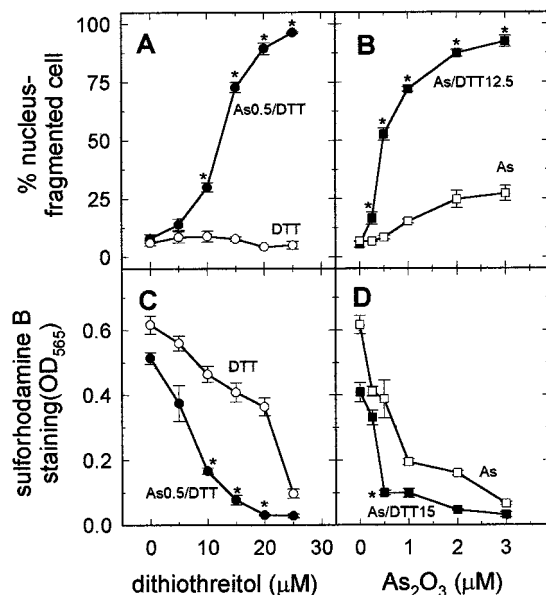


Fig. 3. Effects of treatment with As_2O_3 and DTT on nuclear fragmentation and growth in NB4 cells. Cells were treated for 24 h with various concentrations of DTT (○), 0.5 μM As_2O_3 plus DTT (●), various concentrations of As_2O_3 (□), or As_2O_3 plus 12.5 μM DTT (■). After fixation and staining, the cells were examined for nuclear integrity (A and B). Cells were treated for 48 h with various concentrations of DTT (○), 0.5 μM As_2O_3 plus various concentrations of DTT (●; As₂O₃/DTT), various concentrations of As_2O_3 (□; As), or various concentrations of As_2O_3 plus 15 μM DTT (■; As/DTT15). The cell density was estimated by sulforhodamine B staining as described in *Materials and Methods* (C and D). Results are from three experiments. **p* < .05 in Student's *t* test by comparing the value of treatment with As_2O_3 plus DTT to the additive value of As_2O_3 and DTT alone.

suppressive effect, in the concentration range from 12.5 to 25 μM, DTT enhanced As_2O_3 -induced nuclear fragmentation in a concentration-dependent manner (Fig. 3A). By varying the concentration of As_2O_3 , the enhancing effect of DTT on As_2O_3 -induced nuclear fragmentation was again observed (Fig. 3B). To confirm the observation that DTT enhances the toxic effect of As_2O_3 , we carried out a cell-growth inhibition test by treating NB4 cells for 48 h with various concentrations of As_2O_3 or DTT alone and in combination. The results indicate very clearly that DTT and As_2O_3 acted synergistically to inhibit the growth of NB4 cells and a 48-h treatment with DTT >25 μM or with As_2O_3 >3 μM decreased the cell number nearly to 0 (Fig. 3, C and D).

We then tested the effects of other thiol compounds on the As_2O_3 -induced nuclear fragmentation in NB4 cells. The results showed that in combined treatment with 0.5 μM As_2O_3 , no apparent enhancing effects were observed with monothiol compounds, such as β-mercaptoethanol or glutathione, or with a dithiol compound, such as dimercaptosuccinic acid (Fig. 4, A–C); however, the dithiol compound 2,3-dimercaptopropanol showed an enhancing effect (Fig. 4D). We also tested whether these thiol compounds could enhance apoptosis in combination with higher concentrations of As_2O_3 . The results indicate that dimercaptopropanol could enhance apoptosis in combination with As_2O_3 >0.5 μM, β-mercaptoethanol could enhance apoptosis in combination with As_2O_3 >1 μM, glutathione could enhance apoptosis in combination with As_2O_3 at 2 μM; however, dimercaptosuccinic acid did not show an enhancement (Fig. 4E). The effect of DTT in combination with sodium arsenite on the nuclear fragmentation in NB4 cells was also tested. The result indicates that

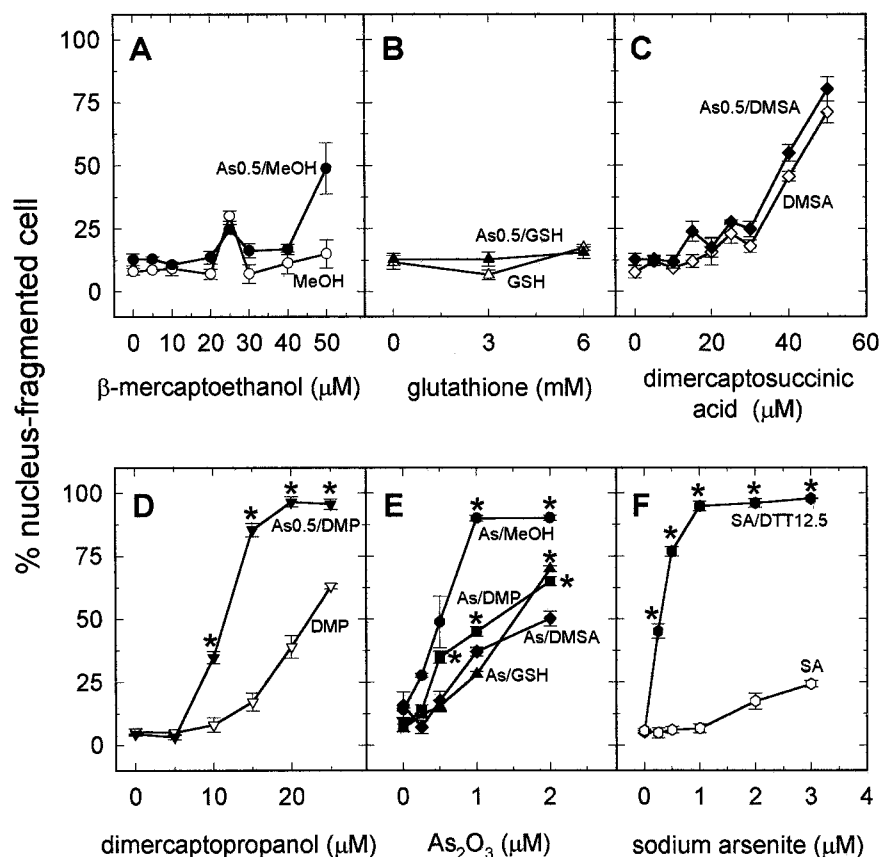


Fig. 4. Effects of treatment with trivalent inorganic arsenic compounds in combination with thiol compounds on nuclear fragmentation in NB4 cells. Cells were treated for 24 h with various concentrations of thiol compounds alone (open symbols), thiol compounds plus 0.5 μM As_2O_3 (closed symbols) (A, B, C, and D), various concentrations of As_2O_3 alone (□), As_2O_3 plus 50 μM β-mercaptoethanol (●), As_2O_3 plus 6 mM glutathione (▲), As_2O_3 plus 20 μM dimercaptosuccinic acid (◆), As_2O_3 plus 10 μM dimercaptopropanol (■) (E), various concentrations of sodium arsenite alone (○), or sodium arsenite plus 12.5 μM DTT (●) (F).

DTT also markedly enhanced the nuclear fragmentation induced by sodium arsenite (Fig. 4F).

Reactive Oxygen Species Are Involved in As₂O₃- and As₂O₃-Plus-DTT-Induced Apoptosis. We previously reported that reactive oxygen species are involved in sodium arsenite-induced apoptosis in Chinese hamster ovary cells (Wang et al., 1996). Therefore, we tested the effects of various oxidant modulators on As₂O₃-plus-DTT-induced nuclear fragmentation in NB4 cells. The results indicate that sodium pyruvate (a hydrogen peroxide scavenger), sodium selenite (a glutathione peroxidase activator), and catalase could reduce, whereas diethyldithiocarbamate (a superoxide dismutase inhibitor) and mercaptosuccinic acid (a glutathione peroxidase inhibitor) could enhance the As₂O₃-plus-DTT-induced nuclear fragmentation (Fig. 5, A–E). However, 3-aminotriazole (a catalase inhibitor) in the concentration range from 5 to 40 mM had no effect (Fig. 5F). The nuclear fragmentation induced by As₂O₃ alone was decreased by sodium pyruvate, sodium selenite, and catalase (Fig. 6A) and increased by diethyldithiocarbamate and mercaptosuccinic acid. However, 3-aminotriazole had no effect (Fig. 6B). Recently, we also presented evidence to show that nitric oxide is involved in sodium arsenite-induced micronuclei and DNA strand breaks in Chinese hamster ovary cells (Gurr et al., 1998; Lynn et al., 1998). Therefore, we tested the effects of nitric oxide synthase inhibitors, *N*_ω-nitro-L-arginine methyl ester and *S*-methyl-L-thiocitrulline, on As₂O₃- as well as As₂O₃-plus DTT-induced nuclear fragmentation in NB4 cells. The results indicate that *N*_ω-nitro-L-arginine methyl ester in the concentration range from 5 to 40 μM and *S*-methyl-L-thio-

citrulline from 5 to 40 μM had no effect on the As₂O₃- or As₂O₃-plus-DTT-induced apoptosis (Fig. 7, A and B). The results so far suggest that reactive oxygen species rather than nitric oxide are involved in As₂O₃- as well as As₂O₃-plus-DTT-induced nuclear fragmentation in NB4 cells. To further test this hypothesis, we studied the ability of sodium nitrosopruside, a nitric oxide-generating agent, and paraquat, a superoxide-generating agent, to induce nuclear fragmentation. The results indicate that a 24-h treatment with 0.1 to 1.0 mM sodium nitrosopruside did not induce nuclear fragmentation, whereas paraquat > 75 μM did (Fig. 7C). These results are consistent with the view that reactive oxygen species rather than nitric oxide are involved in NB4 apoptosis.

Confirmation from DNA Ladder and Caspase. We used DNA laddering analysis to provide additional confirmation of the above results. DNA ladders were observed in extracts from NB4 cells treated with As₂O₃ at concentrations of 1, 2, 3, and 4 μM for 24 h (data not shown), whereas a 24-h treatment with 20 μM phenylarsine oxide did not result in

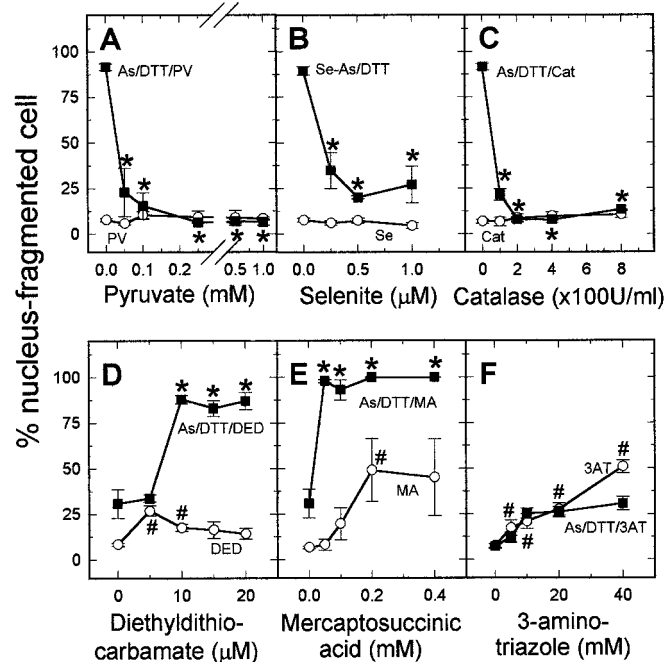


Fig. 5. Effects of pyruvate, selenite, catalase, diethyldithiocarbamate, mercaptosuccinic acid, and 3-aminotriazole on nuclear fragmentation of As₂O₃-plus-DTT-treated NB4 cells. Cells were treated for 24 h (25 h for selenite) with various concentrations of modulator alone (open symbols), or with As₂O₃ plus DTT plus modulator (selenite was added 1 h before As₂O₃ and DTT; closed symbols). In A, B, and C, 0.5 μM As₂O₃ and 15 μM DTT were used; in D, E, and F, 0.5 μM As₂O₃ and 10 μM DTT were used. * and # indicate *p* < .05 in Student's *t* test by comparing the value of treatment with versus without modulator or the value of treated versus untreated cells.

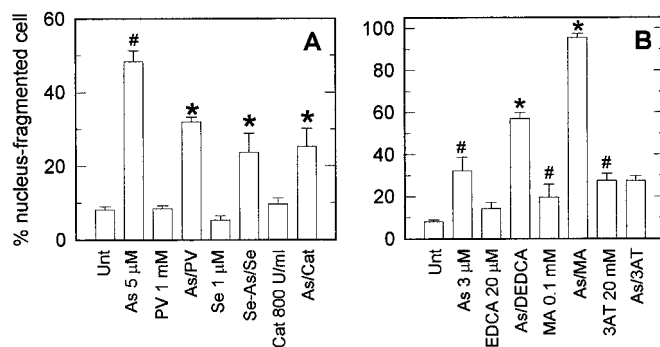


Fig. 6. Effects of pyruvate, selenite, catalase, mercaptosuccinic acid, diethyldithiocarbamate, and 3-aminotriazole on nuclear fragmentation of As₂O₃-treated NB4 cells. Cells were treated for 24 h with As₂O₃ alone, modulator alone, or As₂O₃ plus modulator. A, 5 μM As₂O₃, 1 mM sodium pyruvate, 1 μM selenite, and 800 U/ml catalase were used. B, 3 μM As₂O₃, 0.1 mM mercaptosuccinic acid, 20 μM diethyldithiocarbamate, and 20 mM 3-aminotriazole were used. Mean ± S.E. are from three experiments for treatments with As₂O₃, pyruvate plus As₂O₃, selenite, selenite plus As₂O₃, and mercaptosuccinic acid. # and * indicate *p* < .05 in Student's *t* test by comparing treated versus untreated cells or treatment with As₂O₃ alone versus As₂O₃ plus modulator.

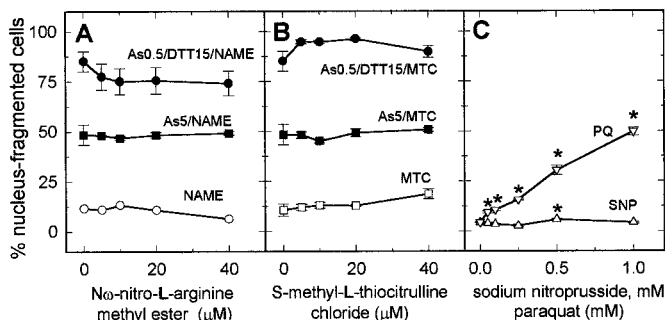


Fig. 7. Effects of nitric oxide synthase inhibitors, nitric oxide generator, and superoxide generator on nuclear fragmentation of As₂O₃ plus DTT-treated NB4 cells. A and B, cells were treated for 24 h with various concentrations of *N*_ω-nitro-L-arginine methyl ester (○), *S*-methyl-L-thiocitrulline (□), As₂O₃ plus inhibitor (■), or 0.5 μM As₂O₃ plus 15 μM DTT plus inhibitor (●). C, cells were treated for 24 h with various concentrations of sodium nitrosopruside (△) or paraquat (▽).

Discussion

In this article, data from nuclear fragmentation, DNA laddering, and caspase induction collectively show that DTT can enhance the As_2O_3 -induced apoptosis in NB4 cells. Because dithiol compounds are effective antidotes for arsenic poisoning, DTT is not expected to enhance the activity of As_2O_3 . The enhancing effect of DTT on the As_2O_3 toxicity as demonstrated in the present experiments is somewhat similar to the recent reports that DTT enhances the expression of arsenite-induced stress proteins (Kato et al., 1997) and that melarsoprol induces apoptosis in several leukemia cell lines (Konig et al., 1997; Wang et al., 1998). Melarsoprol is an organic arsenic compound synthesized by complexing melarsen oxide with a dithiol compound, 2,3-dimercaptopropanol (Ercoli and Wilson, 1948). Our results also show that dimercaptopropanol could enhance As_2O_3 -induced nuclear fragmentation in NB4 cells (Fig. 4, D and E).

Similar to the complexing of melarsen oxide with 2,3-dimercaptopropanol to produce melarsoprol, As_2O_3 may complex with DTT to produce a new compound that has a higher potency in inducing apoptosis in NB4 cells than As_2O_3 . In fact, sodium arsenite can complex with DTT to produce a stable precipitate; the crystal structure of this complex has also been determined (Cruse and James, 1972). Our results also show that DTT markedly enhanced the sodium arsenite-induced nuclear fragmentation in NB4 cells (Fig. 4F). Therefore, it is possible that As_2O_3 may also complex with DTT to produce a new compound. Complexation of inorganic trivalent arsenic with cysteine or glutathione has been shown to

DNA ladder (Fig. 7, lane 19). A 24-h treatment with 0.5 μM As_2O_3 alone or 15 μM DTT alone did not result in DNA ladder; however, DNA ladders resulted from the combined treatment with 0.5 μM As_2O_3 plus 15 μM DTT (Fig. 8, lanes 13–15). DNA ladders resulted from treatments with 0.25 and 2.0 mM dimethylarsinic acid, but 15 μM DTT did not increase the DNA ladder in 0.25 mM dimethylarsinic acid-treated cells (Fig. 8, lanes 23 to 25). The As_2O_3 - and As_2O_3 -plus-DTT-induced DNA laddering could be reduced by sodium pyruvate or sodium selenite but enhanced by mercaptosuccinic acid (Fig. 8, lanes 4–10 and 15–18).

The interleukin-1 β family of proteases is responsible for the specific cleavage of a set of structural and regulatory proteins that leads to apoptosis (Casciola Rosen et al., 1996). We measured the activity of DEVDase, which represents caspase-3 and caspase-7 (Thornberry et al., 1997), to provide further support for the above results. In a preliminary experiment, we treated NB4 cells with 0.5 μM As_2O_3 plus 20 μM DTT for various times and measured the DEVDase activity. The result showed a peak activity in cells treated for 18 h (data not shown). Thus, DEVDase activity was measured after an 18-h treatment in subsequent experiments. The results presented in Fig. 9 indicate that DEVDase activity decreased in cells treated with 20 μM phenylarsine oxide but increased in cells treated with 3.0 μM As_2O_3 . DEVDase activity of As_2O_3 -treated cells could be decreased by selenite and pyruvate and increased by mercaptosuccinic acid. DTT could enhance DEVDase activities of As_2O_3 -treated cells and the activities could also be decreased by selenite and pyruvate and increased by mercaptosuccinic acid.

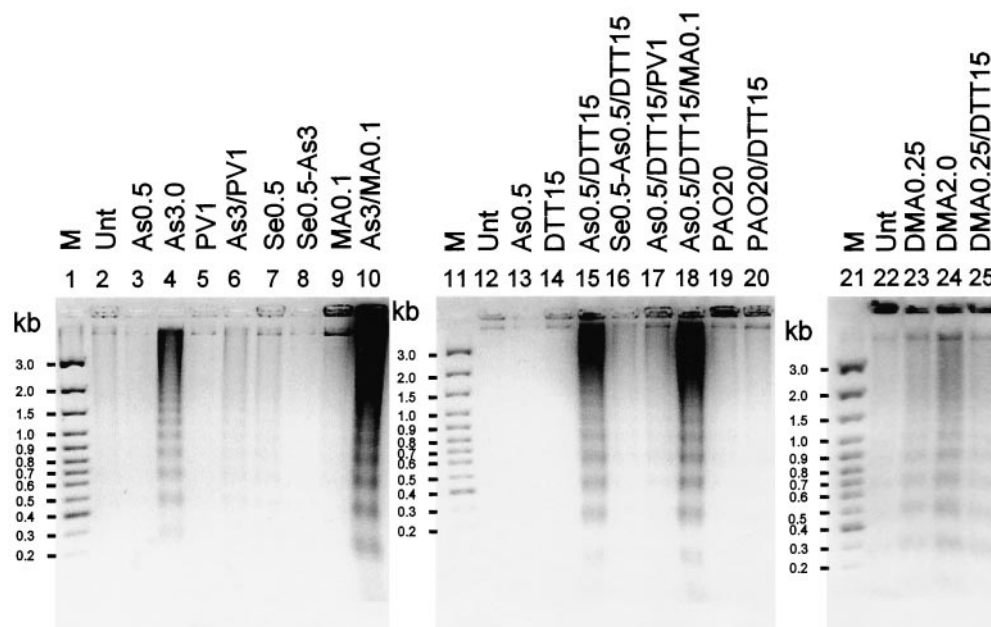


Fig. 8. Effects of treatment with As_2O_3 , phenylarsine oxide, dimethylarsinic acid, DTT, and oxidant modulators on DNA ladder. Unless specified, cells were treated for 24 h with As_2O_3 alone, modulator alone, or As_2O_3 plus modulator. Lane 1, DNA size marker; lane 2, extract from untreated cells; lane 3, extract from cells treated with 0.5 μM As_2O_3 ; lane 4, extract from cells treated with 3.0 μM As_2O_3 ; lane 5, 1 mM sodium pyruvate; lane 6, 3.0 μM As_2O_3 plus 1 mM pyruvate; lane 7, 0.5 μM selenite; lane 8, 0.5 μM selenite was added for 1 h and then 3.0 μM As_2O_3 was added for another 24 h; lane 9, 0.1 mM mercaptosuccinic acid; lane 10, 3.0 μM As_2O_3 plus 0.1 mM mercaptosuccinic acid; lane 11, DNA size marker; lane 12, untreated; lane 13, 0.5 μM As_2O_3 ; lane 14, 15 μM DTT; lane 15, 0.5 μM As_2O_3 plus 15 μM DTT; lane 16, 0.5 μM selenite was added for 1 h and then 0.5 μM As_2O_3 plus 15 μM DTT were added for another 24 h; lane 17, 0.5 μM As_2O_3 plus 15 μM DTT plus 1 mM pyruvate; lane 18, 0.5 μM As_2O_3 plus 15 μM DTT plus 0.1 mM mercaptosuccinic acid; lane 19, 20 μM phenylarsine oxide; lane 20, 20 μM phenylarsine oxide plus 15 μM DTT; lane 21, DNA size marker; lane 22, untreated; lane 23, 0.25 mM dimethylarsinic acid; lane 24, 2.0 mM dimethylarsinic acid; lane 25, 0.25 mM dimethylarsinic acid plus 15 μM DTT.

produce inhibitors of glutathione reductase that are several-fold more potent than the parental arsenical (Styblo et al., 1997). We speculate that DTT may complex with As_2O_3 to form a potent inhibitor for glutathione reductase, which could alter the redox status of cells. Alternatively, DTT may facilitate the influx of As_2O_3 into cells, or the new compound of As_2O_3 -DTT complex may have a higher cellular uptake than As_2O_3 . More research is needed to elucidate the mechanisms of why DTT in combination with As_2O_3 or sodium arsenite acts synergistically in inducing apoptosis in NB4 cells. Continuous research along this line may lead to the development of new therapeutic strategies for treating leukemia.

The present results show that both As_2O_3 alone and As_2O_3 -plus-DTT-induced apoptosis could be modulated by pyruvate, selenite, diethyldithiocarbamate, and mercaptosuccinic acid. These results are consistent with the notion that reactive oxygen species are involved in both As_2O_3 alone and As_2O_3 -plus-DTT-induced apoptosis. The view that reactive oxygen species are involved in arsenite-induced apoptosis is also consistent with the previous reports (Wang et al., 1996; Watson et al., 1996; Chen et al., 1998). In supporting this view, arsenite has also been shown to oxidize dichlorofluo-

rescein (Wang et al., 1996; Chen et al., 1998; Gurr et al., 1998); oxidative stress is known to activate the caspase and cause apoptosis (Hampton and Orrenius, 1997). Therefore, As_2O_3 probably induces apoptosis by up-regulating the caspase genes via a reactive oxygen species pathway. However, in the present experiments, catalase was very effective in reducing the As_2O_3 -plus-DTT-induced nuclear fragmentation but was less effective in reducing the nuclear fragmentation induced by As_2O_3 alone. In contrast, the catalase inhibitor 3-aminotriazole increased neither As_2O_3 alone nor As_2O_3 -plus-DTT-induced nuclear fragmentation. Catalase was effective in reducing the arsenite-induced apoptosis in NIH3T3 cells (Chen et al., 1998) but was ineffective in reducing the arsenite-induced apoptosis in Chinese hamster ovary cells (Wang et al., 1996) and human neutrophils (Watson et al., 1996). The variable results with catalase may be caused by the different cell types used, but this is not the reason in the present experiments.

The results showing that As_2O_3 -induced nuclear fragmentation was not affected by inhibitors of nitric oxide synthase and that nitric oxide generator did not induce nuclear fragmentation suggest that nitric oxide probably is not involved in As_2O_3 -induced apoptosis in NB4 cells. This observation is consistent with the report of Chen et al. (1998). Arsenite has been shown to induce DNA strand breaks and micronuclei via the generation of nitric oxide (Gurr et al., 1998; Lynn et al., 1998). Nitric oxide has been reported to prevent the increase of and to directly inhibit caspase-3-like activity in hepatocytes (Kim et al., 1997). Therefore, if treatment with As_2O_3 increases nitric oxide in NB4 cells, then this pathway would be expected to decrease rather than increase apoptosis.

The present results also suggest that binding to the vicinal thiols of tyrosine phosphatase may not be the mechanism by which As_2O_3 induces apoptosis in NB4 cells. This notion comes from the observations that DTT enhanced As_2O_3 -induced apoptosis and phenylarsine oxide, a phosphatase inhibitor that has strong thiol-binding activity, did not induce apoptosis in NB4 cells, whereas a weak thiol-binding agent, dimethylarsinic acid, did. The observation that phenylarsine oxide did not induce apoptosis in NB4 cells is consistent with the report that phenylarsine oxide is an inhibitor of caspases (Takahashi et al., 1997). Our result also shows that phenylarsine oxide reduced As_2O_3 -plus-DTT-induced apoptosis (data not shown). In addition, phenylarsine oxide may also prevent apoptosis by inhibiting superoxide generation (Li and Guillory, 1997). Therefore, the available data indicate opposite effects of As_2O_3 and phenylarsine oxide on apoptosis, caspase activity, and superoxide generation. However, still more direct evidence is needed to exclude the involvement of vicinal thiol-binding in As_2O_3 -induced apoptosis in NB4 cells. Recent research indicates that arsenite inhibits DNA repair at micromolar levels, whereas millimolar levels of arsenite are required to inhibit purified DNA repair enzymes (Lynn et al., 1997; Hu et al., 1998). Pyruvate dehydrogenase is an enzyme that is supersensitive to arsenite (Hu et al., 1998); however, our unpublished results (Hsien-Tsung Lai and K.-Y.J.) indicate that phenylarsine oxide inhibits the activity of pyruvate dehydrogenase at a much lower concentration range than arsenite. This discussion led to the suggestion that inorganic trivalent arsenic may not be as potent a vicinal thiol-binding agent as previously recognized (not as

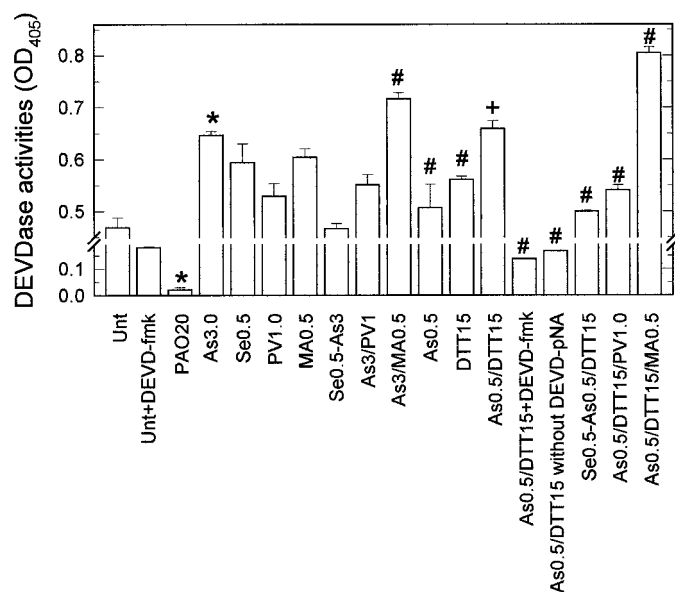


Fig. 9. Effects of treatment with As_2O_3 , DTT, and oxidant modulators on caspase activity. Unless specified, 1×10^6 NB4 cells were treated for 18 h with one chemical alone or with 2 to 3 chemicals in combination. NB4 cells at 1×10^6 were untreated (Unt) or treated for 18 h with 20 μM phenylarsine oxide (PAO20), 3.0 μM As_2O_3 (AS 3.0), 0.5 μM selenite (Se0.5), 1.0 mM pyruvate (PV1.0), 0.5 mM mercaptosuccinic acid (MA0.5), 3.0 μM As_2O_3 plus 1 mM pyruvate (As3/PV1), 3.0 μM As_2O_3 plus 0.5 mM mercaptosuccinic acid (As3/MA0.5), 0.5 μM As_2O_3 (As0.5), 15 μM DTT (DTT15), 0.5 μM As_2O_3 plus 15 μM DTT (As0.5/DTT15), 0.5 μM As_2O_3 plus 15 μM DTT plus 1 mM pyruvate (As0.5/DTT15/PV1.0), or 0.5 μM As_2O_3 plus 15 μM DTT plus 0.1 mM mercaptosuccinic acid (As0.5/DTT15/MA0.5). In Se0.5-As3 and Se0.5-As0.5/DTT15, 0.5 μM selenite was added for 1 h and then 3.0 μM As_2O_3 or 0.5 μM As_2O_3 plus 15 μM DTT were added for another 18 h. The cells were then lysed and DEVDase activities were determined as described in *Materials and Methods*. DEVD-pNA and DEVD-fmk were substrate and specific inhibitor for DEVDase, respectively. Unless specified, DEVD-pNA 50 μM was added in each assay, whereas DEVD-fmk 5 μM was added only in some specified assays. Results are means and S.E. of two experiments. *, #, and + indicate $p < .05$ in Student's t test by comparing with absorbance values from untreated cells, treatment without oxidant modulator, or the additive value of As_2O_3 and DTT alone, respectively.

potent as phenylarsine oxide, in any case). Therefore, arsenite may not express its toxicity directly by inactivating the proteins through binding to their vicinal thiol groups. Although superoxide and/or nitric oxide have been shown to be involved in arsenite-induced poly(ADP-ribosylation), micronuclei, gene mutation (Hei et al., 1998), and apoptosis, it still is possible that arsenite may bind to cellular vicinal thiols to regulate the generation of these molecules.

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