Arsenic trioxide inhibits growth of As4.1 juxtaglomerular cells via cell cycle arrest and caspase-independent apoptosis

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Arsenic trioxide inhibits growth of As4.1 juxtaglomerular cells via cell cycle arrest and caspase-independent apoptosis. Am J Physiol Renal Physiol 293: F511–F520, 2007. First published May 16, 2007; doi:10.1152/ajprenal.00385.2006.—We investigated the in vitro effects of arsenic trioxide on cell growth, cell cycle regulation, and apoptosis in As4.1 juxtaglomerular cells. Arsenic trioxide inhibited the growth of As4.1 cells with an IC50 of ~5 μM. Arsenic trioxide induced S phase arrest of the cell cycle and very efficiently stimulated apoptosis in As4.1 cells, as evidenced by flow cytometric detection of sub-G1 DNA content, annexin V binding assay, and 4′,6-diamidino-2-phenylindole staining. This apoptotic process was accompanied by the loss of mitochondrial transmembrane potential (ΔΨm), a decrease in Bcl-2, the activation of caspase-3, and cleavage of poly(ADP-ribose) polymerase. However, all of the caspase inhibitors tested in this experiment failed to rescue As4.1 cells from arsenic trioxide-induced cell death in view of sub-G1 cells and annexin V positive-staining cells. However, a caspase-8 inhibitor (Z-IETD-FMK) noticeably decreased the loss of ΔΨm in arsenic trioxide-treated cells. When we examined the changes in reactive oxygen species (ROS), H2O2, or O2•− in arsenic trioxide-treated cells, H2O2 was significantly decreased and O2•− was increased. In addition, we detected a decreased GSH content in arsenic trioxide-treated cells. Taken together, we have demonstrated that arsenic trioxide as a ROS generator potently inhibited the growth of As4.1 JG cells through S phase arrest of the cell cycle and caspase-independent apoptosis.

reactive oxygen species; mitochondria

REACTIVE OXYGEN SPECIES (ROS) include hydrogen peroxide (H2O2), nitric oxide (NO), superoxide anion (O2•−), hydroxyl radical (•OH), and peroxynitrite (ONOO−) and have recently been implicated in the regulation of many important cellular events, including transcription factor activation, gene expression, differentiation, and cellular proliferation (1, 2, 12). ROS are formed as by-products of mitochondrial respiration or precise oxidases, including NADPH oxidase, xanthine oxidase (XO), and certain arachidonic acid oxygenases (52). A change in the redox state of the tissue implies a change in ROS generation or metabolism. Principal metabolic pathways include SOD, which is expressed as extracellular, intracellular, and mitochondrial isoforms. These isoforms metabolize O2•− to H2O2. Further metabolism by peroxidases that include catalase and glutathione peroxidase yields O2 and H2O (46). Cells possess antioxidant systems to control the redox state, which is important for their survival. Excessive production of ROS gives rise to the activation of events that lead to death or survival in several types of cells (5, 10, 40, 44). The exact mechanisms involved in cell death induced by ROS are not yet known, and the protective effect mediated by some antioxidants remains controversial.

Arsenic trioxide, a common environmental toxin, has recently been reported to induce complete remission in patients with relapsed or refractory acute promyelocytic leukemia (APL) without severe marrow suppression (41). Although the mechanism of the antileukemic effect of arsenic trioxide is not well understood, it is known that arsenic trioxide is able to degrade a PML protein and a PML/RARα fusion protein in APL with a (15;17) (4, 37). More recently, it has been shown that the antiproliferative effect of arsenic trioxide is not limited to APL cells but can also be observed in a variety of hematological malignancies without having the PML/RARα fusion protein (30, 45, 50), which suggests that the antiproliferative effect of arsenic trioxide might be independent of a PML or PML/RARα fusion protein. Accumulating evidence has indicated that arsenic trioxide can regulate many biological functions such as cell proliferation, apoptosis, differentiation, and angiogenesis in various cell lines (28). Furthermore, arsenic trioxide as a mitochondrial toxin induces a loss of mitochondrial transmembrane potential (14, 17, 30) and, as such, it induces the generation of ROS (22, 24, 28). These phenomena could trigger the apoptosis of target cells. Therefore, it is thought that arsenic trioxide induces apoptosis in tumor cells by affecting the mitochondria and ROS generation.

Juxtaglomerular (JG) cell tumors (also known as reninomas), first described in the late 1960s (21, 33), are rare benign tumors of the kidney. About 100 cases have been described to date. Reninomas are known to arise from JG cells. Clinically, reninoma patients suffer from headaches, polyuric, nocturia, and dizziness, among other symptoms. Hypertension is a sign in almost all patients, and laboratory findings of hyperreninemia, hyperaldosteronism, and hypokalemia are characteristic. A malignant juxtaglomerular cell tumor was recently described (11). It has been speculated that the kidney and juxtaglomerular apparatus (JGA) contain a ROS-generating system that is responsive to angiotensin II (46, 47). The ROS in the JGA-related cells are related to the regulation of blood pressure (46, 47). However, the role of ROS in kidney cell death, especially in JG cells, has not been evaluated in the investigation of apoptosis. Notably, arsenic trioxide is a common environmental toxin. Toxic effects of arsenic trioxide usually result from ingestion. Initial glomerular damage leading to proteinuria is common following high arsenic exposure. Tubular necrosis

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and degeneration, oliguria with proteinuria, hematuria, and acute renal failure are also frequently observed. The As4.1 cell line has been used as a model for JG cells. This cell line was isolated from kidney neoplasm in a transgenic mouse containing a renin SV40 T-antigen transgene (39). Therefore, understanding the molecular mechanism of kidney cell death, especially that of JG, by arsenic trioxide is an important topic of further research.

In the present study, we evaluated the effects of arsenic trioxide on the inhibition of As4.1 cells and investigated its mechanism.

MATERIALS AND METHODS

Cell culture. As4.1 cells (ATCC no. CRL-2193) are a renin-expressing clonal cell line derived from the kidney neoplasm of a transgenic mouse (39). Cell cultures were maintained in humidified room air containing 5% CO2 at 37°C. As4.1 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin (GIBCO BRL, Grand Island, NY). Cells were routinely grown in 100-mm plastic tissue culture dishes (Nunc, Roskilde, Denmark) and were harvested with a solution of trypsin-EDTA when they were in a logarithmic phase of growth. Cells were maintained at the above-described culture conditions for all experiments.

Reagents. Arsenic trioxide was purchased from Sigma-Aldrich (St. Louis, MO) and was dissolved in 1.65 M NaOH at 1 × 10−1 M as a stock solution. A pan caspase inhibitor (Z-VAD-FMK), caspase-3 inhibitor (Z-DEVD-FMK), caspase-8 inhibitor (Z-IETD-FMK), and caspase-9 inhibitor (Z-LEHD-FMK), all of which were obtained from R&D Systems (Minneapolis, MN), were dissolved in DMSO (Sigma).

Growth inhibition assay. The in vitro growth inhibition effect of arsenic trioxide on As4.1 cells was determined by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye absorbance of living cells as previously described (30). In brief, cells (2 × 104 cells/well) were first seeded in 96-well microtiter plates (Nunc). After exposure to the drug for 24, 48, or 48 h, 50 μl of MTT (Sigma) solution (2 mg/ml in PBS) was added to each well, and the plates were incubated for an additional 3 or 4 h at 37°C. MTT solution in medium was aspirated off. To achieve solubilization of the formazan crystal formed in viable cells, 100 or 200 μl of DMSO was added to each well. The optical density of each well was measured at 570 nm using a microplate reader (Spectra MAX 340, Molecular Devices, Sunnyvale, CA). Each plate contained multiple wells of a given experimental condition and multiple control wells. This procedure was replicated for two to four plates/condition.

Cell cycle and sub-G1 analysis. The cell cycle and sub-G1 distribution were determined by staining DNA with propidium iodide (PI; Sigma) as previously described (29). In brief, 1 × 106 cells were incubated with the designated doses of arsenic trioxide for 24–48 h. Cells were then washed in PBS and fixed in 70% ethanol. The percentages of cells in the different phases of the cell cycle or having the sub-G1 distribution were determined by flow cytometry.

Western blot analysis. Cells were incubated with the designated doses of arsenic trioxide for 48 h. The cells were then washed in PBS and suspended in five volumes of lysis buffer [20 mM HEPES (pH 7.9), 20% glycerol, 200 mM KCl, 0.5 mM EDTA, 0.5% NP-40, 0.5 mM DTT, 1% protease inhibitor cocktail; Sigma]. The lysates were then collected and stored at −20°C until further use. Supernatant protein concentrations were determined by the Bradford method. Supernatant samples containing 40 μg of total protein were resolved by 8, 12.5, or 15% SDS-PAGE gel, depending on the target protein sizes, transferred onto Immobilon-P polyvinylidene difluoride membranes (Millipore) by electroblotting, and probed with anti-p53, anti-Bax, anti-Bcl-2, anti-caspase-3, anti-β-tubulin, and anti-pol (ADP-ribose) polymerase (PARP; Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies. The membrane blots were developed by using an ECL kit (Amersham, Arlington Heights, IL).

Annexin V/FITC staining. Apoptosis was determined by staining cells with annexin V-FITC and PI labeling because annexin V can identify the externalization of phosphatidylserine during the progression of apoptosis and, therefore, can detect cells in early stages of apoptosis. To quantitate the apoptosis of cells, prepared cells were washed twice with cold PBS and then resuspended in 500 μl of binding buffer [10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, 2.5 mM CaCl2] at a concentration of 1 × 106 cells/ml. Five microliters of annexin V-FITC (PharMingen, San Diego, CA) and 10 μl of 20 μg/ml PI were then added to these cells, which were analyzed with a FACStar flow cytometer (Becton Dickinson). Viable cells were negative for both PI and annexin V; apoptotic cells were positive for annexin V and negative for PI, while late apoptotic dead cells displayed both high annexin V and PI labeling. Nonviable cells, which underwent necrosis, were positive for PI and negative for annexin V.

Measurement of mitochondrial membrane potential. Mitochondrial membranes were monitored using rhodamine 123 fluorescent dye, a cell-permeable cationic dye that preferentially enters into mitochondria based on the highly negative mitochondrial membrane potential (ΔΨm). Depolarization of ΔΨm results in the loss of rhodamine 123 from the mitochondria and a decrease in intracellular fluorescence. In brief, cells were washed twice with PBS and incubated with rhodamine 123 (0.1 μg/ml; Sigma) at 37°C for 30 min. PI (1 μg/ml) was subsequently added, and the staining intensity of rhodamine 123 and PI was determined by flow cytometry.

Morphological analysis of apoptosis by staining with 4′,6-diamidino-2-phenylindole (DAPI) staining. The cells were cultured in eight-chamber glass slides (LabTek, Nunc, Naperville, IL) containing 10% FBS in the absence or presence of arsenic trioxide (10 μM). After 48-h incubation, the slides were then rinsed with PBS and fixed in 80% ethanol for at least 30 min. Cellular DNA was stained with 1 μg/ml DAPI dissolved in PBS for 30 min. The slides were then visualized on a Carl Zeiss fluorescence microscope. DAPI permeates the plasma membrane and yields blue chromatin. Viable cells display normal nuclear size and blue fluorescence, whereas apoptotic cells show condensed chromatin and fragmented nuclei.

Quantification of caspase-3 activity. The activity of caspase-3 was assessed using caspase-3 Colorimetric Assay Kits (R&D Systems), which are based on the spectrophotometric detection of the color reporter molecule p-nitroanilide (pNA) after cleavage from the labeled substrate DEVD-pNA (caspase-3) as an index. In brief, cells were incubated with the designated dose of arsenic trioxide for 48 h. The cells were then washed in PBS and suspended in 5 volumes of lysis buffer (20 mM HEPES, pH 7.9, 20% glycerol, 200 mM KCl, 0.5 mM EDTA, 0.5% NP-40, 0.5 mM DTT, 1% protease inhibitor cocktail; Sigma). The lysates were then collected and stored at −20°C until use. Protein concentration was determined by the Bradford method. Supernatant samples containing 100 μg of total protein were used for determination of caspase-3 activity. These are added to each well in 96-well microtiter plates (Nunc) with the DEVD-pNA at 37°C for 1–2 h. The optical density of each well was measured at 405 nm using a microplate reader (Spectra MAX 340, Molecular Devices). Each plate contained multiple wells of a given experimental condition and multiple control wells. The activity of caspase-3 was expressed in arbitrary absorbance units (absorbance at a wavelength of 405 nM).

Detection of intracellular H2O2 and O2− concentration. Intracellular H2O2 concentration was detected by means of an oxidation-sensitive fluorescent probe dye, 2′,7′-dichlorodihydrofluorescein di-
acetate (H$_2$DCFDA; Invitrogen Molecular Probes, Eugene, OR). H$_2$DCFDA was deacetylated intracellularly by nonspecific esterase, which was further oxidized by cellular peroxides to a fluorescent compound, 2,7-dichlorofluorescein (DCF). Dihydroethidium (DHE) (Invitrogen Molecular Probes) is a fluorogenic probe that is highly selective for superoxide anion radical detection. DHE is cell permeable and reacts with the superoxide anion to form ethidium, which then intercalates in the deoxyribonucleic acid, thereby exhibiting a red fluorescence. In brief, cells were incubated with the designated doses of arsenic trioxide combined with or without caspase inhibitors for 24–48 h. Cells were then washed in PBS and incubated with 20 μM H$_2$DCFDA or 5 μM DHE at 37°C for 30 min according to the instructions of the manufacturer. DCF fluorescence and red fluorescence were detected by a FACStar flow cytometer (Becton Dickinson). For each sample, 5,000 or 10,000 events were collected. H$_2$O$_2$ and O$_2$•⁻ production was expressed as mean fluorescence intensity (MFI), which was calculated by CellQuest software.

**Detection of intracellular GSH.** Cellular GSH levels were analyzed using 5-chloromethylfluorescein diacetate (CMFDA; Molecular Probes). CMFDA is a useful, membrane-permeable dye used to determine levels of intracellular glutathione (16, 27, 31). In brief, cells were incubated with the designated doses of arsenic trioxide combined with or without caspase inhibitors for 24–48 h. Cells were then washed in PBS and incubated with 5 μM CMFDA at 37°C for 30 min according to the instructions of the manufacturer. CMF fluorescence was detected by a FACStar flow cytometer (Becton Dickinson) and then analyzed using CellQuest software. For each sample, 5,000 or 10,000 events were collected.

**Statistical analysis.** Results represent means ± SE of at least two or three independent experiments. Microsoft Excel or Instat software (GraphPad Prism4, San Diego, CA) was used to analyze the data. Student’s t-test or one-way ANOVA with post hoc analysis using Tukey’s multiple comparison test was used for parametric data. Statistical significance was defined as *P* < 0.05.

**RESULTS**

**Effect of arsenic trioxide on inhibition of growth of As4.1 JG cells.** We examined the effect of the arsenic trioxide on the growth of As4.1 cells using an MTT assay. Dose-dependent inhibition of cell growth was observed in As4.1 cells with an IC$_{50}$ of 5 μM following treatment with arsenic trioxide for 48 h (Fig. 1A).

![Fig. 1](http://ajprenal.physiology.org/)

*Fig. 1.* Effects of arsenic trioxide (ATO) on growth inhibition of As4.1 cells in vitro. Exponentially growing cells were treated with the indicated concentrations of ATO for 48 h. A: cell growth inhibition was assessed by MTT assays. B: graph shows the changes in cell cycle phase distribution by ATO. C: cell cycle and sub-G1 analysis were measured by DNA flow cytometric analysis and analyzed using ModFit software (Verity Software). D: graph shows the sub-G1 percentages of M1 region from C. E: cells were treated with or without ATO (10 μM) for 48 h, stained with DAPI, and observed under a fluorescence microscope (×400). Arrows indicate the apoptotic nuclei with condensed chromatin. *P* < 0.05 compared with the ATO-untreated control group.
Suppression of As4.1 cell growth by arsenic trioxide can be explained by its ability to arrest cells in specific phases of the cell cycle. As shown in Fig. 1B, DNA flow cytometric analysis indicated that the percentage of As4.1 cells at the S phase of the cell cycle was significantly elevated from ~30 to 45% at 72 h following exposure to arsenic trioxide (1, 5, and 10 μM). Exposure to higher concentrations of arsenic trioxide did not alter the cell cycle distribution compared with control cells. It seems that higher concentrations of this drug cause cell death rather than cell cycle arrest.

We performed an in vitro apoptosis detection assay to determine whether arsenic trioxide-treatment could induce apoptosis in As4.1 cells. As shown in Fig. 1, C and D, arsenic trioxide increased the sub-G1 population in a dose-dependent manner at 48 h. Following exposure to 20 μM arsenic trioxide, the percentages of As4.1 cells in the sub-G1 population were elevated to ~50%. To characterize the cell death induced by arsenic trioxide, we examined the nuclear morphologies of dying cells using the fluorescent DNA-binding agent DAPI. As4.1 cells treated with 5 or 10 μM arsenic trioxide at 48 h displayed typical morphological features of apoptotic cells, i.e., condensed nuclei (Fig. 1E) with no cytoplasm stained for β-tubulin and separated apoptotic bodies (data not shown).

Effects of arsenic trioxide on plasma membrane, ∆Ψ_m, and apoptotic-related proteins in As4.1 cells. To further confirm and evaluate the induction of apoptosis, we stained cells with annexin V and PI. As with the percentages of cells in the sub-G1 group determined by flow cytometry, the proportion of annexin V-staining cells in the arsenic trioxide-treated cells was increased in a dose-dependent manner (Fig. 2, A and B). We could also detect As4.1 cells with a slight amount of necrosis (annexin-negative and PI-positive proportion cells).

Concerning the relationship between Bcl-2 and Bax regulation during the arsenic trioxide-induced apoptosis, Bcl-2 protein was decreased in a dose-dependent manner at 48 h (Fig. 2C). However, Bax levels were constitutively expressed in the absence of significant changes in its protein level (Fig. 2C), which suggests that apoptosis induced by arsenic trioxide may be mediated through the downregulation of an antiapoptotic protein, Bcl-2, in As4.1 cells. In addition, both Bcl-2 and Bax are transcriptional targets for the tumor suppressor protein p53, which induces cell cycle arrest or apoptosis in response to DNA damage (8). As shown in Fig. 2C, the amount of p53 protein was markedly increased in the As4.1 cells treated with even 1 μM arsenic trioxide. As4.1 cells treated with 1 μM arsenic trioxide showed S phase arrest of the cell cycle but did...
not show apoptosis. Taken together, these results indicate that the cell cycle arrest and apoptosis of As4.1 cells induced by this agent may be strongly dependent on p53 status.

To elucidate the effect of arsenic trioxide on ΔΨm, cells were treated with increasing doses for 48 h. The percentage of rhodamine 123 negative-staining cells showed a very similar pattern to that of the annexin-positive cells on treatment with arsenic trioxide (Fig. 2, D and E). Following exposure to 10 μM arsenic trioxide, the percentage of rhodamine 123 negative-staining cells was elevated to ~60%. Next, we wanted to determine whether caspase-3 might be activated during the induction of apoptosis by arsenic trioxide, because caspase-3 plays an essential role as an executor in apoptosis. We did not observe that the 32-kDa precursor (procaspase-3) was decreased by the doses of arsenic trioxide used in this study (1, 3, and 7 μM) (Fig. 2F). However, we observed the significant activation of caspase-3 in As4.1 cells treated with 7 μM arsenic trioxide (Fig. 2G). In regard to PARP protein, which is a major substrate for executed caspases and a hallmark of apoptosis, Western blotting showed that the intact 116-kDa moiety of PARP was degraded in arsenic trioxide-treated As4.1 cells (Fig. 2F). These results indicate conclusively that arsenic trioxide-induced apoptosis in As4.1 cells is accompanied by the loss of ΔΨm and PARP degradation.

Effects of caspase inhibitors on arsenic trioxide-induced apoptosis and cell cycle. To determine which caspases are required for the induction of apoptosis by arsenic trioxide, we treated cells with a pan caspase inhibitor (Z-VAD-FMK), caspase-3 inhibitor (Z-DEVD-FMK), caspase-8 inhibitor (Z-IETD-FMK), and caspase-9 inhibitor (Z-LEHD-FMK) at a concentration of 12.5 or 25 μM. Inhibitors for caspase-3, caspase-8, and caspase-9 did not block apoptotic events in arsenic trioxide-treated As4.1 cells at 48 h, as determined by assessing the sub-G1 population and annexin V-positive staining (Fig. 3). Instead, pan caspase inhibitor Z-VAD-FMK at a concentration of 25 μM seemed to intensify the level of apoptosis of As4.1 cells treated with arsenic trioxide. With regard to ΔΨm, none of the caspase inhibitors tested in our experiments (12.5 μM) rescued As4.1 cells from the loss of ΔΨm induced by arsenic trioxide (Fig. 4). However, caspase-8 inhibitor Z-IETD-FMK at a concentration of 25 μM was likely to decrease the loss of ΔΨm in arsenic trioxide-treated cells (Fig. 4), while the 12.5 μM concentration of inhibitors for caspases tested in our experiment did not. In regard to the cell cycle distribution in the presence of caspase inhibitors, these inhibitors could not significantly alter the changes in cell cycle distribution caused by arsenic trioxide (7 μM) (data not shown).
Effect of arsenic trioxide on ROS and GSH production in As4.1 cells. To elucidate the involvement of H$_2$O$_2$ in arsenic trioxide-induced As4.1 cell death, we assessed the H$_2$O$_2$ levels using H$_2$DCFDA fluorescence. As shown in Fig. 5, A and C, H$_2$O$_2$ levels were significantly decreased in As4.1 cells exposed to arsenic trioxide for 48 h. The pattern of decreased H$_2$O$_2$ levels caused by this drug (1 and 10 $\mu$M) was detected as early as 20, 30, 50, 70, 90, and 240 min after treatment (data not shown). In regard to the effects of caspase inhibitors on H$_2$O$_2$ levels, these inhibitors could not significantly alter H$_2$O$_2$ levels by arsenic trioxide (7 $\mu$M) (Fig. 5, A and C).

Next, we attempted to detect changes to O$_2^{-}$ in arsenic trioxide-induced As4.1 cell death. Red fluorescence derived from DHE, reflecting O$_2^{-}$ accumulation, was increased in the arsenic trioxide-treated cells (Fig. 5, B and D). The accumulation of O$_2^{-}$ was observed as early as 30 min after exposure of cells to 1 $\mu$M arsenic trioxide (data not shown). It is possible that the increase in O$_2^{-}$ by arsenic trioxide is necessary at an early step of apoptosis in As4.1 cells. In regard to the changes in O$_2^{-}$ levels by caspase inhibitors, inhibitors for pan caspase and caspase-8 seemed to slightly decrease O$_2^{-}$ levels increased by arsenic trioxide (Fig. 5, B and D), but inhibitors for caspase-3 and caspase-9 did not (Fig. 5, B and D).

Cellular GSH has been shown to be crucial for regulation of cell proliferation, cell cycle progression, and apoptosis (32, 34). Therefore, we analyzed the changes in GSH levels in As4.1 cells by using CMF fluorescence. The M1 population of As4.1 cells showed the percentage of cells with depleted GSH content. Arsenic trioxide (7 $\mu$M) significantly elevated the percentages of cells residing in the M1 population at 48 h (38 vs. 8%) (Fig. 6), indicating the depletion of intracellular GSH content of As4.1 cells by arsenic trioxide. The decrease in intracellular GSH content was observed as early as 20 min after exposure to 10 $\mu$M arsenic trioxide (data not shown). Inhibitors for caspase-3, -8, and -9 did not significantly amplify the amount of GSH decreased by arsenic trioxide (Fig. 6). Instead, pan caspase and caspase-9 inhibitors were likely to enhance the depletion of intracellular GSH content in arsenic trioxide-treated As4.1 cells.

DISCUSSION

We focused on the apoptotic roles of arsenic trioxide as a ROS generator in As4.1 JG cells. We also have demonstrated that arsenic trioxide inhibits the proliferation of As4.1 cells by inducing cell cycle arrest and triggering apoptosis. The susceptibility to arsenic trioxide in these cells is lower than that in leukemia and myeloma cell lines (30, 37) and is similar to that in solid tumors such as human small-cell lung cancer, ovarian cancer, colon cancer, cervical cancer, renal cell carcinoma, and breast cancer cell lines (17, 25). The differing susceptibilities to arsenic trioxide of cell lines might result from the different origins of the cells.

Our cell cycle analysis has revealed that low doses of arsenic trioxide (1–10 $\mu$M) were able to induce S phase arrest of As4.1 cells at 48 h. It is known that arsenic trioxide induces G$_1$ and/or G$_2$M phase arrest depending on cell type (17, 26, 30). Our result may be the first report to indicate that arsenic trioxide can induce S phase arrest. In response to DNA damage, p53 induces cell cycle arrest or apoptosis and regulates Bax and Bcl-2 protein expression (8). In our experiment, the expression of p53 was dramatically increased in the As4.1 cells treated with 1 $\mu$M arsenic trioxide. Because 1 $\mu$M arsenic trioxide could not trigger apoptosis, but induced significant S phase arrest of the cell cycle, p53 induction by 1 $\mu$M arsenic trioxide acted as a mediator for an S phase checkpoint. Taken together, these data suggest that the molecular mechanisms of cell cycle arrest of arsenic trioxide have great variety.

In addition, our data showed that arsenic trioxide potently induced apoptosis in a dose-dependent manner in As4.1 JG cells. To gain insight into the molecular mechanisms involved in apoptosis caused by arsenic trioxide, expression of the apoptosis-related proteins and changes in $\Delta\Psi_{m}$ were assessed in As4.1 cells. It has been reported that nonorganic (arsenic trioxide) or organic (melarsoprol) arsenic compounds induce apoptosis through the downregulation of Bcl-2 protein. Similarly, we showed that induction of apoptosis was accompanied by an elevation in the Bax-to-Bcl-2 ratio, more specifically a decrease in Bcl-2 protein. It is conceivable that the induction of p53 by a higher dose of arsenic trioxide upregulates the ratio of Bax to Bcl-2, resulting in the triggering of apoptosis. These results imply that cell growth inhibition by arsenic trioxide...
occurs in a p53-dependent manner in As4.1 JG cells. It has been suggested that a high ratio of Bax to Bcl-2 can cause the collapse of \( \Psi_m \) in target cells, resulting in the release of cytochrome c and apoptosis (49). According to our data, arsenic trioxide induced the loss of \( \Psi_m \) in As4.1 cells. These results support the idea that arsenic trioxide primarily causes damage to the mitochondria of target cells and then allows progression to the next step of apoptosis.

In the cytosol, cytochrome c forms an apoptosome that is composed of Aaf-1 and procaspase-9, resulting in activation of caspase-9. Caspase-9 then activates the effector caspases, including procaspase-3, to execute the process of apoptosis (7). Caspase activation, especially that of caspase-3, is thought to be important for the process of arsenic trioxide-induced cell death in vitro and in vivo (17, 22–24, 28, 30). Correspondingly, caspase-3 was activated and PARP protein was degraded by arsenic trioxide in our experiments, although we did not observe that the 32-kDa precursor (procaspase-3) was decreased at the indicated doses of arsenic trioxide. To determine which caspases are required for the induction of apoptosis by arsenic trioxide, we incubated arsenic trioxide-treated As4.1 cells with caspase inhibitors. Interestingly, caspase inhibitors for pan-caspase and caspase-8 failed to reduce cell death induced by arsenic trioxide, as evidenced by annexin V-positive staining (Supplementary Fig. 1A; all supplementary material for this study can be found online at the AJP-Renal Physiology web site). Furthermore, these inhibitors intensified apoptosis in As4.1 cells treated with arsenic trioxide. It is possible that higher concentrations of caspase inhibitors are required for attenuation of apoptosis induced by arsenic trioxide or TNF-\( \alpha \) treatment in As4.1 cells. However, the 12.5 \( \mu \)M concentration of caspase inhibitors for caspase-3, -8, or -9 could very efficiently prevent the arsenic trioxide-induced apoptosis in Calu-6 lung carcinoma cells at 72 h (unpublished observations). Although we also cannot rule out the possibility that other caspases such as caspase-6 and caspase-7 are exclusively required for the induction of apoptosis in As4.1 cells, our results suggest that cell death induced by arsenic trioxide or TNF-\( \alpha \) in As4.1 cells is not dependent on the activation of caspase. In accordance with our results, caspase-independent death by arsenic trioxide has been reported by other groups (20, 35, 36). The mechanism by which arsenite induces this caspase-independent death remains enigmatic. Candidates for induction of caspase-independent death include apoptosis-inducing factor (19), endonuclease G (43), and ROS

**Fig. 5. Effects of caspase inhibitors on reactive oxygen species (ROS) production in ATO-treated As4.1 cells.** Exponentially growing cells were treated with the indicated caspase inhibitors (25 \( \mu \)M) in addition to 7 \( \mu \)M ATO for 48 h. H\(_2\)O\(_2\) (A) and O\(_2\)•\(^{-}\) (B) levels were determined using a FACStar flow cytometer as described in MATERIALS AND METHODS. Graphs show the levels of mean 2,7-dichlorofluorescein (DCF) fluorescence in A (C) and mean dihydroethidium (DHE) fluorescence in B (D). * \( P < 0.05 \) compared with the control group.
It is noteworthy that apoptosis-inducing factor mediates cell death by arsenic trioxide through a caspase-independent pathway in human cervical cancer cells (20).

With regard to $\Delta \Psi_m$, only the caspase-8 inhibitor at the concentration of 25 $\mu$M noticeably decreased the loss of $\Delta \Psi_m$. These results suggest that caspase-8 activation may show a strong involvement in the perturbation of $\Delta \Psi_m$ in arsenic trioxide-treated As4.1 cells, although the inhibition of this caspase alone cannot be sufficient to ultimately prevent apoptosis caused by arsenic trioxide. In addition, caspase-8 inhibitor Z-IETD did not alter the loss of $\Delta \Psi_m$ in TNF-α-treated As4.1 cells (Supplementary Fig. 1B). Activation of caspase-8 in arsenic trioxide-induced apoptosis has been reported in gastric cancer cells (23) and myeloma cells (26). Additionally, the combination of arsenic trioxide and TNF-related apoptosis-inducing ligand synergistically enhanced cleavage of caspase-8 in leukemic cells (42). The results of Liu et al. (26) suggest that, in the cells expressing functional p53, the initiator caspase-9 is the principal caspase activated by arsenic trioxide, leading to caspase-3 activation and apoptosis. In contrast, caspase-8 and -10 are the principal caspases activated by arsenic trioxide in cells without functional p53 (26). If these data are accurate, it is conceivable that arsenic trioxide perturbs $\Delta \Psi_m$ through the activation of caspase-8 in As4.1 cells, because As4.1 cells were originally derived from a kidney neoplasm in a transgenic mouse containing a renin SV40 T-antigen transgene (39).

Arsenic trioxide can disturb the natural oxidation and reduction equilibrium in cells, leading to an increase in ROS by a variety of redox enzymes, including flavoprotein-dependent, superoxide-producing enzymes such as NADPH oxidase (6, 22, 24, 28). Arsenic trioxide decreases GSH levels and increases intracellular ROS levels in certain APL cells (9). Therefore, to elucidate the involvement of ROS such as H$_2$O$_2$ and O$_2$. in arsenic trioxide-induced As4.1 cell death, we assessed these ROS levels by using H$_2$DCFDA and DHE fluorescence. Our data showed that the intracellular H$_2$O$_2$ level was not increased in the arsenic trioxide-treated As4.1 cells. This result is not consistent with other reports, which indicated that increased intracellular H$_2$O$_2$ played an important role in arsenic trioxide-induced As4.1 cells (9). These discrepancies are likely to result from cell-type specificity and/or different methods used for detection of H$_2$O$_2$ levels. Next, we checked out
whether or not another ROS, O$_2^•$-, was increased by arsenic trioxide. Arsenic trioxide enhanced the O$_2^•$- content in As4.1 cells. It is possible that arsenic trioxide directly or indirectly inhibited SOD, resulting in increased O$_2^•$- and decreased H$_2$O$_2$ levels in As4.1 cells. The inhibitors for pan caspase and caspase-8 seemed to slightly decrease O$_2^•$- levels that had originally been increased by arsenic trioxide, but this phenomenon could not be related to the reduction of apoptosis by arsenic trioxide. An pattern of increased O$_2^•$- following treatment with arsenic trioxide was reported in esophageal cancer SHEE85 cells (38), but this pattern was not observed in arsenic trioxide-treated acute myelogenous leukemia HL-60 cells (15) and renal cell carcinoma ACHN cells (48). These discrepancies also may result from cell-type specificity and/or different methods used for detection of O$_2^•$- levels. Although changes in ROS induced by arsenic trioxide act as very important mediators in the induction of apoptosis in target cancer cells, the precise roles of changes in ROS induced by this drug must be defined more clearly. With regard to intracellular GSH, this is a main nonprotein antioxidant in the cell, and it could clear away the superoxide anion free radicals and provide electrons for enzymes such as glutathione peroxidase, which reduces H$_2$O$_2$ to H$_2$O. It has been reported that intracellular GSH content has a decisive effect on arsenic trioxide-induced apoptosis (9, 23, 24, 48). Similarly, our results indicate that intracellular GSH content is depleted by arsenic trioxide in As4.1 cells. These results suggest that intracellular GSH levels are tightly related to a factor in arsenic trioxide-induced cell death.

In summary, we have demonstrated that arsenic trioxide as a generator of ROS, especially O$_2^•$-, potently inhibited the growth of As4.1 JG cells through S phase arrest of the cell cycle and caspase-independent apoptosis.

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ARSENIC TRIOXIDE INHIBITS GROWTH OF As4.1 CELLS


