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Intracellular GSH Level is a Factor in As4.1 Juxtaglomerular Cell Death by Arsenic Trioxide

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Abstract Arsenic trioxide has been known to regulate many biological functions such as cell proliferation, apoptosis, differentiation, and angiogenesis in various cell lines. We investigated the involvement of GSH and ROS such as H_2O_2 and $O_2^{\cdot -}$ in the death of As4.1 cells by arsenic trioxide. The intracellular ROS levels were changed depending on the concentration and length of incubation with arsenic trioxide. The intracellular $O_2^{\cdot -}$ level was significantly increased at all the concentrations tested. Arsenic trioxide reduced the intracellular GSH content. Treatment of Tiron, ROS scavenger decreased the levels of ROS in 10 μM arsenic trioxide-treated cells. Another ROS scavenger, Tempol did not decrease ROS levels in arsenic trioxide-treated cells, but slightly recovered the depleted GSH content and reduced the level of apoptosis in these cells. Exogenous SOD and catalase did not reduce the level of ROS, but did decrease the level of $O_2^{\cdot -}$. Both of them inhibited GSH depletion and apoptosis in arsenic trioxide-treated cells. In addition, ROS scavengers, SOD and catalase did not alter the accumulation of cells in the S phase induced by arsenic trioxide. Furthermore, JNK inhibitor rescued some cells from arsenic trioxide-induced apoptosis, and this inhibitor decreased the levels of $O_2^{\cdot -}$ and reduced the GSH depletion in these cells. In summary, we have demonstrated that arsenic trioxide potently generates ROS, especially $O_2^{\cdot -}$, in As4.1 juxtaglomerular cells, and Tempol, SOD, catalase, and JNK inhibitor partially rescued cells from arsenic trioxide-induced apoptosis through the up-regulation of intracellular GSH levels. J. Cell. Biochem. 104: 995–1009, 2008.

Key words: arsenic trioxide; ROS; cell cycle; apoptosis; As4.1; ROS scavenger; SOD; catalase; GSH; JNK inhibitor

Reactive oxygen species (ROS) include hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot -}$), and hydroxyl radical ($^{\cdot}OH$). ROS have recently been implicated in the regulation of many important cellular events, including transcription factor activation, gene expression, differentiation, and cellular proliferation [Bubici et al., 2006]. ROS are formed as byproducts of mitochondrial respiration or oxidases, including nicotine adenine diphosphate (NADPH) oxidase, xanthine oxidase (XO), and certain arachidonic acid oxygenases [Zorov et al., 2006]. A change in the redox state of the tissue implies a change in ROS generation or metabolism. Principal metabolic pathways include superoxide dismutase (SOD), which is expressed as extracellular, intracellular, and mitochondrial isoforms. These pathways metabolize $O_2^{\cdot -}$ to H_2O_2. Further metabolism by peroxidases that include catalase and glutathione peroxidase yields O_2 and H_2O [Wilcox, 2002]. Cells possess antioxidant systems to control the redox state, which is important for their survival. Excessive

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Abbreviation used: ROS, reactive oxygen species; NADPH, nicotine adenine diphosphate; XO, xanthine oxidase; SOD, superoxide dismutase; APL, acute promyelocytic leukemia; JGCT, juxtaglomerular cell tumors; FBS, fetal bovine serum; PI, propidium iodide; PBS, phosphate buffer saline; FITC, fluorescein isothiocyanate; SOD, superoxide dismutase; H_2DCFDA, 2′,7′-Dichlorodihydrofluorescein diacetate; DHE, dihydroethidium; GSH, glutathione; CMFDA, 5-chloromethylfluorescein diacetate.Assistant Professor.

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production of ROS gives rise to an activation of events that lead to death and survival in several types of cells [Chen et al., 2006; Dasmahapatra et al., 2006; Wallach-Dayan et al., 2006]. The accurate mechanisms involved in cell death induced by ROS are not fully understood and the protective effect mediated by some antioxidants remains controversial.

Arsenic trioxide (As_2O_3), 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 [Soignet et al., 1998]. 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 *t*(15;17) [Shao et al., 1998]. 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 and solid tumor cell lines without having the PML/RARα fusion protein [Park et al., 2000; Haga et al., 2005; Kong et al., 2005; Woo et al., 2006], which suggests that the antiproliferative effect of arsenic trioxide might be independent on a PML or PML/RARα fusion protein. Accumulating evidence indicates that arsenic trioxide could regulate many biological functions such as cell proliferation, apoptosis, differentiation, and angiogenesis in various cell lines [Miller et al., 2002]. Arsenic trioxide can disturb the natural oxidation and reduction equilibrium in cells, leading to an increase of ROS by a variety of redox enzymes, including flavoprotein-dependent superoxide-producing enzymes such as NADPH oxidase [Miller et al., 2002; Kim et al., 2006; Li et al., 2006]. Arsenic trioxide decreases glutathione (GSH) and increases intracellular ROS levels in certain APL cells [Dai et al., 1999]. Further, as a mitochondrial toxin, As_2O_3 induces a loss of mitochondrial transmembrane potential in cells [Park et al., 2000; Haga et al., 2005]. These phenomena could trigger the apoptosis of target cells. Therefore, it is thought that As_2O_3 may induce apoptosis in tumor cells by affecting the mitochondria, as well as the generation of ROS and the depletion of GSH. Arsenic trioxide is an important, 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 and degeneration, oliguria with proteinuria, hematuria, and acute renal failure are also frequently observed. Therefore, understanding the molecular mechanism of kidney cell death by arsenic trioxide is an important subject.

As4.1 cells have been used as a model for juxtaglomerular cells. This cell line was isolated from kidney neoplasm in a transgenic mouse containing a renin SV40 T-antigen transgene [Sigmund et al., 1990]. We recently found that arsenic trioxide inhibits the growth of As4.1 cells with an IC_{50} of about 5 μM. Arsenic trioxide efficiently induced apoptosis, as evidenced by flow cytometric detection of sub-G1 DNA content, annexin V binding assay, and DAPI staining. This apoptotic process was accompanied by a loss of mitochondrial membrane potential ($\Delta\Psi_m$), Bcl-2 down-regulation, and PARP degradation [Han et al., 2007a]. In the present study, we evaluated the involvement of GSH and ROS such as H_2O_2 and $O_2^{\cdot -}$ in arsenic trioxide-induced As4.1 cell death, and investigated whether ROS scavengers and JNK inhibitor reduce arsenic trioxide-induced As4.1 cell death.

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 [Sigmund et al., 1990]. Cell cultures were maintained in humidified room air containing 5% CO_2 at 37°C. As4.1 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (GIBCO BRL, Grand Island, NY).

Reagents

Arsenic trioxide was purchased from Sigma–Aldrich Chemical Company (St. Louis, MO), and was dissolved in 1.65 M NaOH at 1×10^{-1} M as a stock solution. The cell-permeable $O_2^{\cdot -}$ scavengers, 4-hydroxy-TEMPO (4-hydroxyl-2,2,6,6-tetramethylpierydine-1-oxyl) (Tempol), Tiron (4,5-dihydroxyl-1,3-benzededisulfonic acid), Trimetazidine (1-[2,3,4-trimethoxibenzyl]-piperazine), and NAC (N-acetylcysteine) were obtained from Sigma. These were dissolved in designated solution buffer at 1×10^{-1} M as a

stock solution. SOD and catalase were obtained from Sigma and dissolved in 50 mM potassium phosphate buffer at 4,733 U/ml. JNK inhibitor (420119), MEK inhibitor (PD98059), and p38 inhibitor (SB203580) were purchased from Calbiochem. These were dissolved in DMSO solution buffer at 1×10^{-2} M as a stock solution. Each inhibitor was used at a concentration of 10 μM. Cells were pretreated with inhibitors for 10 min prior to treatment with arsenic trioxide. All of the stock solutions were wrapped in foil and kept at 4°C or −20°C.

Cell Cycle and Sub-G_1 Analysis

Cell cycle and sub-G_1 distribution were determined by staining DNA with propidium iodide (PI; Sigma) as previously described [Park et al., 2003]. PI is a fluorescent biomolecule that can be used to stain DNA. In brief, 1×10^6 cells were incubated with the designated doses of arsenic trioxide combined with or without ROS scavenger for 48 h. Cells were then washed in phosphate-buffered saline (PBS) and fixed in 70% ethanol. Cells were again washed with PBS and then incubated with PI (10 μg) with simultaneous treatment of RNase at 37°C for 30 min. The percentages of cells in the different phases of the cell cycle or having sub-G_1 DNA content were measured with a FACStar flow cytometer (Becton Dickinson, San Jose, CA) and analyzed by using Lysis II and CellFit software (Becton Dickinson) or ModFit software (Verity Software, Inc.).

Annexin V/PI Staining

Apoptosis was determined by staining cells with annexin V-fluorescein isothiocyanate (FITC) or -phycoerythrin (PE) and PI labeling, as annexin V can be used to identify the externalization of phosphatidylserine during the progression of apoptosis and, therefore, can be used to detect cells during early phases of apoptosis as previously described [Park et al., 2007a]. PI can be also used to differentiate necrotic, apoptotic, and normal cells. This agent is membrane-impermeant and is generally excluded from viable cells. In brief, 1×10^6 cells were incubated with arsenic trioxide with or without ROS scavenger or MAPK inhibitor for 48 h. The 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 $CaCl_2$) at a concentration of 1×10^6 cells/ml. Then, 5 μl of annexin V-FITC or -PE (PharMingen, San Diego, CA) and PI (0.1 μg/ml) were 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, whereas late apoptotic dead cells displayed both high annexin V and PI labeling. Non-viable cells, which underwent necrosis, were positive for PI and negative for annexin V.

Measurement of Mitochondrial Membrane Potential ($\Delta\Psi_m$)

Mitochondrial membrane was monitored using the fluorescent dye, Rhodamine 123, a cell-permeable cationic dye that preferentially enters into mitochondria based on highly negative mitochondrial membrane potential ($\Delta\Psi_m$) as previously described [Park et al., 2000]. Depolarization of mitochondrial membrane potential ($\Delta\Psi_m$) results in the loss of Rhodamine 123 from the mitochondria and a decrease in intracellular fluorescence. In brief, 1×10^6 cells were incubated with arsenic trioxide with or without ROS scavenger or MAPK inhibitor for 48 h. Cells were washed twice with PBS and incubated with Rhodamine 123 (0.1 μg/ml; Sigma) at 37°C for 30 min. The percentages of cells showing Rhodamine 123 negative staining were determined by flow cytometry.

Detection of Intracellular ROS and $O_2^{\cdot -}$ Concentration

Intracellular general ROS such as H_2O_2, $^{\cdot}OH$ and $ONOO^-$ concentration were detected by means of an oxidation-sensitive fluorescent probe dye, 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCFDA) (Invitrogen Molecular Probes, Eugene, OR) as previously described [Han et al., 2007b; Park et al., 2007b]. H_2DCFDA was deacetylated intracellularly by nonspecific esterase, which was further oxidized by cellular peroxides to the fluorescent compound 2,7-dichlorofluorescein (DCF). Dihydroethidium (DHE) (Invitrogen Molecular Probes) is a fluorogenic probe that is highly selective for superoxide anion radical ($O_2^{\cdot -}$) among ROS. DHE is cell-permeable, and reacts with superoxide anion to form ethidium, which in turn intercalates in the deoxyribonucleic acid, thereby exhibiting a red fluorescence as previously described [Han et al., 2007b; Park et al., 2007b]. In brief, cells were incubated with the designated doses of arsenic trioxide combined

with or without ROS scavengers or MAPK inhibitors for 48 h. Cells were then washed in PBS and subsequently incubated with 20 μM H_2DCFDA 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). In particular, DCF fluorescence intensity was significantly increased in H_2O_2-treated As4.1 cells (Supplement 1). For each sample, 5,000 or 10,000 events were collected. ROS and $O_2^{\cdot -}$ levels were expressed as mean fluorescence intensity (MFI), which was calculated using CellQuest software.

Detection of Intracellular Glutathione (GSH)

Cellular GSH levels were analyzed using 5-chloromethylfluorescein diacetate (CMFDA, Molecular Probes). CMFDA is a useful, membrane-permeable dye that is used to determine intracellular glutathione levels [Macho et al., 1997]. In brief, cells were incubated with the designated doses of arsenic trioxide combined with or without ROS scavenger or MAPK inhibitor for 48 h. Cells were then washed in PBS and subsequently incubated with 5 μM CMFDA at 37°C for 30 min according to the instructions of the manufacturer. Cytoplasmic esterases convert nonfluorescent CMFDA to fluorescent 5-chloromethylfluorescein, which can then react with glutathione. CMF fluorescence was detected by a FACStar flow cytometer (Becton Dickinson), and was calculated using CellQuest software. For each sample, 5,000 or 10,000 events were collected.

Statistical Analysis

Results represent the mean of at least three independent experiments; bar, SD. Microsoft Excel or Instat software (GraphPad Prism4, San Diego, CA) was used to analyze the data. Student's *t*-test or one-way analysis of variance (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 ROS and GSH Production in As4.1 Cells

H_2DCFDA fluorescence dye was used to assess the production of intracellular ROS in arsenic trioxide-treated As4.1 cells. As shown in Figure 1A,B, intracellular ROS levels were significantly increased in As4.1 cells treated with 1 μM arsenic trioxide at 48 h. Treatment with arsenic trioxide also increased

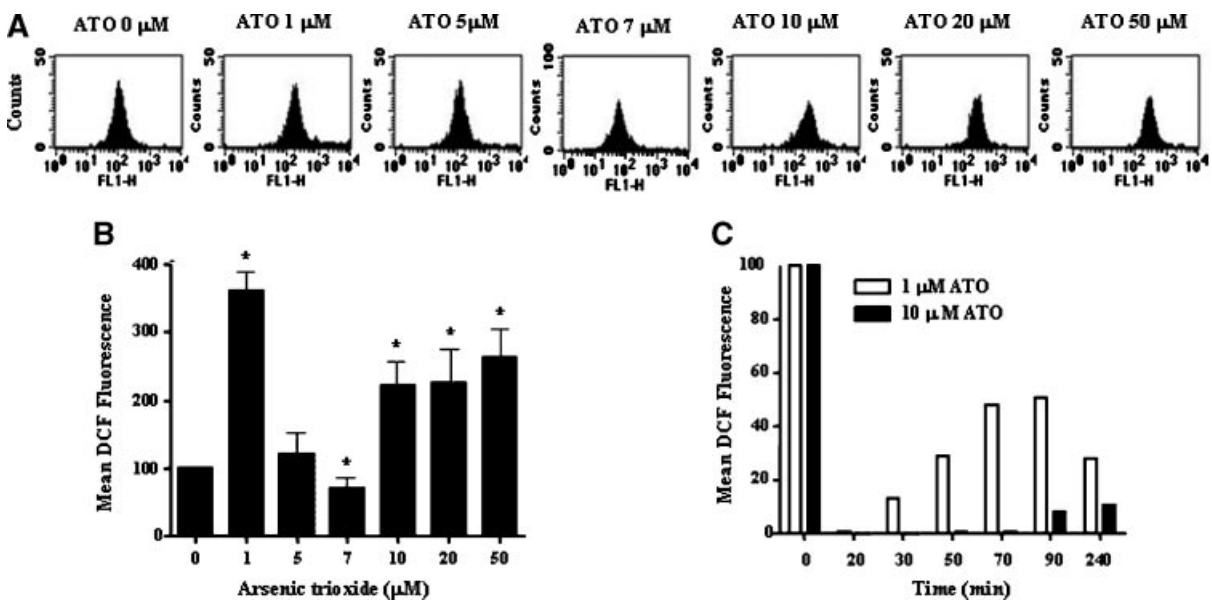

Fig. 1. Effects of arsenic trioxide on the production of ROS in As4.1 cells. **A**: Exponentially growing cells were treated with the indicated concentration of arsenic trioxide for 48 h. The intracellular H_2O_2 level was determined by a FACStar flow cytometer as described in Materials and Methods Section. **B**: Graph shows the levels of mean DCF fluorescence of A. **C**: Graph shows the intracellular H_2O_2 level for the designated amount of time. ATO stands for arsenic trioxide.*$P < 0.05$ as compared with the control group.

intracellular H_2O_2 levels among ROS (Supplement 2). However, cells treated with 7 μM arsenic trioxide clearly showed a decrease in ROS levels compared with untreated control cells. This decrease was observed several times. Additionally, at arsenic trioxide concentrations over 10 μM, ROS levels were increased (Fig. 1A,B). As demonstrated in Figure 1C, a decreased pattern in ROS levels by this drug (1 or 10 μM) was clearly detected in less than 0.5 h. Then, a slow increase of ROS levels induced by 1 μM arsenic trioxide was observed until about 90 min. However, its maximum level was approximately 50% of that of untreated control cells. In regard to the ROS levels induced by 10 μM arsenic trioxide, the increasing phenomenon started at a time of 90 min.

Next, we assessed changes of intracellular $O_2^{\cdot -}$ levels in the arsenic trioxide-treated As4.1 cells. In As4.1 cells treated with arsenic trioxide (1–50 μM), red fluorescence derived from DHE, which reflects the accumulation of $O_2^{\cdot -}$, was significantly increased more than five times that of the control cells (Fig. 2A,B). The accumulation of $O_2^{\cdot -}$ was observed at the early time of 30 min after the initiation of exposure of 10 μM arsenic trioxide (Fig. 2C). Its maximum level was reached at about 90 min, and was more than 10 times higher than that of control cells. Additionally, the level of $O_2^{\cdot -}$ in As4.1 cells treated with 1 μM arsenic trioxide was significantly increased over the time of 50 min, but its levels were lower than those of 10 μM arsenic trioxide-treated cells.

Cellular GSH has been shown to be crucial for regulation of cell proliferation, cell cycle progression and apoptosis [Schnelldorfer et al., 2000]. Therefore, we analyzed changes in the GSH levels of As4.1 cells by using CMF fluorescence. The M1 population of As4.1 cells showed intracellular GSH depleted cells. Arsenic trioxide significantly elevated the percentages of cells residing in the M1 population in a dose-dependent manner at 48 h (Fig. 3), indicating the depletion of intracellular GSH content of As4.1 cells by arsenic trioxide. Treatment with 5 μM arsenic trioxide mildly depleted the GSH content in As4.1 cells. Noteworthy changes in the depletion of intracellular GSH content were observed at arsenic trioxide concentrations of approximately 5–10 μM. The decrease of intracellular GSH content was observed at an early time of 20 min following exposure to 10 μM arsenic trioxide (data not shown).

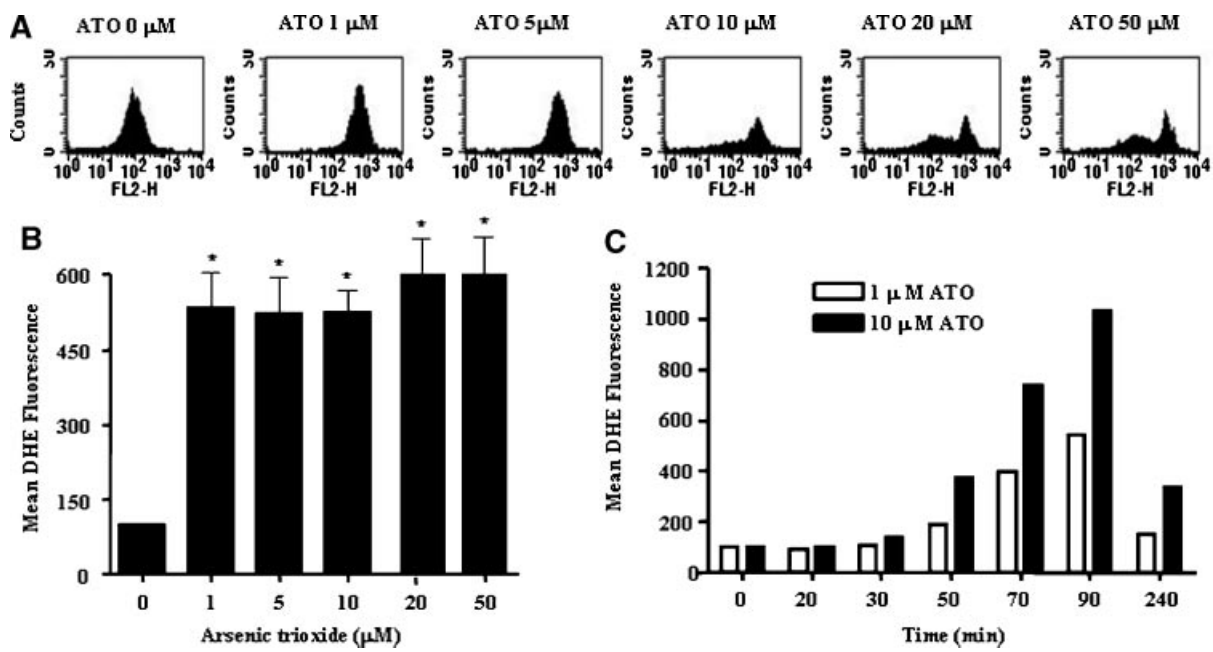

Fig. 2. Effects of arsenic trioxide on the production of ROS, $O_2^{\cdot -}$, in As4.1 cells. **A**: Exponentially growing cells were treated with the indicated concentration of arsenic trioxide for 48 h. The intracellular $O_2^{\cdot -}$ level was determined by a FACStar flow cytometer as described in Materials and Methods Section. **B**: Graph shows the levels of mean DHE fluorescence of A. **C**: Graph shows the intracellular $O_2^{\cdot -}$ level for the designated amount of time. $^*P < 0.05$ as compared with the control group.

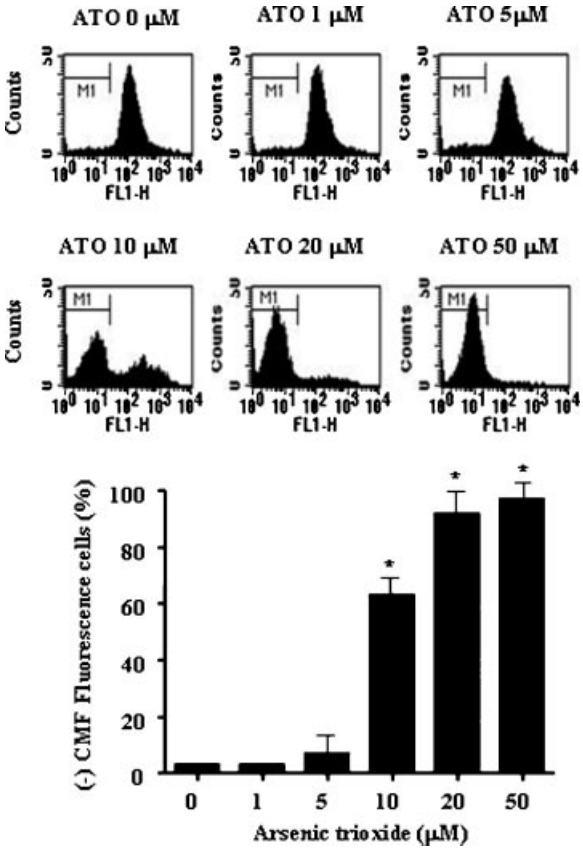

Fig. 3. Effects of arsenic trioxide on the production of GSH in As4.1 cells. Exponentially growing cells were treated with the indicated concentration of arsenic trioxide for 48 h. The intracellular GSH level was determined by a FACStar flow cytometer as described in Materials and Methods Section. Intracellular GSH depleted cells are indicated by the percent of CMF negative fluorescence (M1 region) cells of each figure. Graph shows the percent of intracellular GSH depleted cells of the above figures. $*P < 0.05$ as compared with the control group.

Effects of ROS Scavengers on ROS Production, GSH Depletion, and Apoptosis in Arsenic Trioxide-Treated As4.1 Cells

To determine whether ROS production and GSH depletion in arsenic trioxide-treated As4.1 cells were changed by ROS scavengers, cell-permeable ROS scavengers, Tempol and Tiron [Yamada et al., 2003], and a well-known antioxidant, NAC, were co-incubated with the arsenic trioxide-treated As4.1 cells for 48 h. An anti-ischemic and metabolic agent, Trimetazidine, was also used as an indirect antioxidant [Stanley and Marzilli, 2003]. As shown in Figure 4A,D, the increased level of ROS induced by 10 μM arsenic trioxide was not decreased by Tempol (50 μM), Trimetazidine (50 μM), and NAC (50 μM). However, Tiron significantly decreased ROS levels in 10 μM arsenic trioxide-treated As4.1 cells. In regard to the $O_2^{\cdot -}$ levels in relation to these ROS scavengers, Tempol, Trimetazidine, and NAC (50 μM) actually augmented the $O_2^{\cdot -}$ levels in arsenic trioxide-treated cells (Fig. 4B,E), while Tiron did not alter the $O_2^{\cdot -}$ levels in arsenic trioxide-treated cells. To measure the precise intracellular fluorescence level of ROS, we used only the cells residing in the R2 region, which could be considered cells with intact plasma membrane (Fig. 4A,B). In addition, only the Tiron significantly decreased ROS levels in As4.1 cells treated with 1 μM arsenic trioxide to a level below that of control cells. None of the ROS scavengers used in this experiment significantly altered the $O_2^{\cdot -}$ levels in 1 μM arsenic trioxide-treated cells (data not shown). The scavengers did not decrease the depletion of GSH content in As4.1 cells treated with arsenic trioxide (10 μM). Only the Tempol showed a very slight increase of GSH content in arsenic trioxide-treated cells (Fig. 4C,F).

Next, we examined whether these scavengers (50 μM) could prevent arsenic trioxide-induced As4.1 cell death. Only the Tempol could decrease the sub-G1 cell population in As4.1 cells treated with arsenic trioxide; the sub-G1 cell population was decreased by approximately 10% (Fig. 5A,D). Other scavengers did not have a similar effect. In view of annexin V positive staining, Tempol, Tiron, and NAC slightly decreased the level of annexin V positive staining in As4.1 cells treated with arsenic trioxide. None of the ROS scavengers could block the loss of mitochondrial membrane potential ($\Delta\Psi_m$). When we used apoptotic cells induced by 8 μM arsenic trioxide for these experiments, similar results were shown (data not shown). Additionally, these ROS scavengers at 0.1, 0.5, and 2.5 mM concentrations did not have significant effects on apoptosis parameters (data not shown). In regard to the cell cycle distribution in relation to ROS scavengers, these scavengers were not able to significantly alter cell cycle distribution changed by arsenic trioxide (10 μM) (Fig. 6).

Effects of SOD and Catalase on ROS Production, GSH Depletion, and Apoptosis in Arsenic Trioxide-Treated As4.1 Cells

Next, to determine whether ROS production and GSH depletion in arsenic trioxide-treated As4.1 cells were changed by exogenous SOD and

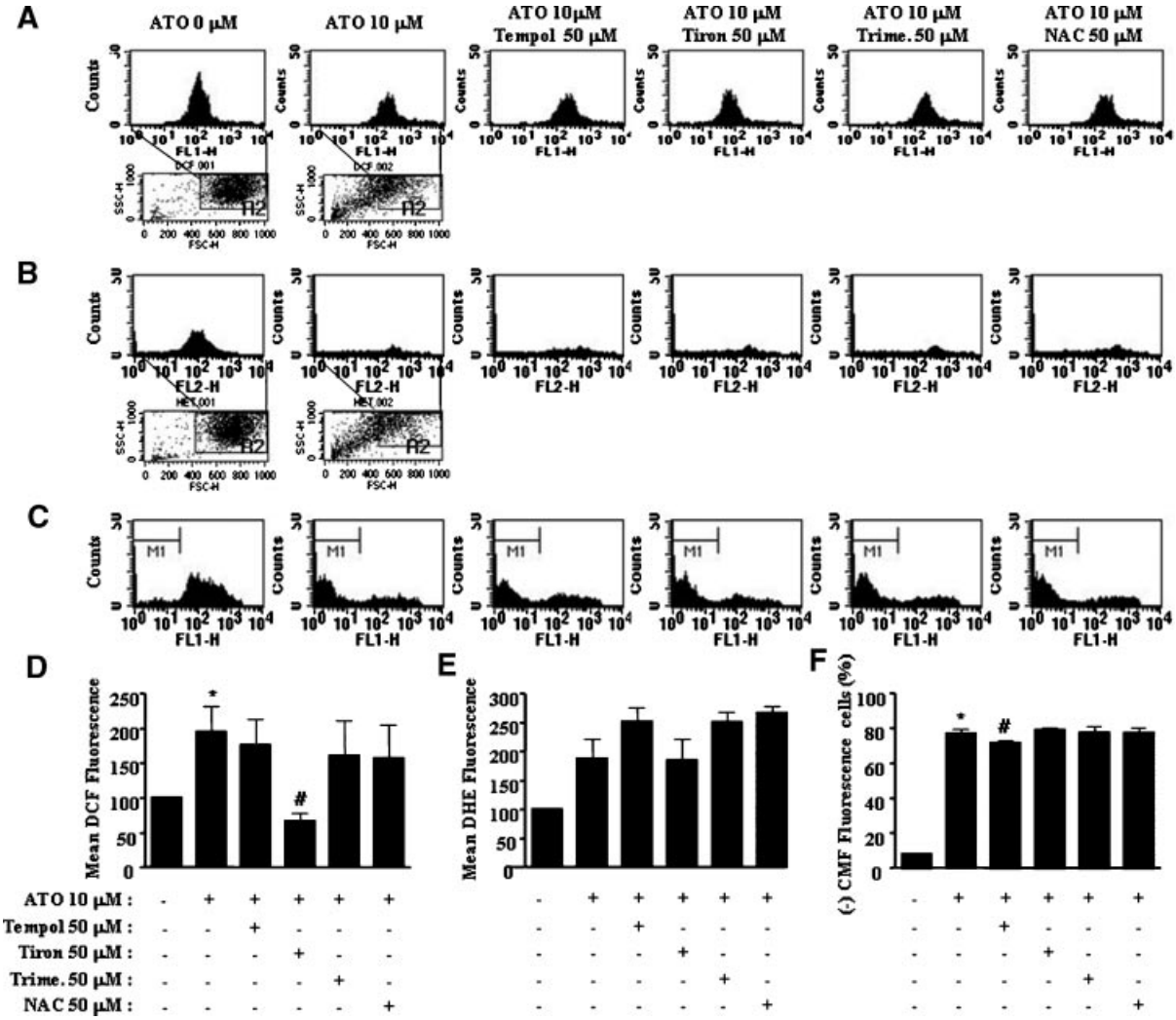

Fig. 4. Effects of ROS scavengers on the production of intracellular ROS and GSH in arsenic trioxide-treated As4.1 cells. Exponentially growing cells were treated with the indicated ROS scavengers in addition to 10 μM of arsenic trioxide for 48 h. **A**: Intracellular ROS levels, which is derived from the cells in the R2 region of the FSC and SSC dot plot. **B**: Intracellular $O_2^{\cdot -}$ level from the cells in the R2 region. **C**: Intracellular GSH level. Graphs show the levels of mean DCF fluorescence of A (**D**), mean DHE fluorescence of B (**E**), and the percent of intracellular GSH depleted cells of C (**F**). $^{*}P < 0.05$ as compared with the control group. $^{\#}P < 0.05$ as compared with the only ATO-treated cells.

catalase, As4.1 cells were treated with arsenic trioxide in the presence or absence of SOD (30 U/ml) and/or catalase (30 U/ml) for 48 h. In this experiment, we used 8 μM arsenic trioxide for the induction of apoptosis in As4.1 cells, because the apoptotic cells induced by 10 μM arsenic trioxide (Fig. 5) were not significantly rescued by SOD and catalase (data not shown). As shown in Figure 7A,D, the level of ROS in As4.1 cells was not altered by 8 μM arsenic trioxide. In addition, neither SOD nor catalase was able to alter the level of ROS in arsenic trioxide-treated As4.1 cells. In regard to the $O_2^{\cdot -}$ levels induced by SOD and catalase, SOD decreased the increased $O_2^{\cdot -}$ levels that were present in arsenic trioxide-treated cells (Fig. 7B,E). Catalase was more effective in reducing the $O_2^{\cdot -}$ levels in arsenic trioxide-treated cells than was SOD. No synergistic effect of SOD and catalase was observed in terms of the reduction of $O_2^{\cdot -}$ levels. SOD or catalase alone did not decrease the basal level of intracellular $O_2^{\cdot -}$ in As4.1 cells. When we assessed the GSH levels of intracellular GSH in arsenic trioxide-treated cells in the presence of SOD and/or catalase, SOD mildly decreased the depletion of GSH content in As4.1 cells treated with arsenic trioxide (8 μM), while catalase

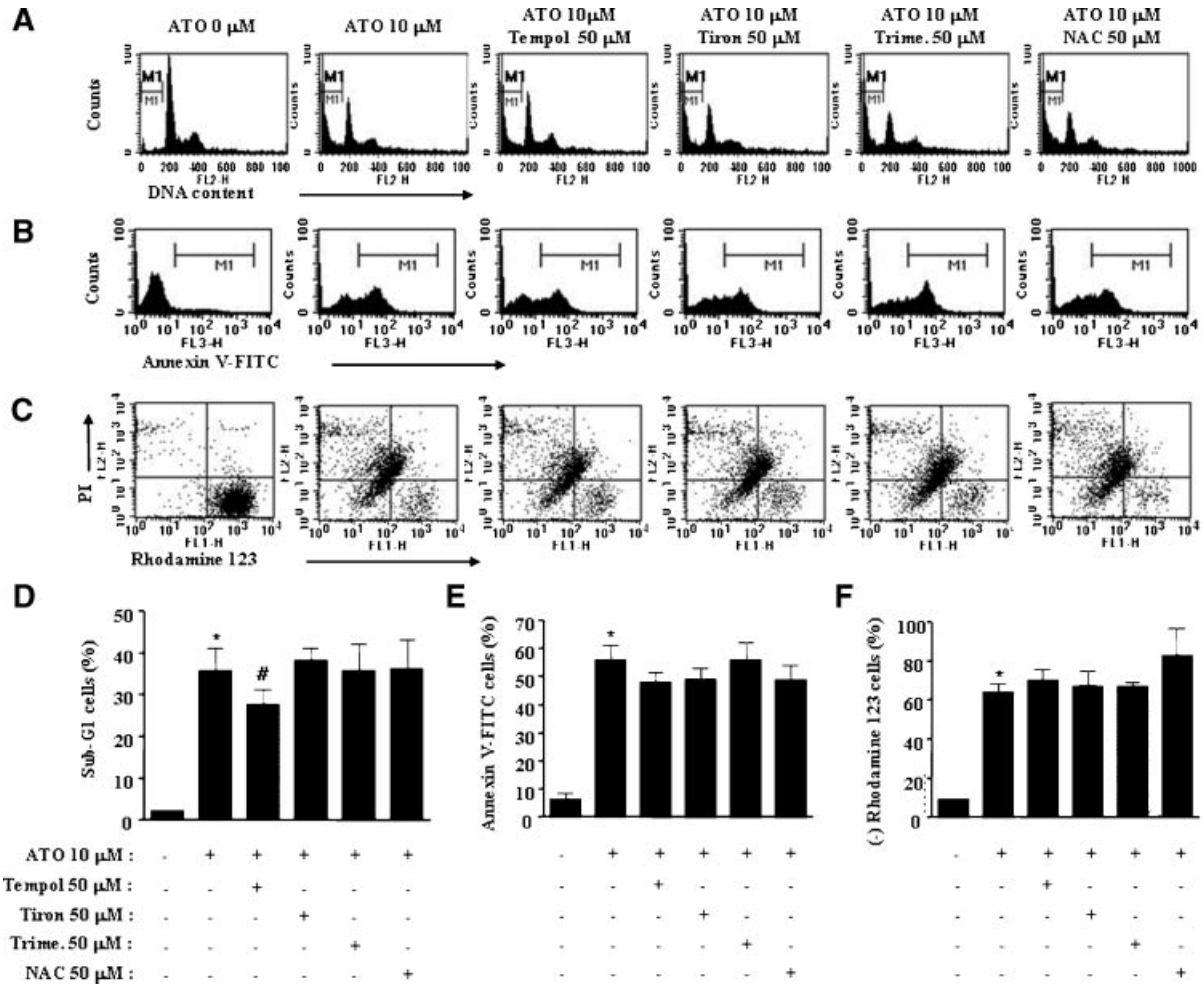

Fig. 5. Effects of ROS scavengers on arsenic trioxide-induced apoptosis. Exponentially growing cells were treated with the indicated ROS scavengers in addition to 10 μM of arsenic trioxide for 48 h. **A**: Sub-G1 cells. **B**: Annexin positive cells. **C**: Rhodamine 123 negative cells were measured by flow cytometric analysis as described in Materials and Methods Section. Graphs show the percentage of sub-G_1 population from A (**D**), annexin positive cells from B (**E**), and Rhodamine 123 negative cells from C (**F**). $^{*}P < 0.05$ as compared with the control group. $^{\#}P < 0.05$ as compared with the only ATO-treated cells.

induced a significant decrease (Fig. 7C,F). No synergistic or addictive effect of SOD and catalase was observed, and SOD or catalase alone did not alter the GSH content in As4.1 cells.

Next, we examined whether SOD and catalase could prevent arsenic trioxide-induced As4.1 cell death. SOD slightly decreased the level of annexin V positive staining in As4.1 cells treated with arsenic trioxide. Catalase was able to strongly decrease the annexin V positive stained cells by about 20% (Fig. 8A,C). In addition, catalase was stronger in reducing the loss of mitochondrial transmembrane potential ($\Delta\Psi_m$) in arsenic trioxide-treated As4.1 cells than was SOD (38% vs. 16%) (Fig. 8B,D). No synergistic or addictive effect of SOD and catalase was observed in relation to the reduction of the loss of mitochondrial transmembrane potential ($\Delta\Psi_m$) in arsenic trioxide-treated As4.1 cells, although the use of SOD and/or catalase strongly conserved mitochondrial transmembrane potential ($\Delta\Psi_m$) in As4.1 cells (Fig. 8B,D). In regard to the cell cycle distribution induced by SOD and catalase, neither SOD nor catalase was able to alter cell cycle distribution changed by arsenic trioxide (8 μM) (Fig. 9). When we used SOD (60 U/ml) and catalase (60 U/ml) in this experiment, these concentrations showed no differences in terms of ROS production, GSH content, apoptosis parameters, and cell cycle distribution in com-

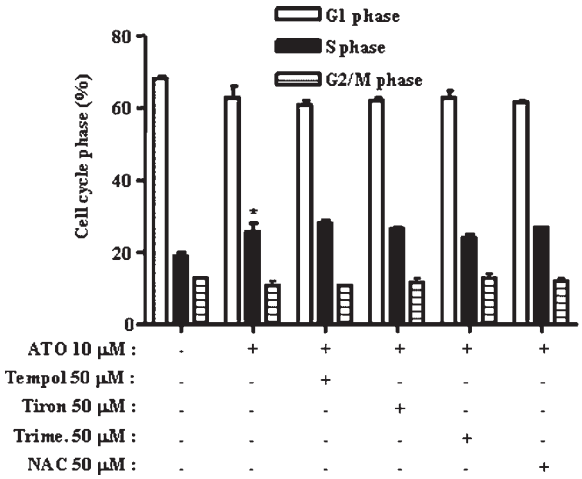

Fig. 6. Effects of ROS scavengers on cell cycle distribution in arsenic trioxide-treated As4.1 cells. Exponentially growing cells were treated with the indicated ROS scavengers in addition to 10 μM of arsenic trioxide for 48 h. Graph shows the cell cycle distribution following treatment with arsenic trioxide and ROS scavengers. $^{*}P < 0.05$ as compared with the control group.

parison those shown following exposure to lower concentrations of SOD (30 U/ml) and catalase (30 U/ml) (data not shown).

Effects of MEK, JNK, and p38 Inhibitor on ROS Production, GSH Depletion, and Apoptosis in Arsenic Trioxide-Treated As4.1 Cells

Next, we investigated whether ROS production and GSH depletion in arsenic trioxide-treated As4.1 cells were changed by MEK, JNK, or p38 inhibitor. As4.1 cells were treated with arsenic trioxide in the presence or absence of MEK, JNK, or p38 inhibitor for 48 h. As shown in Figure 10A, MEK inhibitor slightly increased the level of ROS in arsenic trioxide-treated As4.1 cells. JNK and p38 inhibitors did not alter the ROS levels in these cells (Fig. 10A). In regard to $O_2^{\cdot -}$ levels, JNK inhibitor reduced the $O_2^{\cdot -}$ levels in arsenic trioxide-treated As4.1 cells (Fig. 10B). MEK inhibitor very slightly reduced the $O_2^{\cdot -}$ levels in these cells, but p38 inhibitor did not significantly alter the levels in these cells (Fig. 10B). When we assessed the

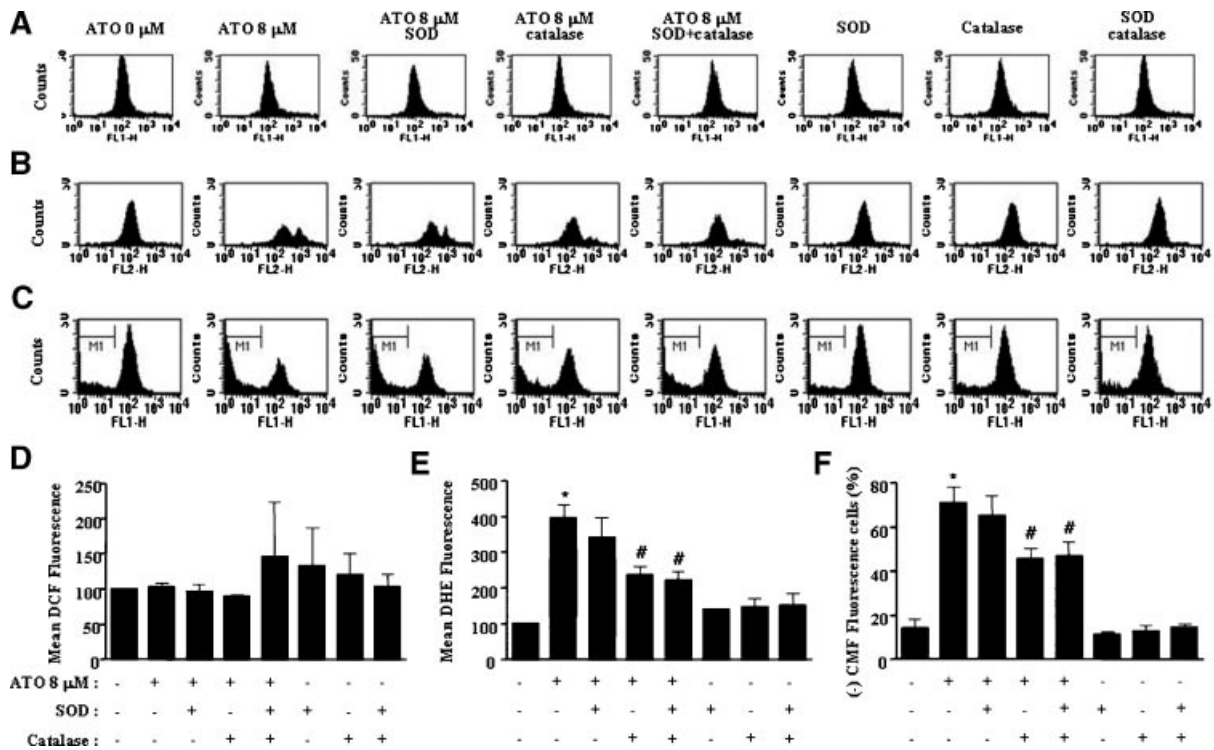

Fig. 7. Effects of SOD and catalase on intracellular ROS and GSH production in arsenic trioxide-treated As4.1 cells. Exponentially growing cells were treated with SOD, catalase, and arsenic trioxide (8 μM) for 48 h. **A**: Intracellular ROS levels. **B**: Intracellular $O_2^{\cdot -}$ level. **C**, Intracellular GSH level. Graphs show the levels of mean DCF fluorescence of A (**D**), mean DHE fluorescence of B (**E**), and the percent of intracellular GSH depleted cells of C (**F**). $^{*}P < 0.05$ as compared with the control group. $^{\#}P < 0.05$ as compared with the only ATO-treated cells.

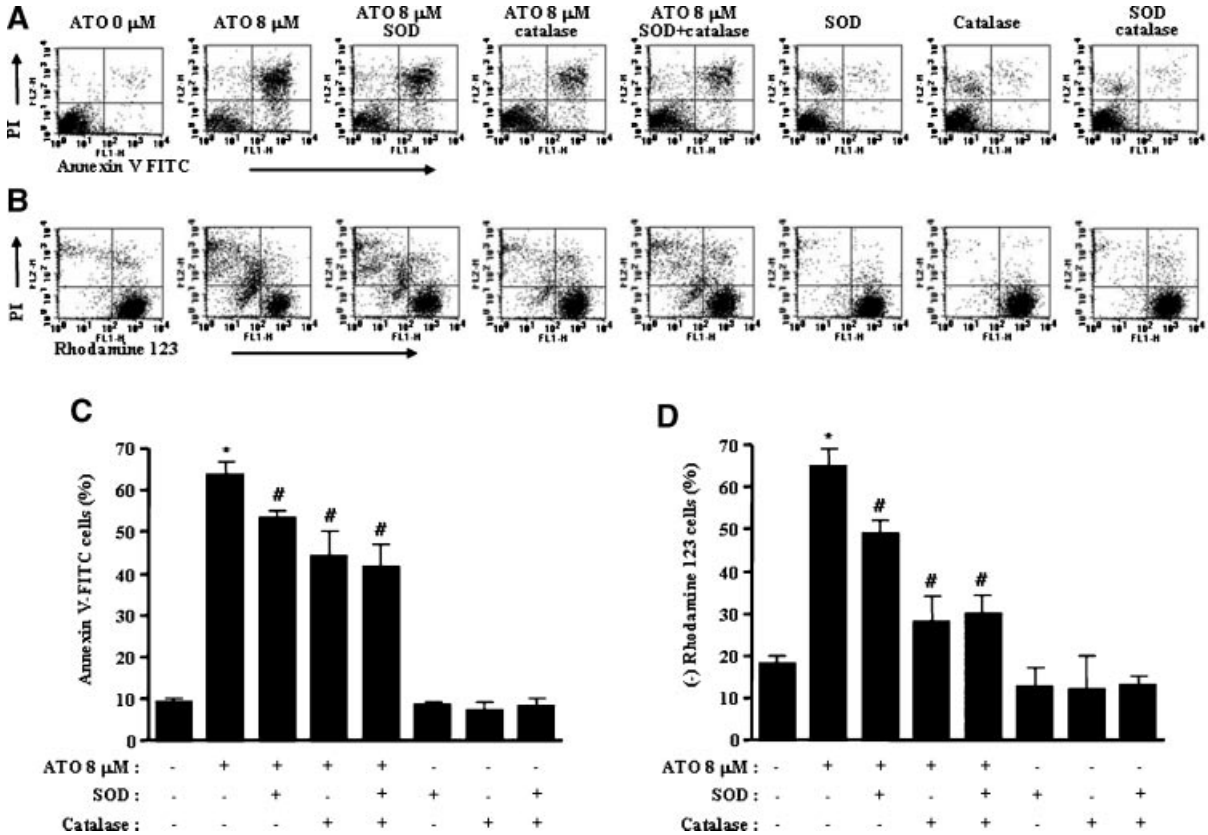

Fig. 8. Effects of SOD and catalase on arsenic trioxide-induced apoptosis. Exponentially growing cells were treated with SOD, catalase, and arsenic trioxide (8 μM) for 48 h. **A**, Annexin positive cells and **B**, Rhodamine 123 negative cells were measured by flow cytometric analysis as described in Materials and Methods Section. Graphs show the percentage of annexin positive cells from A (**C**) and Rhodamine 123 negative cells from B (**D**). $^{*}P < 0.05$ as compared with the control group. $^{\#}P < 0.05$ as compared with the only ATO-treated cells.

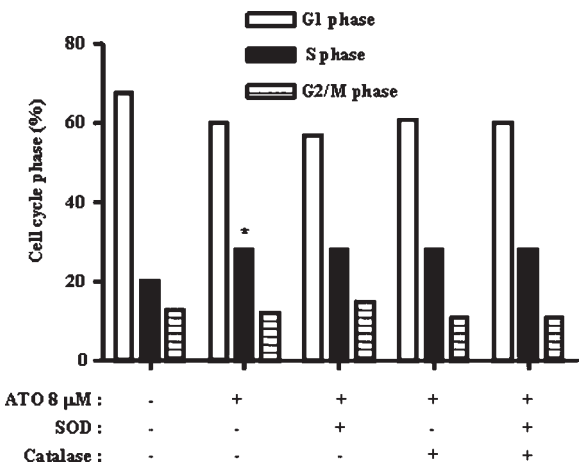

Fig. 9. Effects of SOD and catalase on cell cycle distribution in arsenic trioxide-treated As4.1 cells. Exponentially growing cells were treated with SOD, catalase, and arsenic trioxide (8 μM) for 48 h. Graph shows the cell cycle distribution by SOD, catalase, and arsenic trioxide. $^{*}P < 0.05$ as compared with the control group.

effects on the levels of intracellular GSH by these inhibitors, JNK inhibitor reduced the depletion of GSH content by about 9% in As4.1 cells treated with arsenic trioxide (Fig. 10C). MEK and p38 inhibitors did not significantly alter the GSH content levels in these cells (Fig. 10C).

Next, we examined whether MEK, JNK, or p38 inhibitor acted to prevent arsenic trioxide-induced As4.1 cell death. JNK inhibitor decreased the levels of annexin V positive staining cells by about 7% in As4.1 cells treated with arsenic trioxide (Fig. 11A). Other inhibitors did not alter the number of annexin V positive staining arsenic trioxide-treated As4.1 cells (Fig. 11A). JNK inhibitor also reduced the loss of mitochondrial transmembrane potential ($\Delta\Psi_m$) by about 13% in arsenic trioxide-treated As4.1 cells (Fig. 11B), while other inhibitors had not effect on the loss of mitochondrial transmembrane potential ($\Delta\Psi_m$) in these cells (Fig. 11B).

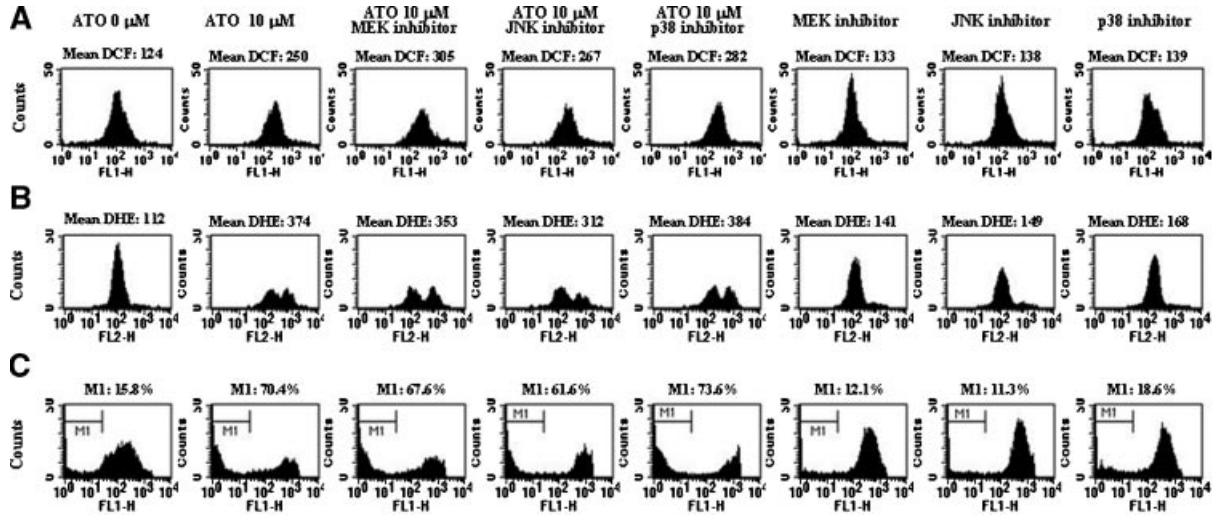

Fig. 10. Effects of MEK, JNK, and p38 inhibitor on intracellular ROS and GSH production in arsenic trioxide-treated As4.1 cells. Exponentially growing cells were treated with arsenic trioxide (10 μM) and/or MEK, JNK, and p38 inhibitor for 48 h. **A**: Intracellular ROS levels is indicated by the value of mean DCF fluorescence in cells in each figure. **B**: Intracellular $O_2^{\cdot -}$ level is indicated by the value of mean DHE fluorescence in cells in each figure. **C**: Intracellular GSH depleted cells are indicated by the percent of CMF negative fluorescence (M1 region) cells of each figure.

DISCUSSION

In this study, we focused on the involvement of GSH and ROS such as H_2O_2 and $O_2^{\cdot -}$ in arsenic trioxide-induced As4.1 cell death and investigated whether ROS scavengers and JNK inhibitor reduce As4.1 cell death, since we recently found that arsenic trioxide inhibited the growth of As4.1 cells with an IC_{50} of about 5 μM using an MTT assay at 48 h [Han et al., 2007a]. The treatment with 5 μM arsenic trioxide induced apoptosis approximately 20%. In this experiment, we used a concentration of 8 or 10 μM arsenic trioxide because these doses were considered to be suitable to differentiate the levels of apoptosis in the presence of arsenic trioxide versus apoptosis in the presence of arsenic trioxide and ROS scavengers or JNK inhibitor.

Our data showed that the intracellular ROS levels decreased or increased depending on the concentration and length of incubation with arsenic trioxide in As4.1 cells. In fact, 7 or 8 μM arsenic trioxide could efficiently induce apoptosis in As4.1 cells without causing an increase in the intracellular ROS levels. In contrast,

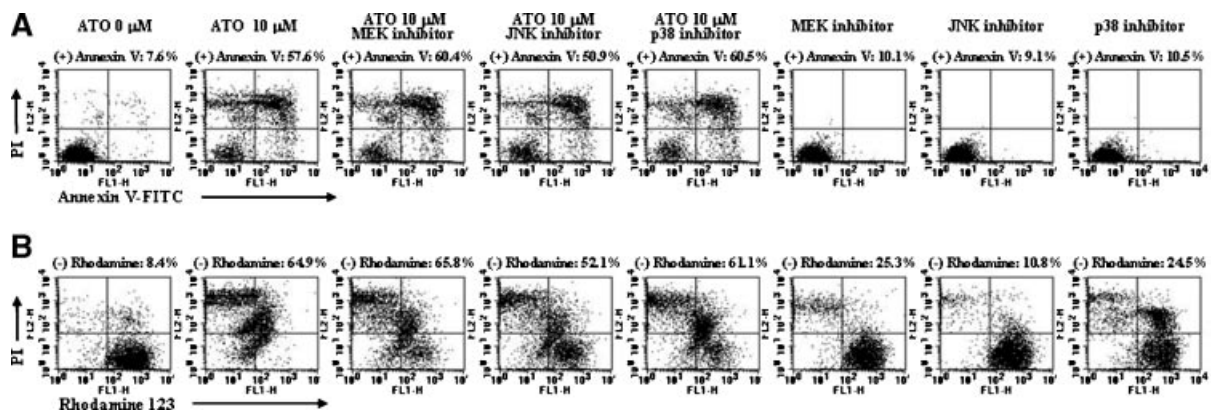

Fig. 11. Effects of MEK, JNK, and p38 inhibitor on arsenic trioxide-induced apoptosis. Exponentially growing cells were treated with arsenic trioxide (10 μM) and/or MEK, JNK, and p38 inhibitor for 48 h. **A**: Percentage of annexin positive cells in each figure. **B**: Percentage of Rhodamine 123 negative cells in each figure.

treatment with 1 μM arsenic trioxide strongly increased the levels of ROS in As4.1 cells, but did not significantly induce apoptosis (data not shown). It is possible that arsenic trioxide induces ROS production without the induction of apoptosis at this low concentration. These results are not consistent with other reports, which have shown that increased intracellular ROS such as H_2O_2 plays an important role in arsenic trioxide-induced cell death in cervical cancer cells [Kang et al., 2004], APL cells [Jing et al., 1999], hepatocellular carcinoma HepG2 [Li et al., 2006], and glioblastoma A172 cells [Haga et al., 2005]. Data from Haga et al. [2005] also showed that accumulation of H_2O_2 was detected in arsenic trioxide-treated glioblastoma T98G cells, although apoptosis did not occur in these cells. These discrepancies are likely to result from cell-type specificity and/or different methods used to detect ROS such as H_2O_2 levels. We investigated whether the intracellular changes of ROS levels induced by ROS scavengers prevent the cell death induced by 10 μM arsenic trioxide. Treatment with Tiron was found to significantly reduce the level of ROS in As4.1 cells treated with 10 μM arsenic trioxide, but this decrease was not proportional to the arsenic trioxide-induced decrease in the number of sub-G1 cells, which suggests that the changes of intracellular ROS by arsenic trioxide were not directly related to apoptosis in As4.1 cells. This notion could be supported by the finding that SOD and catalase significantly lessen apoptosis induced by arsenic trioxide without altering intracellular ROS levels. Therefore, further studies are needed to verify the role of intracellular ROS in arsenic trioxide-induced cell death.

It has been reported that the increased pattern of $O_2^{\cdot -}$ induced by arsenic trioxide was reported in esophageal cancer SHEE85 cells [Shen et al., 2003], but this pattern was not observed in arsenic trioxide-treated acute myelogenous leukemia HL-60 cells [Han et al., 2005] and renal cell carcinoma ACHN cells [Wu et al., 2004]. Therefore, we assessed whether the intracellular levels of $O_2^{\cdot -}$ were increased by arsenic trioxide, and found that arsenic trioxide markedly enhanced the $O_2^{\cdot -}$ content in As4.1 cells at 48 h. We also detected a striking accumulation of $O_2^{\cdot -}$ in arsenic trioxide-treated cells in less than 30 min. It is possible that arsenic trioxide directly or indirectly inhibited SOD, resulting in increased $O_2^{\cdot -}$ levels in the As4.1 cells. To test this possibility, we used Tempol and Tiron as stable SOD mimetics [Greenstock and Miller, 1975; Yamada et al., 2003]. As shown in our results, Tempol and Tiron were not able to decrease $O_2^{\cdot -}$ levels in arsenic trioxide-treated As4.1 cells. This non-effect of Tempol and Tiron as $O_2^{\cdot -}$ scavengers likely results from the peculiarity of As4.1 cells, since we have detected that these scavengers significantly decreased $O_2^{\cdot -}$ levels in arsenic trioxide-treated Calu-6 human lung carcinoma cells (data not shown). A well-known antioxidant, NAC and an anti-ischemic and metabolic agent, Trimetazidine also did not reduce the $O_2^{\cdot -}$ levels, while SOD was able to slightly reduce the $O_2^{\cdot -}$ levels. Taken together, the induction of $O_2^{\cdot -}$ levels in As4.1 cells by arsenic trioxide could not simply be explained by the decreased activity of SOD. Interestingly, SOD and catalase were able to slightly reduce the $O_2^{\cdot -}$ levels, but the reducing proportion of apoptotic cells by these was significant. These data suggest that besides the intracellular level of $O_2^{\cdot -}$, other redox indicators such as GSH are required for explaining the arsenic trioxide-induced apoptosis in As4.1 cells.

With regard to intracellular GSH, which is a main non-protein antioxidant in the cell, it was able to clear away the superoxide anion free radical and provide electrons for enzymes that reduce H_2O_2 to H_2O, such as glutathione peroxidase. It has been reported that the intracellular GSH content has a decisive effect on arsenic trioxide-induced apoptosis [Dai et al., 1999; Kitamura et al., 2000; Wu et al., 2004; Li et al., 2006]. These data demonstrate that the apoptotic effects of arsenic trioxide are inversely proportional to GSH content. Likewise, our result clearly indicates the depletion of intracellular GSH content by arsenic trioxide in As4.1 cells. These results support the suggestion that intracellular GSH levels are tightly related to a factor in arsenic trioxide-induced cell death. In fact, only the Tempol slightly recovered the depleted GSH and decreased the levels of apoptosis in arsenic trioxide-treated cells without the reduction of intracellular $O_2^{\cdot -}$ levels. When we measured the expression of a proapoptotic protein, Bax, in arsenic trioxide-treated cells in the presence of Tempol, we observed that Bax expression was decreased in Tempol-treated cells (data not shown), which suggests that Tempol inhibits apoptosis to a small extent through the down-regulation of

Bax. In regard to the regulation of GSH by SOD and catalase, catalase was stronger in terms of the recovery of the GSH depletion induced by arsenic trioxide compared to SOD. This result is consistent with the notion that a redox system can maintain a normal intracellular GSH level by regulating catalase and peroxidase activity rather than that of SOD. Such a proportion of GSH content by SOD and catalase in arsenic trioxide-treated As4.1 cells could also be applicable to the apoptosis index levels such as annexin V positive staining and the loss of mitochondrial transmembrane potential ($\Delta\Psi_m$). In fact, catalase had a strong anti-apoptotic capacity compared to SOD. This ability was supported by the observation that the cleavage of poly(ADP-ribose) polymerase (PARP) protein in the arsenic trioxide-treated cells was repressed by catalase, but not by SOD (data not shown). This result indicates the importance of GSH levels in arsenic trioxide-induced cell death. Interestingly, we could not observe the synergistic or additive effect of SOD and catalase on the ROS, GSH, and apoptosis parameters. These results suggest that catalase works downstream of SOD and intracellular GSH levels regulated by catalase is a critical step in triggering apoptosis. In terms of the cell cycle distribution following treatment with ROS scavengers, SOD, and catalase, none of these were able to alter S phase accumulation of cells induced by arsenic trioxide, which indicates that the changes to ROS levels and GSH content by these factors are not tightly related to the regulation of the cell cycle in As4.1 cells.

It has been reported that arsenic trioxide-induced apoptosis involves stress-mediated pathways such as activation of JNK and ERK [Ramos et al., 2006; Potin et al., 2007]. Therefore, we investigated whether ROS production, GSH depletion, and apoptosis in arsenic trioxide-treated As4.1 cells were changed by MEK, JNK, or p38 inhibitor. In particular, treatment with JNK inhibitor decreased apoptosis in arsenic trioxide-treated cells. Our result supports the report that the activation of JNK is a crucial mediator of arsenic trioxide-induced apoptosis [Davison et al., 2004; Potin et al., 2007]. However, this anti-apoptotic effect of JNK inhibitor was not accompanied by the reduction of ROS in arsenic trioxide-treated As4.1 cells. In regard to $O_2^{\cdot -}$ levels, JNK inhibitor reduced the $O_2^{\cdot -}$ levels in arsenic trioxide-treated As4.1 cells. MEK inhibitor also very slightly reduced the $O_2^{\cdot -}$ levels, but did not reduce apoptosis in arsenic trioxide-treated As4.1 cells. These results suggest that ROS levels in arsenic trioxide-treated As4.1 cells are not wholly related to apoptosis, but are at least partially related to apoptosis. We also rule out the possibility that the production of ROS by arsenic trioxide may be a standby marker of arsenic trioxide treatment. Concerning the levels of intracellular GSH following treatment with these inhibitors, JNK inhibitor significantly reduced the depletion of GSH content in As4.1 cells treated with arsenic trioxide, which provides additional support for the notion that intracellular GSH levels are tightly related to a factor in arsenic trioxide-induced cell death.

It is speculated that the kidney and juxtaglomerular apparatus (JGA) contain an ROS-generating system that is responsive to angiotensin II [Wilcox, 2002, 2003]. The ROS in the JGA-related cells are related to blood pressure regulation [Wilcox, 2002, 2003]. However, the roles of ROS in kidney cell death, especially JG cells, have not been evaluated in inspections of apoptosis. Therefore, understanding the molecular mechanism of kidney cell death by ROS generators, especially arsenic trioxide, is an important subject, since arsenic trioxide is a common environmental toxin and initial glomerular damage leading to proteinuria is common following high arsenic exposure.

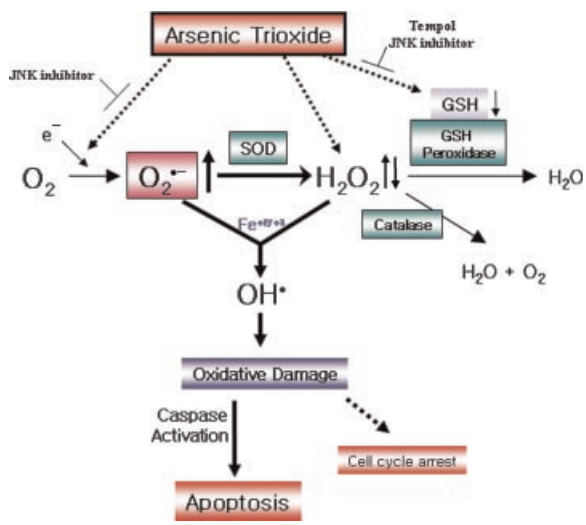

Fig. 12. The scheme for arsenic trioxide-induced As4.1 cell death through ROS and GSH. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

In summary, we have demonstrated that arsenic trioxide potently generates ROS, especially $O_2^{\cdot -}$, in As4.1 JG cells, and have also shown that Tempol, SOD, catalase, and JNK inhibitor partially rescues cells from arsenic trioxide-induced apoptosis through the up-regulation of intracellular GSH levels (Fig. 12).

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