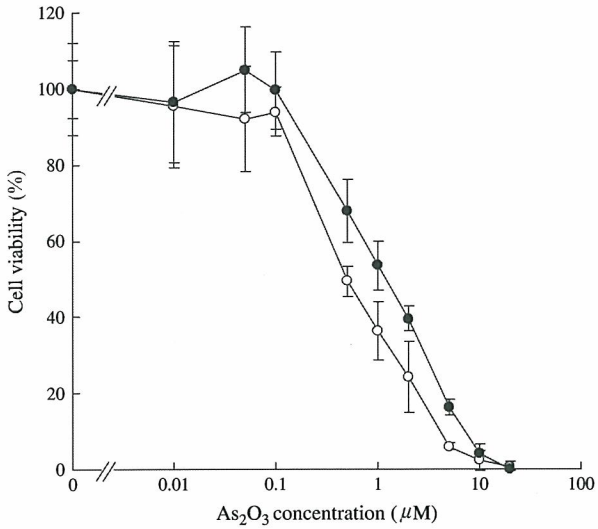

Figure 2. Viability of mock- or *HMOX1*-transfected HEK293 cells exposed to various concentration of As_2O_3. Cells were treated for 48 hours with 0.01 to 20 μM As_2O_3, and cell viability was measured using the Premix WST-1 cell proliferation assay system. The assay was performed in quadruplicate with essentially similar results. (○) mock-transfected HEK293 cells, (⇑) *HMOX1*-transfected HEK293 cells. Data are presented as the mean ± standard error. Cell viability was determined as the optical density at 440 to 600 nm relative to that of the sample treated with 0 μM As_2O_3.

53.5%, 39.5%, and 16.2%, respectively, which was greater than that of the mock-transfected HEK293 cells at each concentration of arsenic trioxide (49.3%, 36.3%, 24.5%, and 6.0%, respectively). Thus, *HMOX1* may confer a cytoprotective effect against arsenic trioxide.

Arsenic trioxide-induced cell death is associated with ROS

HMOX1 is induced by oxidative stress, therefore, we examined whether oxidative stress, especially ROS such as superoxide anion or hydrogen peroxide, could damage HEK293 cells. After incubation with 1 and 2 mU/mL xanthine oxidase and 1 mM hypoxanthine for 24 hours, cell survival was 37.1% and 5.7%, respectively (data not shown). Similarly, after incubation with 20 and 40 μM hydrogen peroxide for 24 hours, cell survival was 63.5% and 32.3%, respectively (data not shown). Thus, superoxide anion and hydrogen peroxide showed dose-dependent cytotoxicity in HEK293 cells.

To determine which ROS might be related to arsenic trioxide-induced cytotoxicity, HEK293 cells were treated with arsenic trioxide, superoxide anion, or hydrogen peroxide in the presence or absence of Tiron (a superoxide anion scavenger), DPI (an inhibitor of NAD(P)H oxidase), superoxide dismutase (an enzyme that catalyzes hydrogen peroxide formation from superoxide anion), and catalase (an enzyme that catalyzes the conversion of hydrogen peroxide to oxygen and water) (Fig. 7). Tiron and catalase significantly protected HEK293 cells from arsenic trioxide-induced cell death, whereas superoxide dismutase significantly enhanced arsenic trioxide cytotoxicity (Fig. 3A). Tiron and catalase significantly protected HEK293 cells from superoxide anion-induced cell death, whereas superoxide dismutase significantly enhanced superoxide anion cytotoxicity (Fig. 3B). Catalase significantly protected HEK293 cells from hydrogen peroxide-induced cell death; Tiron showed no effect on cell viability, and superoxide dismutase significantly enhanced hydrogen peroxide cytotoxicity (Fig. 3C). The viability of HEK293 cells in the presence of 0.05, 0.1, and 0.2 μM DPI was 81%, 79.1%, and 74.1%, respectively. DPI did not affect hydrogen peroxide-induced toxicity and only slightly inhibited arsenic trioxide cytotoxicity. However, DPI significantly prevented superoxide anion cytotoxicity (Fig. 3D). These data suggest that arsenic trioxide induced both superoxide anion and hydrogen peroxide generation through the activation of NAD(P)H oxidase.

To confirm the existence of superoxide anion in arsenic trioxide-exposed HEK293 cells, superoxide anion was detected with MCLA, a selective chemiluminescent reagent for superoxide anion [24]. The generation of superoxide anion was accelerated by the addition of arsenic trioxide, suggesting that arsenic trioxide enhances production of superoxide anion (Fig. 4).

α-Lipoic acid attenuates arsenic oxide's cytotoxicity in PRCC and HEK293 cells

Cytotoxicity of arsenic trioxide appears to be, at least in part, attributable to ROS, therefore, we hypothesized that some antioxidants may protect against arsenic trioxide cytotoxicity. We examined the ability of α-lipoic acid to act as such an antioxidant. In PRCC, the viability following exposure to 1, 2, 5, 10, and 20 μM arsenic trioxide was recovered by the addition of α-lipoic acid (Fig. 5A). Similarly, the viability of HEK293 cells after treatment with 0.5, 1, 2, and 5 μM arsenic trioxide was regained by the addition of α-lipoic acid (Fig. 5A). Thus, α-lipoic acid showed cytoprotective activity against arsenic trioxide-induced cell injury, supporting our hypothesis that the mechanism of arsenic trioxide cytotoxicity is at least partly attributable to ROS.

To confirm the antioxidant activity of α-lipoic acid, HEK293 cells exposed to arsenic trioxide and/or α-lipoic acid were assayed for the presence of superoxide anion. Arsenic trioxide-induced superoxide anion generation was suppressed after adding α-lipoic acid (Fig. 5B), demonstrating an antioxidant effect of α-lipoic acid against superoxide anion production.

α-Lipoic acid significantly protected HEK293 cells from arsenic trioxide-induced cell death, slightly but not significantly protected cells from superoxide anion-induced cell death, and had no effect on hydrogen peroxide-induced cell death (Fig. 5C).

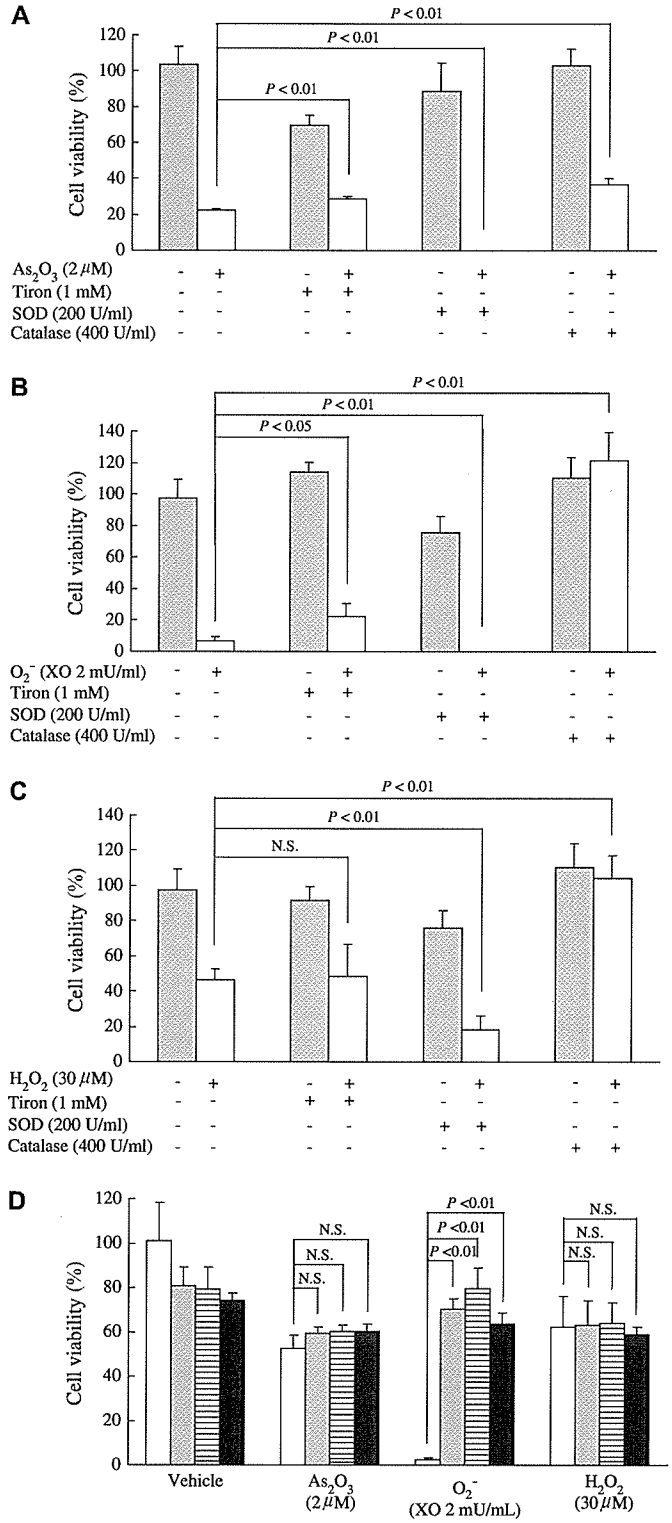

Figure 3. Effect of inhibitors of reactive oxygen species (ROS)-producing enzymes on As_2O_3, O_2^-, and H_2O_2. (**A**) Cells were treated for 48 hours with 2 μM As_2O_3 in the presence or absence of 1 mM Tiron, 200 U/mL superoxide dismutase (SOD), or 400 U/mL catalase. (**B**) Cells were treated for 24 hours with 1 mM hypoxanthine and 2 mU/mL xanthine oxidase (XO) in the presence or absence of 1 mM Tiron, 200 U/mL SOD, or 400 U/mL catalase. (**C**) Cells were treated for 24 hours with 30 μM H_2O_2 in the presence or absence of 1 mM Tiron, 200 U/mL SOD, or 400 U/mL catalase. (**D**) Cells were treated for 24 hours with 2 μM As_2O_3, 2 mU/mL XO, or 30 μM H_2O_2 at 0.05 to 0.2 μM of DPI. Open bar, 0 μM DPI; gray bar, 0.05 μM DPI; striped bar, 0.1 μM DPI; closed bar, 0.2 μM DPI. Cell viability was measured using the Premix WST-1 cell proliferation assay system. The assay was performed in quadruplicate with essentially similar results. Data are presented as the mean ± standard error. NS = not significant ($p > 0.05$).

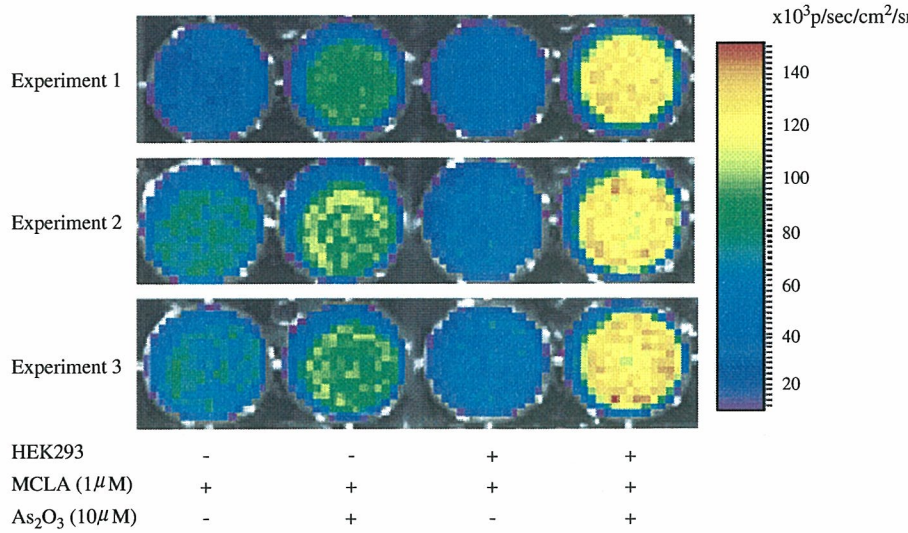

Figure 4. Production of O_2^- in HEK293 cells exposed to As_2O_3. Cells suspended in 0.9% NaCl were treated with or without 10 μM As_2O_3. O_2^- was visualized by adding 1 μM 2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo[1,2-a]pyrazine-3-one (MCLA) and using an IVIS imaging system. Three independent experiments were performed.

HL-60 cells and NB4 cells were treated with arsenic trioxide in the presence or absence of α-lipoic acid. The concentration-cell viability curves were similar between in presence and in absence of α-lipoic acid in both cells (Fig. 6A and B). In KMS12BM cells and U266 cells, there were also no significant differences of cell viability in both treatments (Fig. 6C and D). Thus, α-lipoic acid did not have a significant effect on arsenic trioxide cytotoxicity in both human acute promyelocytic leukemia cell lines (HL-60 and NB4 cells) and human multiple myeloma cell lines (KMS12BM and U266 cells).

Discussion

Arsenic is well known as an environmental toxin associated with increased risk of cancer and cardiovascular disease in humans [25]. Recently, arsenic trioxide has been used therapeutically to induce remission of relapsed or refractory acute promyelocytic leukemia and developed for treatment of multiple myeloma, and arsenic trioxide has been reported to cause renal toxicity in clinical use. In this study, we analyzed gene-expression patterns in PRCC and HEK293 cells after exposure to arsenic trioxide in order to identify the genes related to renal toxicity. Classification of the 73 differentially expressed genes produced five gene clusters (Table 1). In these genes, *HMOX1* encodes heme oxygenase 1, which is an essential enzyme in heme catabolism and cleaves heme to form biliverdin. Heme oxygenase activity is induced by its substrate, heme, and by oxidative stress. *HMOX1* gene expression was upregulated in a time- and dose-dependent manner in PRCC and HEK293 cells after arsenic trioxide exposure (Fig. 1A and B), which confirmed previous findings [26]. Heme oxygenase 1 protein was also detected in arsenic trioxide-treated PRCC and HEK293 cells (Fig. 1C). Menzel et al. reported that the level of heme oxygenase 1 in human lymphoblastoid cells treated with arsenite increased in a dose-related manner [27]. Morgan et al. also showed that oxidative stress induced *HMOX1* gene expression in a human hepatocellular carcinoma cell line, HepG2 [28]. Thus, *HMOX1* may be strongly associated with toxicity in various organs, including the kidney. Poss and Tonegawa [29] reported a cytoprotective effect of *HMOX1* against oxidative stress in cells from heme oxygenase 1-deficient mice], whereas Miralem et al. [30] reported that *HMOX1* did not influence arsenite-induced apoptosis in cells infected with *HMOX1* small interfering RNA. These results indicate the controversy surrounding the *HMOX1* response to oxidative stress. In the present study, HEK293-*HMOX1* cells were significantly resistant to arsenic trioxide (Fig. 2).

Given that *HMOX1* is induced by oxidative stress [28], we assumed that arsenic trioxide would cause renal injury through oxidative stress. Indeed, superoxide anion and hydrogen peroxide suppressed HEK293 cell viability, and oxidative stress had the potential to cause cytotoxicity in HEK293 cells. Chen et al. [31] proposed the following sequence (diagrammed in Fig. 7) as a possible mechanism of arsenite-induced apoptosis in the mouse embryonic fibroblast cell line, NIH/3T3: 1) activated NADPH-oxidase increases the superoxide anion level in cells; 2) superoxide dismutase converts the superoxide anion into hydrogen peroxide; 3) hydrogen peroxide induces the release of cytochrome c into the cytosol, which results in activation of Yama/CPP32 protease, a member of the ICE/ced3 family;

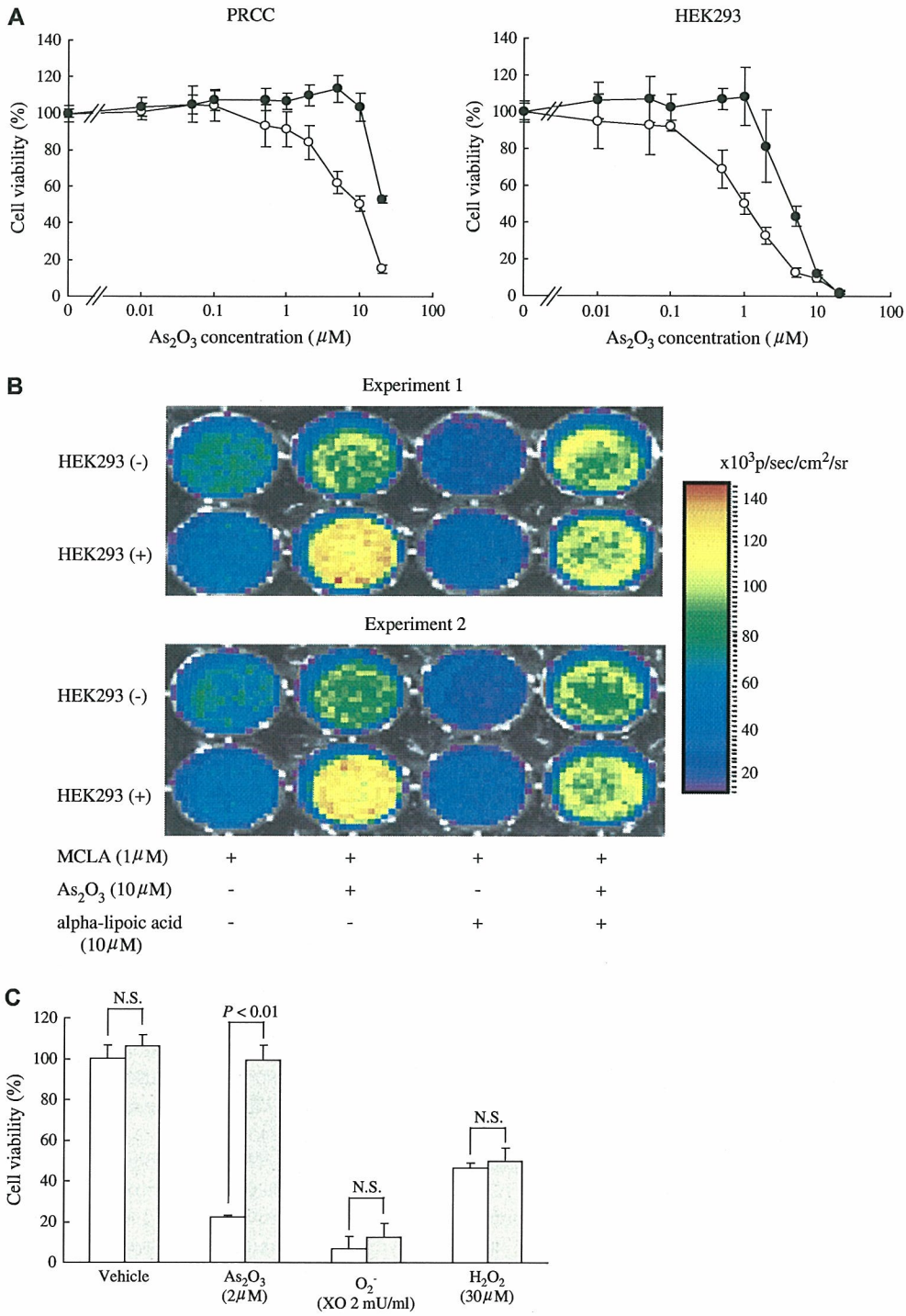

Figure 5. Effect of α-lipoic acid on As_2O_3-induced cytotoxicity in primary renal cortical cells (PRCC) or HEK293 cells. (**A**) Cells were treated for 48 hours with 0.01 to 20 μM As_2O_3 in the presence or absence of 10 μM α-lipoic acid. Cell viability was measured using the Premix WST-1 cell proliferation assay system. The assay was performed in quadruplicate with essentially similar results. (○) Without α-lipoic acid, (⇑) with α-lipoic acid. Data are presented as mean ± standard error. (**B**) Cells suspended in 0.9% NaCl were treated with 10 μM As_2O_3 and/or 10 μM α-lipoic acid. O_2^- was visualized by adding 1 μM 2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo[1,2-a]pyrazine-3-one (MCLA) and using an IVIS imaging system. Two independent experiments were performed. (**C**) Cells were treated with 2 μM As_2O_3 for 48 hours, 1 mM hypoxanthine and 2 mU/mL xanthine oxidase (XO) for 24 hours, or 30 μM H_2O_2 for 24 hours, in the presence or absence of 10 μM α-lipoic acid. Cell viability was measured using the Premix WST-1 cell proliferation assay system. The assay was performed in quadruplicate with essentially similar results. Open bar, without α-lipoic acid; gray bar, with α-lipoic acid. Data are presented as the mean ± standard error. NS = not significant ($p > 0.05$).

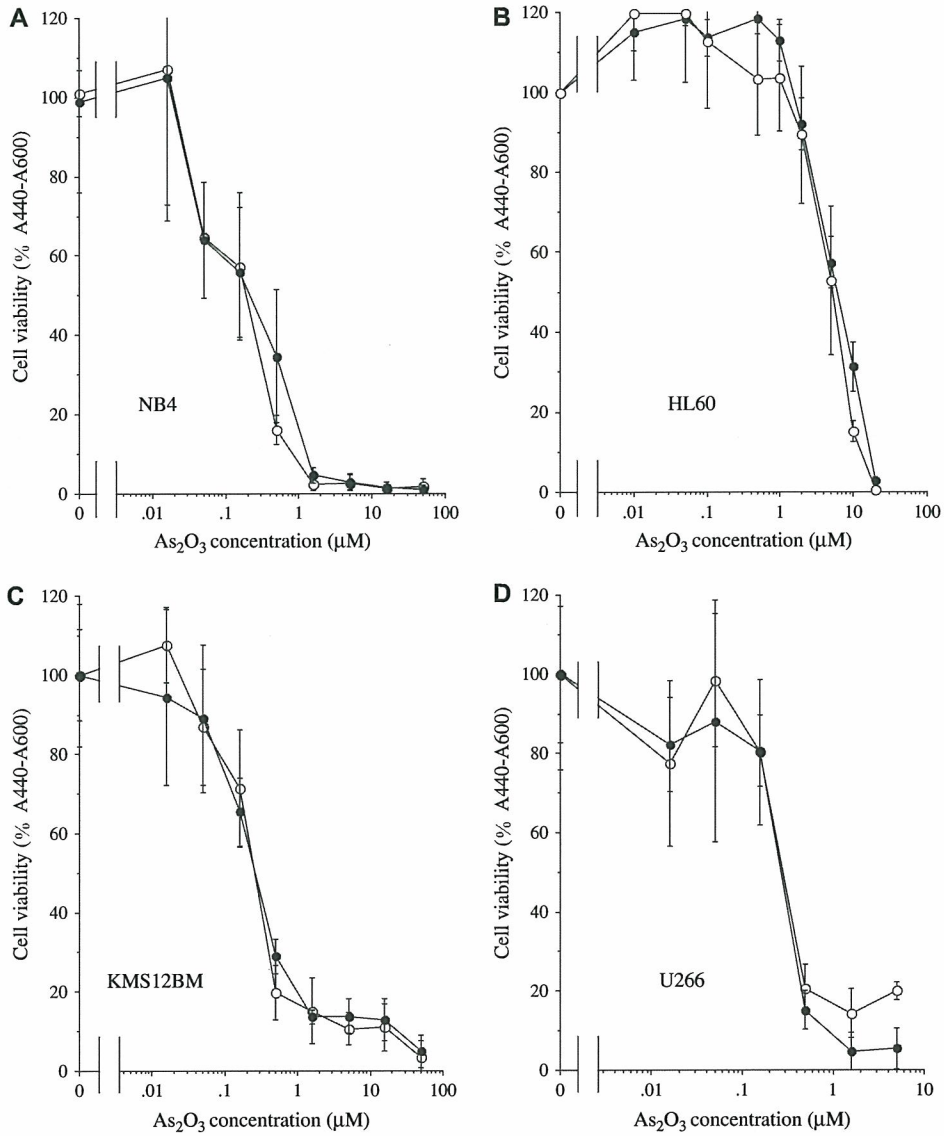

Figure 6. Effect of α-lipoic acid on the viability of As_2O_3-treated NB4 cells, HL-60 cells, KMS12BM cells and U266 cells. Cells were treated for 48 hours with 0.01 to 50 μM As_2O_3 for NB4 cell (**A**), HL-60 cells (**B**), KMS12BM cells, (**C**) and U266 cells (**D**) in the presence or absence of 10 μM α-lipoic acid. Cell viability was measured using the Premix WST-1 cell proliferation assay system. The assay was performed in quadruplicate at least two times with essentially similar results. (○) without α-lipoic acid, (⇑) with α-lipoic acid. Data are presented as the mean ± standard error.

and 4) protease activity degrades the DNA repair enzyme poly(ADP-ribose) polymerase [31]. In the present study, superoxide anion-induced cell death was inhibited by Tiron and catalase, which implies that Tiron is a superoxide anion scavenger and that hydrogen peroxide is produced via the superoxide anion (Fig. 3B). DPI also significantly inhibited superoxide anion-induced cell death, however, this result was attributable to a chemical inhibitory effect of DPI on ROS production (data not shown). Hydrogen peroxide-induced cell death was inhibited only by catalase, which confirms that catalase catalyzes the conversion of cytotoxic hydrogen peroxide into nonreactive, or much less toxic, water and oxygen (Fig. 3C). Arsenic trioxide-induced cell death was inhibited by DPI, Tiron, and catalase, indicating that it might be associated with NAD(P)H oxidase activity and the production of superoxide anion and hydrogen peroxide (Fig. 3A). However, because the inhibitory effect of catalase on arsenic trioxide-induced cell death was much less than that on superoxide anion- or hydrogen peroxide-induced cell death, a toxic mechanism other than ROS production may exist in arsenic trioxide-induced cytotoxicity. Moreover, superoxide anion was detected in arsenic trioxide-treated HEK293 cells, implicating superoxide anion in arsenic trioxide-induced cytotoxicity (Fig. 4).

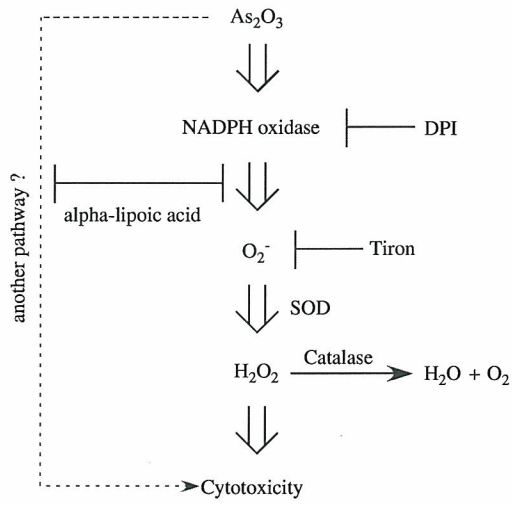

Figure 7. Proposed mechanism of suppression of As_2O_3-induced cytotoxicity by α-lipoic acid. As_2O_3 activates NADPH oxidase and increases the O_2^- level in cells. The O_2^- is then converted to H_2O_2 by superoxide dismutase (SOD), and H_2O_2 induces cytotoxicity through apoptosis. DPI, Tiron, and catalase inhibit this pathway. α-Lipoic acid decreased cytotoxicity by inhibiting this oxidative stress and another pathway.

Karasavvas et al. [32] and Chen et al. [21] reported that antioxidants such as vitamin C and α-tocopherol showed cytoprotective activity against arsenic trioxide [21,32]. In this study, we reveal that another antioxidant, α-lipoic acid, also provides cytoprotection against arsenic trioxide-induced cell death. α-Lipoic acid improved the viability of arsenic trioxide-treated PRCC and HEK293 cells and showed cytoprotective effects in both cell types (Fig. 5A). α-Lipoic acid also reduced the production of superoxide anion in arsenic trioxide-treated HEK293 cells, indicating that α-lipoic acid possesses antioxidant activity that can suppress superoxide anion production to some degree (Fig. 5B).

Moreover, α-lipoic acid demonstrated no cytoprotective effect against superoxide anion or hydrogen peroxide in HEK293 cells (Fig. 5C). Consequently, the cytoprotective effect of α-lipoic acid against arsenic trioxide may be mediated by its inhibition of ROS production, especially upstream of superoxide anion production (Fig. 7). Arsenic trioxide has been used to treat acute promyelocytic leukemia and multiple myeloma; therefore, we examined the effect of α-lipoic acid on arsenic trioxide cytotoxicity in human promyelocytic leukemia cell lines (NB4 cells and HL-60 cells) and multiple myeloma cell lines (KMS12BM cells and U266 cells). These preliminary results suggest that α-lipoic acid does not suppress the antitumor effect of arsenic trioxide (Fig. 6), and that α-lipoic acid has cytoprotective activity only in normal cells and not in tumor cells. This feature suggests that α-lipoic acid might be a suitable agent for treatment or prevention of arsenic trioxide-induced renal toxicity.

In conclusion, arsenic trioxide-induced renal toxicity is strongly associated with increased expression of *HMOX1*, and the cytotoxic mechanism of arsenic trioxide involves ROS production as well as another pathway. As a result of its cytoprotective effect in normal renal cells, but not in leukemia cells, α-lipoic acid may be suitable for treating or preventing arsenic trioxide-induced renal toxicity during therapy for promyelocytic leukemia and multiple myeloma; the clinical application of α-lipoic acid warrants investigation.

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