Upregulation of thioredoxin and its reductase attenuates arsenic trioxide-induced growth suppression in human pulmonary artery smooth muscle cells by reducing oxidative stress

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Abstract. The thioredoxin (Trx) system is an important enzymatic complex involved in cellular redox homeostasis. Arsenic trioxide (ATO; As_2O_3) is known to trigger cell death in vascular smooth muscle cells (VSMCs) via oxidative stress. In the present study, the effects of changes in thioredoxin 1 (Trx1) and Trx reductase1 (TrxR1) on cell growth, death, reactive oxygen species (ROS), and glutathione (GSH) levels were evaluated in ATO-treated human pulmonary artery smooth muscle cells (HPASMCs). ATO inhibited growth and induced cell death in the HPASMCs at 24 h. Overexpression of Trx1 and TrxR1 using adenoviruses attenuated cell growth inhibition caused by ATO and partially prevented cell death. ATO increased ROS levels including the mitochondrial superoxide anion ($O_2^{\bullet-}$) at 5 min. Administration of adTrx1 or adTrxR1 reduced the increased mitochondrial $O_2^{\bullet-}$ level in these cells. HPASMCs treated with Trx1 or TrxR1 siRNA showed increases in ROS levels with or without treatment of ATO at 5 min. Although ATO transiently increased GSH levels at 5 min, Trx1 and TrxR1 siRNAs reduced the increased GSH levels in these cells. In addition, PX-12 (a Trx1 inhibitor) and auranofin (a TrxR1 inhibitor) diminished the cellular metabolism in HPASMCs at 4 h, accompanied by an increase in ROS level and a decrease in GSH level. In conclusion, upregulation of Trx1 and TrxR1 somewhat decreased cell growth inhibition and death in ATO-treated HPASMCs, which was accompanied by reduced oxidative stress.

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Abbreviations: VSMCs, vascular smooth muscle cells; HPASMCs, human pulmonary artery smooth muscle cells; ATO, arsenic trioxide (As_2O_3); ROS, reactive oxygen species; Trx, thioredoxin; TrxR, Trx reductase; SOD, superoxide dismutase; GSH, glutathione; NADPH, nicotinamide adenine dinucleotide phosphate; PX-12, 1-methylpropyl 2-imidazolyl disulfide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; H_2DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; DHE, dihydroethidium; CMFDA, 5-chloromethylfluorescein diacetate

Key words: vascular smooth muscle cells, arsenic trioxide, cell death, thioredoxin, reactive oxygen species, glutathione

Introduction

Vascular smooth muscle cells (VSMCs) are a dynamic constituent of the vasculature. Depending on pathological stimuli, VSMCs are involved in death, hypertrophy, or proliferation associated with hypertension, restenosis, and atherosclerosis (1,2). Reactive oxygen species (ROS) control many cellular processes such as proliferation, migration, apoptosis, and secretion of inflammatory cytokines in VSMCs (1,2). Lung tissues are easily injured by oxidative stress as they are exposed to an oxygen-rich environment. ROS in lung tissues are related to pulmonary vascular physiology. For instance, ROS regulate contractile ability by tuning an intracellular calcium level in human pulmonary artery smooth muscle cells (HPASMCs) (2). ROS are also involved in the development of pulmonary hypertension, ultimately inducing right ventricular failure and death (2). Hence, understanding the physiological or pathophysiological roles of ROS in the VSMCs of lung tissue are important.

Oxygen-derived molecules such as the hydroxyl radical ($^{\bullet}OH$), superoxide anion ($O_2^{\bullet-}$), and hydrogen peroxide (H_2O_2) are principal cellular ROS (3). Cells have various antioxidants that function as redox rheostat, which is imperative for their survival and physiological events. The main metabolic antioxidant enzyme is superoxide dismutase (SOD), which processes $O_2^{\bullet-}$ to H_2O_2 (4). Additional metabolism by catalase or glutathione (GSH) peroxidase produces stable molecules O_2 and H_2O (5). Thioredoxin (Trx) is a small antioxidant protein (~12 kDa) containing redox active cysteine residues at the active site (6). The oxidized disulfide form of Trx is reduced back to the reduced dithiol form by nicotinamide adenine dinucleotide phosphate (NADPH)-dependent Trx reductase (TrxR) (6). Thioredoxin 1 (Trx1) and Trx reductase1 (TrxR1) are usually localized in the cytoplasm; however, Trx2 and TrxR2 are

located in the mitochondria (6). Trx1 is implicated in cell growth, survival, tumor development, and angiogenesis (7-9). In addition, Trx is an anti-inflammatory and antioxidant cytokine involved in various inflammatory disorders, especially pulmonary diseases (10-12). Trx and TrxR can be inhibited by specific drugs. For example, PX-12 (1-methylpropyl 2-imidazolyl disulfide) is an irreversible Trx-1 inhibitor (13,14) and auranofin is a TrxR inhibitor (14). Both PX-12 and auranofin exert antitumor effects in various cancer cells (15-20). Although these drugs do not perfectly and efficiently block the activities of Trx and TrxR, it is plausible to use PX-12 and auranofin to decrease the activities of Trx and TrxR in *in vitro* experiments.

Arsenic is a poison widely distributed throughout the environment and presents an international public health problem. Humans are exposed to arsenic mostly through ingestion of arsenic-contaminated water, food, and air (21). Conversely, inorganic arsenic has long been used to treat several severe diseases, and specifically, arsenic trioxide (ATO; As_2O_3) has recently been shown to be an effective therapeutic drug in certain leukemia patients (21,22). ATO can disturb the natural oxidation and reduction equilibrium in cells by altering various redox enzymes (23,24). ATO, as a mitochondrial toxin, induces a loss of mitochondrial membrane potential (25,26) thereby increasing the production of ROS and consequently triggering apoptosis in target cells (27-29). Currently ATO is considered a TrxR inhibitor (30).

The development of numerous vascular diseases has been associated with chronic arsenic exposure in epidemiological studies (31-34). Blood vessel cells are the most susceptible to arsenic toxicity (31-34). Assimilation of arsenic for a prolonged period leads to pulmonary complications such as cough, bronchitis, and asthma (35). Recently, we reported that ATO induces growth inhibition and death in HPASMCs accompanied by mitochondrial $O_2^{\bullet-}$ increase and GSH depletion (36). In the present study, the roles of Trx1 and TrxR1 proteins in the regulation of cell growth, death, ROS, and GSH levels were investigated in ATO-treated HPASMCs, and the effects of PX-12 and auranofin on cell metabolism, ROS, and GSH levels in HPASMCs were examined.

Materials and methods

Cell culture. Primary HPASMCs obtained from PromoCell GmbH were maintained in a humidified incubator containing 5% CO_2 at 37˚C. HPASMCs were cultured in complete SMC Growth Medium 2 obtained from PromoCell GmbH. Cells were grown in 100-mm plastic tissue culture dishes (Nunc) and washed and detached with HepesBSS (30 mM HEPES), trypsin-EDTA, and trypsin neutralization solution (PromoCell GmbH). For the experiments, HPASMCs between six and seven passages were used.

Reagents. ATO was purchased from Sigma-Aldrich/Merck KGaA and liquefied in 1.65 M NaOH at 10 mM. PX-12 was purchased from Tocris Bioscience and auranofin was obtained from Santa Cruz Biotechnology. Both drugs were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA) at 100 mM as a stock solution.

Infection of cells with adLacZ, adTrx1, and adTrxR1. Overexpression of Trx1 and TrxR1 was accomplished using an adenoviral gene transfer. adLacZ, adTrx1 and adTrxR1 were provided by Dr J. Sadoshima (New Jersey Medical School, Newark, NJ, USA) and infection of cells was performed as previously described (37-39). Briefly, $7x10^5$ cells in 6-well plates (Nunc) were incubated in RPMI-1640 medium (Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS). Cells (~70-80% confluence) in each well were infected with the same titers of control (CTR) adLacZ, adTrx1, or adTrxR1, which were determined using cell culture plague assays. One day later, cells were treated with or without 50 mM ATO for an additional 24 h. The infected cells were collected and used for western blot analysis, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, Annexin V-FITC staining, as well as ROS and GSH level measurements.

Transfection of cells with Trx1 and TrxR1 siRNAs. Gene silencing of Trx1 and TrxR1 was performed as previously described (37-39). A non-specific CTR siRNA duplex (5'-CCU ACGCCACCAAUUUCGU(dTdT)-3'), Trx1 siRNA duplex (5'-GCAUGCCAACAUUCCAGUU(dTdT)-3') and TrxR1 siRNA duplex (5'-GUCGUCUAUGAGAAUGCUU(dTdT)-3') were acquired from the Bioneer Corporation. In brief, $7x10^5$ cells in 6-well plates (Nunc) were incubated in RPMI-1640 medium supplemented with 10% FBS. The next day, cells (~70-80% confluence) in each well were transfected with the CTR, Trx1, and TrxR1 siRNA duplex (80 pmol in Opti-MEM; Gibco; Thermo Fisher Scientific, Inc.) using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen; Thermo Fisher Scientific, Inc.). One day later, cells were treated with or without 50 mM ATO. The transfected cells were used for MTT assays as well as ROS and GSH level measurements.

Western blot analysis. Protein expression levels of Trx1 and TrxR1 in HPASMCs were evaluated using western blotting. Briefly, cells were washed with PBS and lysed in 4 volumes of lysis buffer (Intron Biotechnology). Protein concentrations of clarified lysates were determined using the Bradford method. Protein samples (20 μg per well) were loaded and resolved on 4-15% SDS-PAGE gels, and then transferred to Immobilon-P PVDF membranes (Merck Millipore) using electroblotting. Then, membranes were probed with anti-Trx1 (cat. no. sc-20146), anti-TrxR1 (cat. no. sc-28321) and anti-β-actin (cat. no. sc-47778) antibodies (1,000:1) (Santa Cruz Biotechnology). Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (cat. nos. sc-2357 or sc-2005) (1,000:1) (Santa Cruz Biotechnology). Blots were developed using an EZ-Western Lumi Pico ECL solution kit (DoGen).

Cell growth and metabolism assays. Alterations in cell growth and metabolism in the adenovirus-infected or siRNA-treated HPASMCs with or without ATO, PX-12, or auranofin treatment were indirectly determined by measuring the absorbance of MTT dye (Sigma-Aldrich; Merck KGaA) as previously described (38-41). The reduction of MTT dye to formazan by NAD(P)H-dependent oxidoreductase enzymes depends on the cellular metabolic activity (41). In brief, $3x10^4$ cells per well were seeded in 96-well microtiter plates (Nunc) for MTT assays. After exposure to the indicated amount of ATO, PX-12, or auranofin for 4 or 24 h, 20 μl of MTT solution (2 mg/ml in phosphate-buffered saline, PBS) was added to each well of the

96-well plates (Nunc). The plates were additionally incubated for ~3-4 h at 37˚C. The medium in the plates was withdrawn by pipetting and 100 μl DMSO was added to each well to solubilize the formazan crystals. The optical concentration was measured at 570 nm using a microplate reader (Synergy™ 2; BioTek Instruments Inc.).

Annexin V-FITC staining for the detection of apoptosis. Apoptosis was determined by staining cells with Annexin V-fluorescein isothiocyanate (FITC, Invitrogen; Thermo Fisher Scientific, Inc.; Ex/Em=488/519 nm) as previously described (16,37). Adenovirus-infected HPASMCs were incubated with the indicated amount of ATO for 24 h. Annexin V-FITC stained cells were analyzed with a FACStar flow cytometer (BD Biosciences).

Detection of intracellular ROS levels. ROS levels were measured using oxidation-sensitive fluorescent probe dyes, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA; Ex/Em=495/529 nm; Invitrogen; Thermo Fisher Scientific, Inc.) and dihydroethidium (DHE; Ex/Em=518/605 nm; Invitrogen; Thermo Fisher Scientific, Inc.). DHE is highly selective for $O_2^{\bullet-}$ among ROS. In addition, the mitochondrial $O_2^{\bullet-}$ level was specifically detected using MitoSOX™ Red mitochondrial $O_2^{\bullet-}$ indicator (Ex/Em=510/580 nm; Invitrogen; Thermo Fisher Scientific, Inc.). In brief, $1x10^6$ HPASMCs/ml in a FACS tube (BD Biosciences) were incubated with 20 μM H_2DCFDA, DHE, or MitoSOX™ Red dye for 5, 30, 60, 120 or 180 min. The H_2DCFDA, DHE, and MitoSOX™ Red fluorescence levels were evaluated using a FACStar flow cytometer (BD Biosciences). The ROS levels were expressed as mean fluorescence intensity (MFI), which was calculated using CellQuest software (BD Biosciences). In addition, adenovirus-infected HPASMCs in the presence or absence of ATO for 24 h were incubated with 20 μM H_2DCFDA or DHE at 37˚C for 30 min. H_2DCFDA or DHE fluorescence was assessed using a FACStar flow cytometer (BD Biosciences) and the levels were expressed as MFI.

Detection of intracellular GSH levels. Cellular GSH levels were analyzed using a 5-chloromethylfluorescein diacetate dye (CMFDA, Ex/Em=522/595 nm; Invitrogen; Thermo Fisher Scientific, Inc.) as previously described (42). In brief, $1x10^6$ HPASMCs/ml in a FACS tube (BD Biosciences) were incubated with 5 μM CMFDA for 5, 30, 60, 120 or 180 min. The CMF fluorescence levels were evaluated using a FACStar flow cytometer (BD Biosciences). CMF (GSH) levels were expressed as MFI, which was calculated using CellQuest software (BD Biosciences).

Statistical analysis. Data are expressed as the mean of at least two independent experiments [mean ± standard deviation (SD)]. The data were analyzed using Instat software (GraphPad Prism5; GraphPad Software, Inc.). The Student's t-test or one-way analysis of variance (ANOVA) with post hoc analysis using Tukey's multiple comparison test was performed for parametric data. A P-value <0.05 was considered to indicate statistical significance.

Results

Effects of adTrx1 and adTrxR1 on cell growth and death in ATO-treated HPASMCs. HPASMCs were infected with either adLacZ, adTrx1, or adTrxR1 for the overexpression of Trx1 or TrxR1. In addition, 50 μM ATO and 24 h were used as a suitable dose and time, respectively, due to the confluence of experimental cells. As shown in Fig. 1A, HPASMCs infected with adTrx1 or adTrxR1 exhibited increased Trx1 or TrxR1 protein levels compared with the cells infected with the control adLacZ. Based on MTT assays, 50 μM ATO inhibited the growth of HPASMCs ~25% at 24 h (Fig. 1B). Administration of adTrx1 slightly attenuated cell growth inhibition caused by ATO (Fig. 1B). In addition, adTrxR1 significantly reduced the inhibition of HPASMCs and metabolically increased the reduction of MTT to formazan in the control HPASMCs (Fig. 1B). When HPASMCs were stained with Annexin V-FITC to evaluate cell death, the percentage of Annexin V-stained cells was increased in the ATO-treated HPASMCs at 24 h (Fig. 1C). Both adTrx1 and adTrxR1 slightly prevented the death of ATO-treated HPASMCs (Fig. 1C).

Effects of adTrx1 and adTrxR1 on ROS and GSH levels in ATO-treated HPASMCs. To evaluate intracellular ROS levels in the ATO-treated HPASMCs, H_2DCFDA, DHE and MitoSOX™ Red dyes were used. Since ROS are short live radicals, ROS and GSH levels were assessed at the early time points of 5, 30, 60, 120 or 180 min after ATO treatment. ATO increased ROS (DCF) levels at 5 min in the adLacZ, adTrx1, and adTrxR1-infected HPASMCs when compared with the untreated cells (Fig. 2A). Then, ATO decreased the ROS (DCF) levels to the level of the adLacZ-infected control cells (CTR adLacZ) at ~30-180 min. Control HPASMCs infected with adTrx1 or adTrxR1 showed a gradual decrease in ROS (DCF) levels at 30 min and the decreased levels persisted for 180 min (Fig. 2A). At 24 h, ATO reduced ROS (DCF) levels in the HPASMCs (Fig. 2B). adTrx1 and adTrxR1 attenuated the reduced levels of ROS (DCF) in ATO-treated HPASMCs, and both adenoviruses did not significantly affect the ROS (DCF) levels which remained approximately similar as ROS levels in the control HPASMCs (adLacZ) (Fig. 2B).

Compared with the levels of adLacZ-infected control (CTR adLacZ) cells, ATO increased DHE ($O_2^{\bullet-}$) levels in the adLacZ, adTrx1, and adTrxR1-infected HPASMCs at 5 min (Fig. 3A). In addition, control cells infected with adTrx1 or adTrxR1 showed an increase in DHE ($O_2^{\bullet-}$) levels regardless of ATO at 5 min (Fig. 3A). Then, the ATO-increased DHE ($O_2^{\bullet-}$) levels decreased to the levels of adLacZ-infected control cells at ~120-180 min (Fig. 3A). adTrx1 and adTrxR1 further decreased the DHE ($O_2^{\bullet-}$) levels in ATO-treated cells at ~30-180 min and adTrx1 reduced DHE ($O_2^{\bullet-}$) levels in the control HPASMCs at 120 min (Fig. 3A). In addition, ATO increased mitochondrial $O_2^{\bullet-}$ levels detected using MitoSOX™ Red dye at 5 min whereas adTrx1 and adTrxR1 attenuated the increased levels in these cells (Fig. 3B). adTrx1 and adTrxR1 decreased mitochondrial $O_2^{\bullet-}$ levels in the control HPASMCs at ~30-180 min (Fig. 3B). At 24 h, ATO significantly increased DHE ($O_2^{\bullet-}$) levels in the control HPASMCs (Fig. 3C). Although adTrx1 attenuated the increased DHE ($O_2^{\bullet-}$) levels in ATO-treated cells, the levels were not significantly changed by adTrxR1 (Fig. 3C). In addition, adTrx1 and adTrxR1 did not alter the basal level of DHE ($O_2^{\bullet-}$) in the control HPASMCs (Fig. 3C).

When intracellular GSH levels were measured in adenovirus-infected HPASMCs using a CMFDA dye, 50 μM ATO

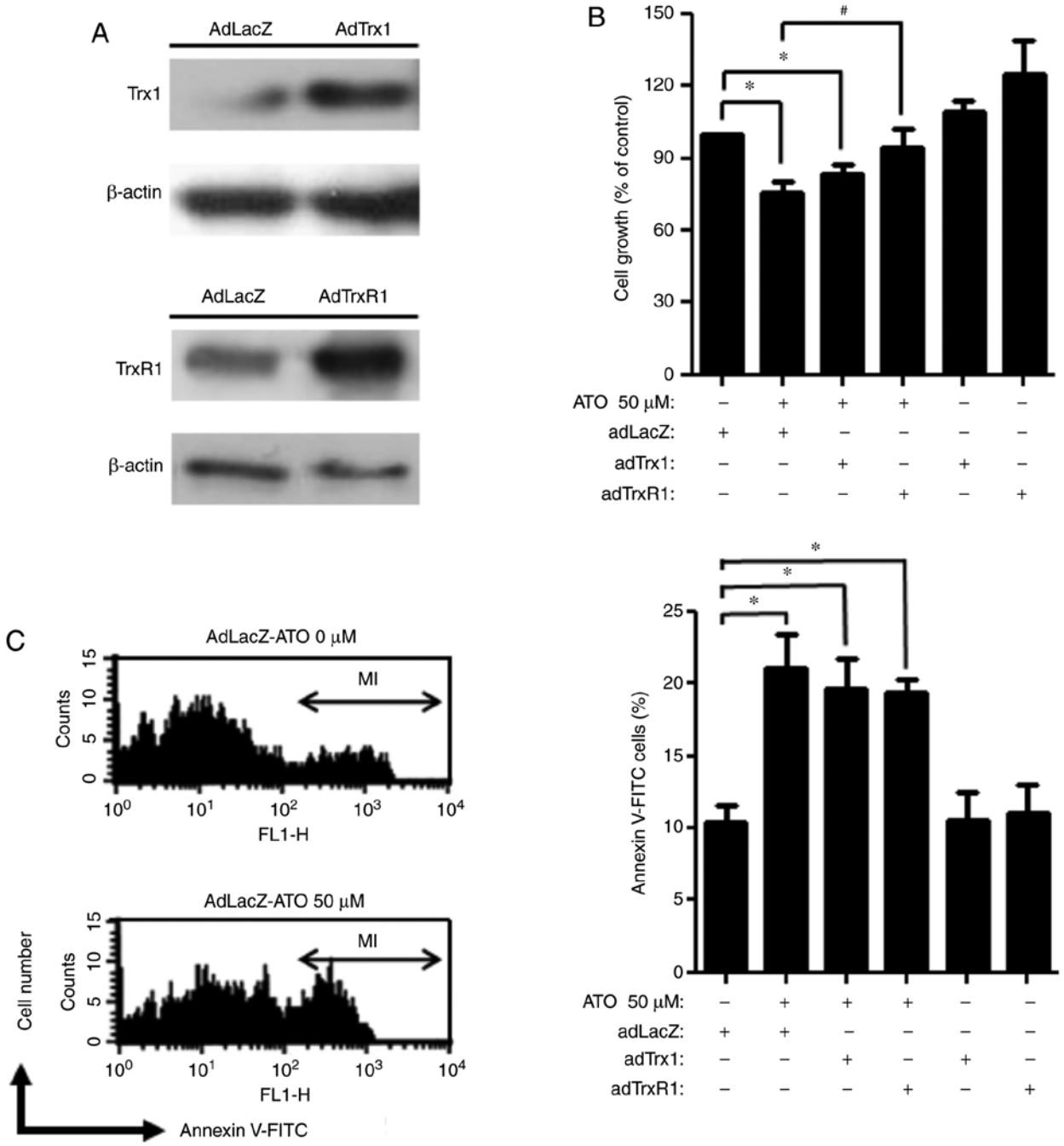

Figure 1. Effects of adTrx1 and adTrxR1 on cell growth and death in HPASMCs. HPASMCs (~70-80% confluence) were infected with control adLacZ, adTrx1 or adTrxR1. Twenty-four hours later, the cells were treated with 50 μM ATO for an additional 24 h. (A) Expression levels of Trx1 and TrxR1 proteins in HPASMCs. (B) Cellular growth changes assessed by the reduction of MTT to formazan for 4 h. (C) Representative images (left) of Annexin V-FITC staining cells, as analyzed by FACStar flow cytometer. M1 regions show the Annexin V-FITC-positive cells. Graph (right) indicates the percentages of Annexin V-FITC-positive cells. *P<0.05 compared with the adLacZ-infected control group; #P<0.05 compared with the adLacZ-infected control cells treated with ATO only. Trx1, thioredoxin 1; TrxR1, Trx reductase1; ATO, arsenic trioxide (As_2O_3); HPASMCs, human pulmonary artery smooth muscle cells.

significantly increased GSH (CMF) levels at 5 min and then gradually decreased to the control levels at 180 min (Fig. 4). adTrx1 and adTrxR1 reduced the increased GSH (CMF) levels in ATO-treated cells at ~5-60 min (Fig. 4). In addition, adTrx1 and adTrxR1 decreased GSH (CMF) levels in the control HPASMCs at ~5-30 min (Fig. 4).

Effects of Trx1 and TrxR1 siRNA on cell metabolism, ROS, and GSH levels in ATO-treated HPASMCs. Next, HPASMCs were transfected with either control, Trx1 siRNA or TrxR1 siRNA for downregulation of the corresponding proteins. As shown in Fig. 5A, Trx1 siRNA suppressed Trx1 expression in the HPASMCs, and TrxR1 siRNA also downregulated the expression of TrxR1 protein. ATO decreased the reduction of MTT to formazan in the control siRNA (CTR siRNA)-treated HPASMCs at 4 h. The metabolism of HPASMCs transfected with Trx1 or TrxR1 siRNA was significantly reduced regardless of ATO (Fig. 5B).

Regarding ROS and GSH levels, HPASMCs treated with Trx1 or TrxR1 siRNA showed increased ROS (DCF) levels

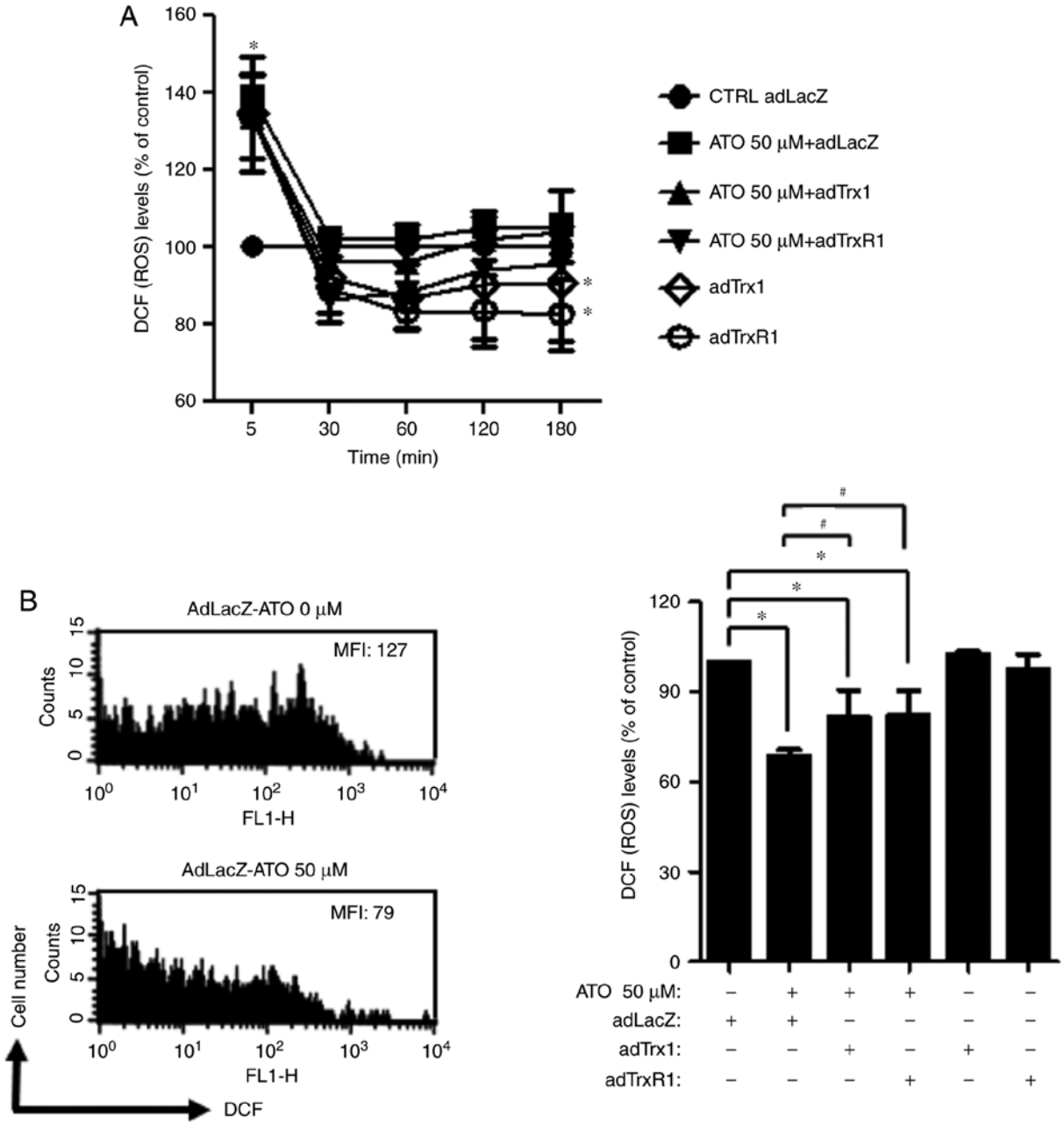

Figure 2. Effects of adTrx1 and adTrxR1 on ROS levels in HPASMCs. HPASMCs (~70-80% confluence) were infected with CTR adLacZ, adTrx1 or adTrxR1. Twenty-four hours later, the cells were treated with 50 μM ATO for the indicated times. ROS levels in HPASMCs were measured using a FACStar flow cytometer. (A) The graph indicate DCF (ROS) levels (% of adLacZ-infected control cells) at the indicated times. (B) Representative images (left) of DCF-staining cells. MFI, mean fluorescence intensity. The graph (right) indicate DCF (ROS) levels (% of adLacZ-infected control cells) at 24 h. *P<0.05 compared with the adLacZ-infected control group; #P<0.05 compared with the adLacZ-infected control cells treated with ATO only. Trx1, thioredoxin 1; TrxR1, Trx reductase1; ATO, arsenic trioxide (As_2O_3); HPASMCs, human pulmonary artery smooth muscle cells; ROS, reactive oxygen species.

with or without ATO at 5 min (Fig. 5C). In particular, TrxR1 siRNA-treated cells had higher ROS (DCF) levels (Fig. 5C). Then, the increased ROS (DCF) levels returned to the levels of control siRNA-treated cells at ~30-180 min (Fig. 5C). Trx1 and TrxR1 siRNAs, specifically Trx1 siRNA, reduced the increased GSH (CMF) levels in the ATO-treated cells at ~5-180 min (Fig. 5D). Comparatively, Trx1 and TrxR1 siRNAs decreased GSH (CMF) levels in the control HPASMCs at ~5-30 min (Fig. 5D).

Effects of PX-12 and auranofin on cell metabolism, ROS, and GSH levels in HPASMCs. Treatment with 30 μM PX-12 and 5 μM auranofin decreased the cellular metabolism of HPASMCs ~40% at 4 h compared with the untreated cells (Fig. 6A). Furthermore, 100 μM PX-12 and 30 μM auranofin nearly ceased the activity of cellular metabolism, indicating that the cells were gradually dying (Fig. 6A). Regarding ROS and GSH levels, 100 μM PX-12 and 30 μM auranofin increased ROS (DCF) levels in HPASMCs at ~30-180 min compared with the control cell (CTR) levels (Fig. 6B). HPASMCs treated with 30 μM PX-12 and 5 μM auranofin showed increased ROS (DCF) levels at ~120-180 min (Fig. 6B). The tested doses of PX-12 and auranofin reduced GSH (CMF) levels in HPASMCs at 30 min and the decreased levels returned to 90% of the control GSH (CMF) levels at 180 min (Fig. 6C).

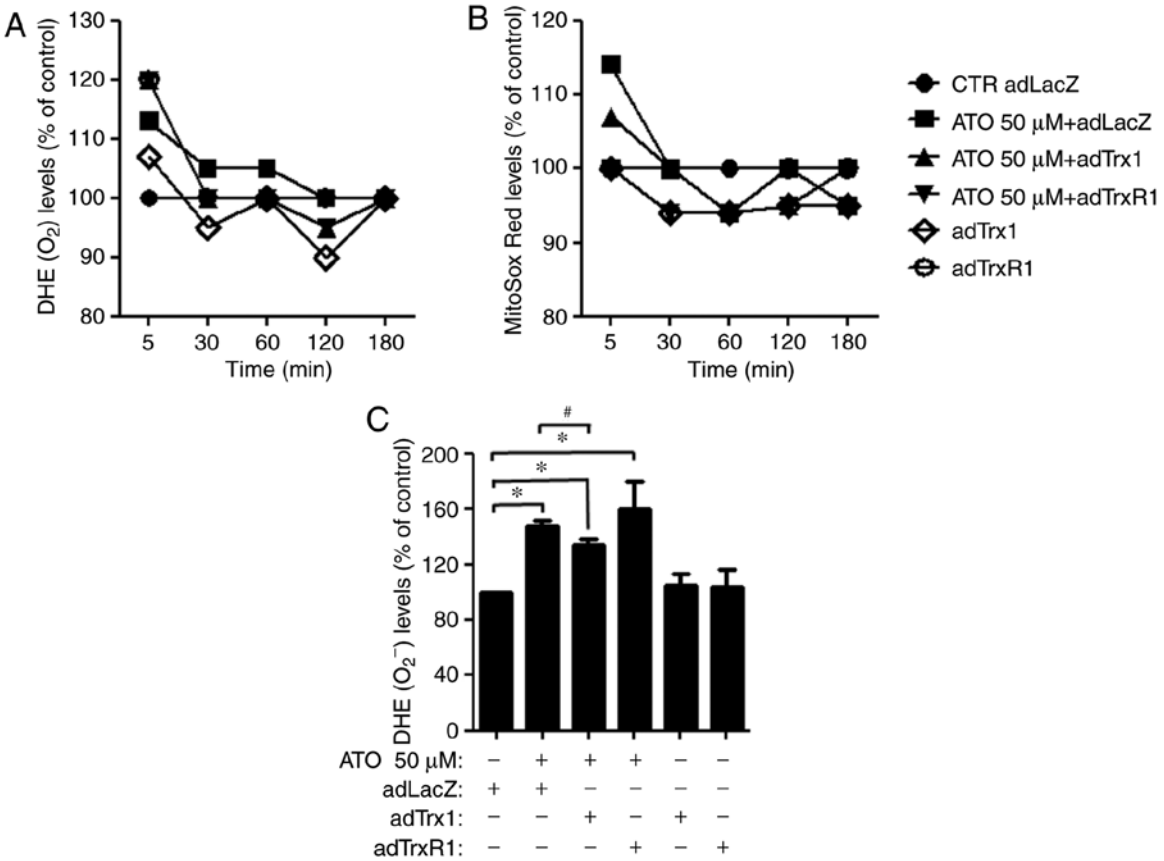

Figure 3. Effects of adTrx1 and adTrxR1 on $O_2^{\bullet-}$ levels in HPASMCs. HPASMCs (~70-80% confluence) were infected with CTR adLacZ, adTrx1, or adTrxR1. Twenty-four hours later, the cells were treated with 50 μM ATO for the indicated times. DHE ($O_2^{\bullet-}$) and MitoSOX™ Red (mitochondrial $O_2^{\bullet-}$) levels in HPASMCs were measured using a FACStar flow cytometer. (A) DHE ($O_2^{\bullet-}$) and (B) MitoSOX™ Red (mitochondrial $O_2^{\bullet-}$) levels compared with the adLacZ-infected control cells at the indicated early times. (C) DHE ($O_2^{\bullet-}$) levels (% of adLacZ-infected control cells) at 24 h. *P<0.05 compared with the adLacZ-infected control group; #P<0.05 compared with the adLacZ-infected control cells treated with ATO only. Trx1, thioredoxin 1; TrxR1, Trx reductase1; ATO, arsenic trioxide (As_2O_3); HPASMCs, human pulmonary artery smooth muscle cells; DHE, dihydroethidium.

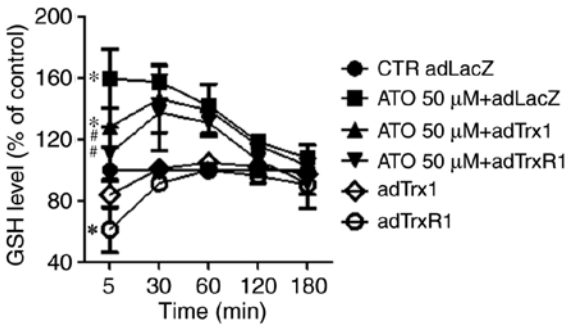

Figure 4. Effects of adTrx1 and adTrxR1 on GSH levels in HPASMCs. HPASMCs (~70-80% confluence) were infected with CTR adLacZ, adTrx1, or adTrxR1. Twenty-four hours later, the cells were treated with 50 μM ATO for the indicated times. GSH levels in HPASMCs were measured using a FACStar flow cytometer. The graph indicates GSH (CMF) levels compared with the adLacZ-infected control cells at the indicated early times. *P<0.05 compared with the adLacZ-infected control group. #P<0.05 compared with the adLacZ-infected control cells treated with ATO only. Trx1, thioredoxin 1; TrxR1, Trx reductase1; ATO, arsenic trioxide (As_2O_3); HPASMCs, human pulmonary artery smooth muscle cells; GSH, glutathione.

Discussion

The thioredoxin (Trx) system consists of Trx, TrxR, and NADPH, an important enzymatic complex that maintains the homeostasis of cellular redox (43). Specifically, thioredoxin 1 (Trx1) and Trx reductase1 (TrxR1) is overexpressed in asthma, acute lung injury, and lung cancer (11,37,44,45). Therefore, the Trx system can be a promising target for the treatment of pulmonary diseases. Recently, it was demonstrated that an environmental agent, arsenic trioxide (ATO), leads to growth inhibition and death in human pulmonary artery smooth muscle cells (HPASMCs) accompanied by mitochondrial $O_2^{\bullet-}$ increase and GSH depletion (36). In addition, PX-12 and auranofin induce the death of lung cancer and mesothelioma cells through oxidative stress (16,19). In the present study, whether changes in Trx1 and TrxR1 expression affect cell growth, death, ROS, and GSH levels in ATO-treated HPASMCs was investigated and the effects of PX-12 and auranofin on cell metabolism and redox status in HPASMCs was analyzed.

HPASMCs infected with adTrx1 and adTrxR1 showed upregulation of Trx1 and TrxR1 proteins. Overexpression of Trx1 and TrxR1, especially TrxR1, attenuated cell growth inhibition caused by ATO. Both adenoviruses moderately prohibited the death of ATO-treated HPASMCs. In our previous studies, adTrx1 was also shown to reduce cell death in suberoylanilide hydroxamic or suberoyl bishydroxamic acid-treated lung and cervical cancer cells (37-39,46). These results support that Trx1 and TrxR1 have anti-apoptotic and

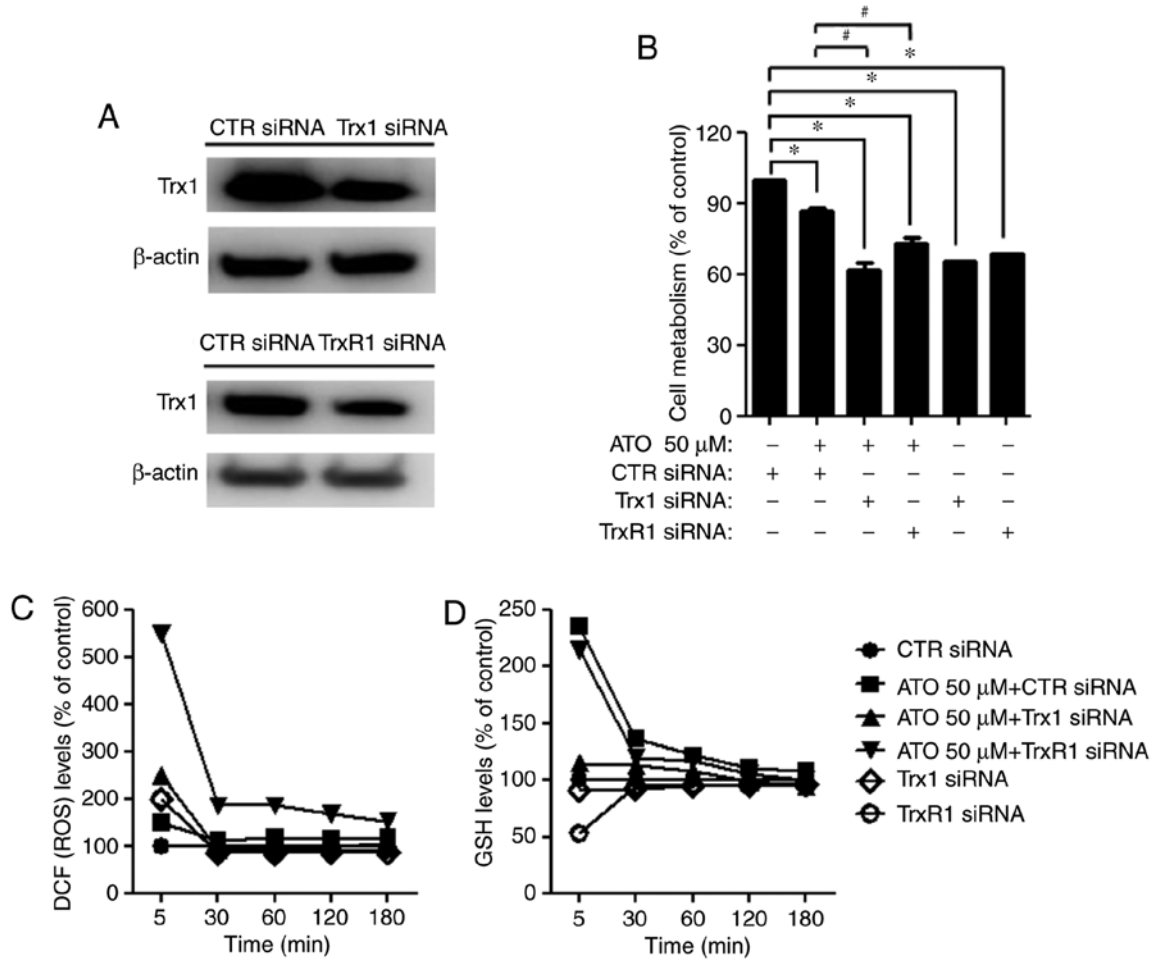

Figure 5. Effects of Trx1 siRNA and TrxR1 siRNA on cell metabolism, ROS, and GSH levels in HPASMCs. HPASMCs (~70-80% confluence) were transfected with CTR siRNA, Trx1 siRNA, or TrxR1 siRNA. Twenty-four hours later, the cells were treated with 50 μM ATO for the indicated times. (A) Expression levels of Trx1 and TrxR1 proteins in HPASMCs. (B) Cellular metabolism changes measured by the reduction of MTT to formazan for 4 h. (C and D) ROS and GSH levels in HPASMCs were measured using a FACStar flow cytometer. DCF (ROS) (C) and GSH (CMF) levels (D) compared with the control siRNA-treated cells at the indicated times. *P<0.05 compared with the control siRNA-treated cells; #P<0.05 compared with the control siRNA-treated cells treated with ATO only. Trx1, thioredoxin 1; TrxR1, Trx reductase1; ATO, arsenic trioxide (As_2O_3); HPASMCs, human pulmonary artery smooth muscle cells; GSH, glutathione; ROS, reactive oxygen species.

cell survival effects (8), which may be important for cellular resistance to toxic agents. ATO diminished the reduction of MTT to formazan in HPASMCs, implying that ATO decreased the cellular metabolism of HPASMCs, consequently reducing the activity of NAD(P)H-dependent oxidoreductase. adTrxR1 efficiently increased cellular metabolism, indicated by the reduction of MTT to formazan in HPASMCs. In contrast, HPASMCs transfected with Trx1 or TrxR1 siRNA showed significant reduction of HPASMC metabolism regardless of ATO. In addition, PX-12 and auranofin, which presumably inhibited the activities of Trx1 and TrxR1, respectively, decreased the cellular metabolism of HPASMCs. Relatively higher doses of PX-12 and auranofin almost terminated the cellular metabolism at 4 h, indicating that the cells were progressively dying. Since Trx can control numerous cell signaling pathways by modulating cell-type-specific transcription factors such as p38, NF-κB, AP-1, p53, and nuclear receptors (11,47-49), Trx is possibly involved in the regulation of basal cellular metabolism in glycolysis, the TCA cycle, and mitochondria to produce ATP or NAD(P)H (50). However, these effects mediated by the Trx system may depend on cell type, co-treatment with drugs as well as basal activity and the level of Trx-related proteins.

ATO increases the production of ROS in numerous cells including SMCs (27-29,36). Likewise, ATO increased $O_2^{\bullet-}$ levels at 24 h in HPASMCs. ATO also increased DCF (ROS) levels including the mitochondrial $O_2^{\bullet-}$ at 5 min. ATO apparently impaired mitochondria, leading to the generation of $O_2^{\bullet-}$, reduction of cell metabolism, and consequent induction of cell death. Mitochondria are the primary energetic subcellular organelles for generating ROS and have $O_2^{\bullet-}$ content approximately 5-10-fold higher than that of the cytoplasm (51). However, at approximately 30-180 min, ATO did not increase ROS levels, including $O_2^{\bullet-}$, compared with the control group cells. Moreover, ATO decreased ROS (DCF) levels at 24 h. It is possible that the fluorescent DCF was not exactly detected due to the cell leakage of the dye and/or the deficiency in the esterase to remove the acetate groups from H_2DCFDA. ATO regulates ROS levels using a variety of redox enzymes (23,24,52) and by causing mitochondrial dysfunction (24,25,53). Therefore, ATO might change ROS levels in HPASMCs differently depending on incubation time and dose. In addition, a relatively low dose

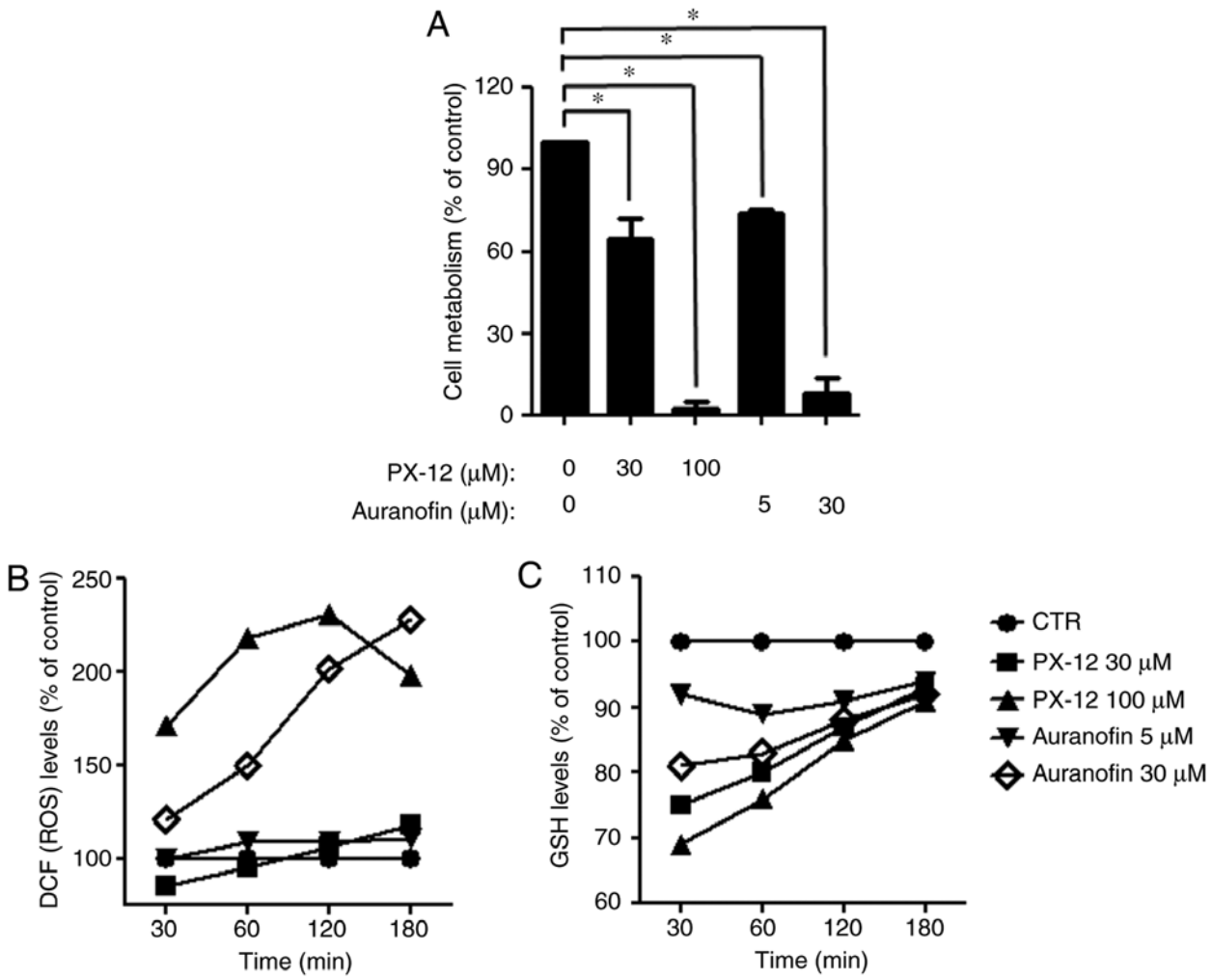

Figure 6. Effects of PX-12 and auranofin on cell metabolism, ROS, and GSH levels in HPASMCs. Approximately 90% growing cells were treated with the indicated amounts of PX-12 and Auranofin for the indicated times. (A) Cellular metabolism changes measured by the reduction of MTT to formazan for 4 h. (B and C) ROS and GSH levels in HPASMCs were measured using a FACStar flow cytometer. DCF (ROS) (B) and GSH (CMF) levels (C) compared with the CTR cells at the indicated times. *P<0.05 compared with the control group. HPASMCs, human pulmonary artery smooth muscle cells; GSH, glutathione; ROS, reactive oxygen species.

of ATO induces a significant oxidative stress as well as a time-dependent differential modulation of antioxidant status in a tissue-specific manner (52,54). Oxidative damage markers such as protein nitration and lipid peroxidation could be very useful to discuss these results in depth. Moreover, if rotenone as a mitochondrial inhibitor were used and the mitochondria were isolated from HPASMCs to study metabolic activity, the results would be more informative.

Administration of adTrx1 or adTrxR1 reduced the increased mitochondrial $O_2^{\bullet-}$ levels in ATO-treated HPASMCs at 5 min. In addition, adTrx1 and adTrxR1 decreased the mitochondrial $O_2^{\bullet-}$ levels in the control HPASMCs at approximately 30-180 min. However, neither adenovirus decreased DCF (ROS) and DHE ($O_2^{\bullet-}$) levels in ATO-treated HPASMCs at 5 min. The altered redox status via overexpression of Trx primarily lead to the maintenance of mitochondrial integrity. Control HPASMCs infected with adTrx1 or adTrxR1 showed a steady decrease in ROS (DCF) levels at approximately 30-180 min. Furthermore, adTrx1 decreased DHE ($O_2^{\bullet-}$) levels in ATO-treated HPASMCs at 24 h and adTrx1 reduced DHE ($O_2^{\bullet-}$) levels in the control HPASMCs at 120 min. In addition, HPASMCs treated with Trx1 or TrxR1 siRNA showed increased ROS (DCF) levels with or without ATO at 5 min. In particular, TrxR1 siRNA-treated cells showed higher ROS (DCF) levels. PX-12 and auranofin increased ROS (DCF) levels in HPASMCs. Although a minimal difference in ROS levels was observed between adTrx1- and ad TrxR1-treated cells, overexpression of Trx generally reduced ROS levels and downregulation of Trx increased ROS levels in HPASMCs. adTrx1 and adTrxR1 did not augment the reduced ROS (DCF) levels in ATO-treated HPASMCs at 24 h; overexpression of Trx most likely increased the basal ROS level by increasing cellular metabolism in these cells.

GSH is a non-protein thiol antioxidant crucial for protecting cells from oxidative stress and has a significant effect on ATO-, PX-12-, and auranofin-induced apoptosis in various cells including HPASMCs (16-20,28,36,55-57). ATO significantly increased GSH (CMF) levels at 5 min and then steadily reduced the levels at 180 min. The short-lived increase of GSH levels was likely due to the defense mechanism against the transiently increased ROS levels caused by ATO. The subsequent decrease of GSH levels was possibly due to its use to decrease

ROS levels. Trx1 and TrxR1 siRNAs decreased GSH (CMF) levels in the control HPASMCs at approximately 5-30 min and both siRNAs reduced the increased GSH (CMF) levels in ATO-treated cells at approximately 5-180 min. Dysregulation of the Trx system by Trx1 and TrxR1 siRNAs failed to produce the basal metabolic reducing agent of NAD(P)H to generate the reduced form of GSH, consequently decreasing GSH levels in these cells. In addition, PX-12 and auranofin reduced GSH (CMF) levels in HPASMCs at 30 min, indicating that down-regulation of the Trx system by siRNA or drugs diminishes the basal cellular metabolism and causes a cellular milieu of comparative oxidative stress. adTrx1 and adTrxR1 attenuated the increased GSH (CMF) levels in ATO-treated cells at approximately 5-60 min. In addition, both adenoviruses lowered the basal GSH (CMF) levels in HPASMCs at approximately 5-30 min. The upregulated activity of the Trx system via adTrx1 and adTrxR1 possibly utilized NADPH during these time periods, which could not renew or create the reduced form of GSH, consequently decreasing GSH levels in these cells.

In summary, upregulation of Trx1 and TrxR1 partially decreased growth inhibition and death in ATO-treated HPASMCs. Generally, overexpression of Trx reduced ROS levels in ATO-treated HPASMCs whereas downregulation of Trx increased ROS levels and decreased GSH levels in these cells. adTrxR1 seemed to efficiently increase cellular metabolism whereas Trx1 or TrxR1 siRNA significantly decreased the cellular metabolism. Both PX-12 and auranofin decreased the cellular metabolism accompanied by an increase in ROS level and a decrease in GSH level. The results from the present study may contribute to research focusing on the Trx system for the treatment or prevention of pulmonary vascular diseases caused by contact with arsenic. Further characterizations of Trx and TrxR in vascular system should be investigated in future using alterative cells due to the difficulty working with primary normal HPASMCs.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

WHP is the sole author of the study responsible for the design, experimental procedures, data collection and analysis and writing of the manuscript. WHP agrees to be accountable for all aspects of the research in ensuring that the accuracy and integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The author declares no competing interests.

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