The effects of N-acetyl cysteine, buthionine sulfoximine, diethyldithiocarbamate or 3-amino-1,2,4-triazole on antimycin A-treated Calu-6 lung cells in relation to cell growth, reactive oxygen species and glutathione

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Abstract. Antimycin A (AMA) inhibits mitochondrial electron transport between cytochrome b and c. We recently demonstrated that AMA inhibits the growth of lung cancer Calu-6 cells and the changes of reactive oxygen species (ROS) and glutathione (GSH) levels affect apoptosis in Calu-6 cells. Here, we examined the effects of N-acetyl-cysteine (NAC, a well known antioxidant), L-buthionine sulfoximine (BSO, an inhibitor of GSH synthesis), diethyl-dithiocarbamate (DDC, an inhibitor of Cu, Zn-SOD) or 3-amino-1,2,4-triazole (AT, an inhibitor of catalase) on AMA-treated Calu-6 cells in relation to cell death, ROS and GSH levels. Treatment with AMA induced cell growth inhibition, apoptosis and the loss of mitochondrial membrane potential (MMP) ($\Delta\Psi_m$) in Calu-6 cells. While the intracellular ROS level was decreased in 50 μM AMA-treated Calu-6 cells, $O_2^{\bullet-}$ levels among ROS were significantly increased. AMA also induced GSH depletion in Calu-6 cells. Treatment with NAC showed decreasing effect on $O_2^{\bullet-}$ levels in AMA-treated cells preventing apoptosis, MMP ($\Delta\Psi_m$) loss and GSH depletion in these cells. BSO significantly increased GSH depletion and apoptosis in AMA-treated cells. While both DDC and AT increased ROS levels in AMA-treated Calu-6 cells, only DDC intensified GSH depletion and apoptosis. BSO and AT increased the ROS level in Calu-6 control cells, but these agents did not induce apoptosis and GSH depletion. In conclusion, our results suggest that GSH depletion rather than ROS level in AMA-treated Calu-6 cells is more tightly related to apoptosis.

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Abbreviations: AMA, antimycin A; ROS, reactive oxygen species; NADPH, nicotine adenine diphosphate; XO, xanthine oxidase; SOD, superoxide dismutase; FBS, fetal bovine serum; PBS, phosphate buffer saline; FITC, fluorescein isothiocyanate; H_2DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; DHE, dihydroethidium; GSH, glutathione; CMFDA, 5-chloromethyl-fluorescein diacetate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI, propidium iodide; DDC; diethyldithiocarbamate, BSO; L-buthionine sulfoximine; AT; 3-amino-1,2,4-triazole, MMP, mitochondrial membrane potential ($\Delta\Psi_m$)

Key words: antimycin A, reactive oxygen species, apoptosis, glutathione, Calu-6

Introduction

Reactive oxygen species (ROS) include hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet-}$) and hydroxyl radical ($^{\bullet}OH$). These molecules regulate many important cellular events, including transcription factor activation, gene expression, differentiation, and cell proliferation (1,2). ROS are formed as by-products of mitochondrial respiration or the action of oxidases, including nicotine adenine diphosphate (NADPH) oxidase, xanthine oxidase (XO) and certain arachidonic acid oxygenases (3). A change in the redox state of a cell or tissue implies a change in ROS generation or metabolism. The principal metabolic pathways include superoxide dismutase (SOD), which is expressed as extracellular, intracellular and mitochondrial isoforms. These isoforms metabolize $O_2^{\bullet-}$ to H_2O_2. Further metabolism by peroxidases, which include catalase and glutathione (GSH) peroxidase, yields O_2 and H_2O (4). GSH is the main non-protein antioxidant in the cell and provides electrons for enzymes such as glutathione peroxidase, which reduce H_2O_2 to H_2O. GSH is crucial for cell proliferation, cell cycle progression and apoptosis (5,6) and is known to protect cells from toxic insult by detoxifying toxic metabolites of drugs and ROS (7). Although cells possess antioxidant systems to control their redox state, excessive production of ROS can be induced and gives rise to the activation of events that lead to death or survival in different cell types (8,9).

Antimycin A (AMA) is a product that is predominantly composed of antimycin A1 and A3, which are derived from *Streptomyces kitazawensis* (10). AMA inhibits succinate dehydrogenase and NADH oxidase, and also inhibits mitochondrial electron transport between cytochrome b and c (11,12). The inhibition of electron transport causes a collapse

of the proton gradient across the mitochondrial inner membrane, thereby breaking down the MMP ($\Delta\Psi_m$) (11,13). This inhibition also results in the production of ROS (13,14). Evidence indicates that either the presence of ROS or the collapse of MMP ($\Delta\Psi_m$) opens the mitochondrial permeability transition pore, which is accompanied by the release of proapoptotic molecules such as cytochrome c into the cytoplasm (15,16). Because AMA acts directly on the mitochondria, AMA-induced apoptosis has been reported in many experiments (17-24).

Lung cancer is a major contributor to the variety cancer death in developed countries. Various novel therapeutic strategies are currently under consideration, as the clinical use of cytotoxic drugs is limited due to intrinsic or acquired resistance and toxicity (25). Studies of the molecular mechanisms of cytotoxic drug action have shed light on the treatment of lung cancer, and novel agents that target specific intracellular pathways related to the distinctive properties of cancer cells continue to be developed.

Recently, we demonstrated that AMA inhibits the growth of lung cancer Calu-6 cells via inducing a G1 arrest of the cell cycle and caspase-dependent apoptosis (26) and the changes of ROS and GSH levels affect apoptosis in AMA-treated Calu-6 cells (27). However, the roles of ROS and GSH in AMA-induced Calu-6 cell death still remain obscure. In the present study, we examined the effects of N-acetylcysteine (NAC, a well known antioxidant), L-buthionine sulfoximine [BSO, an inhibitor of GSH synthesis (28)], diethyldithiocarbamate [DDC, an inhibitor of Cu,Zn-SOD (29)] or 3-amino-1,2,4-triazole [AT, an inhibitor of catalase (30)] on cell growth and death in AMA-treated Calu-6 cells in relation to ROS and GSH levels.

Materials and methods

Cell culture. Human pulmonary adenocarcinoma Calu-6 cells obtained from the ATCC (HTB56) were maintained in a humidified incubator with 5% CO_2 at 37˚C. The cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco-BRL, Grand Island, NY). They were routinely grown in 100-mm plastic tissue culture dishes (Nunc, Roskilde, Denmark) and harvested with a solution of trypsin-EDTA while in logarithmic phase. Cells were maintained in these culture conditions for all experiments.

Reagents. AMA was purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). AMA was dissolved in ethanol at 200 mM as a stock solution. N-acetylcysteine (NAC), L-buthionine sulfoximine (BSO), diethyldithiocarbamate (DDC) and 3-amino-1,2,4-triazole (AT) were also obtained from Sigma. These agents were dissolved in water or ethanol at 100 mM as a stock solution. Cells were pretreated with NAC, BSO, DDC or AT for 30 min prior to treatment with AMA. Ethanol was used as a control vehicle. The stock solution was wrapped in foil and kept at 4˚C or -20˚C.

Cell growth inhibition assay. The effect of drugs on Calu-6 cell growth was determined by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye absorbance of living cells as previously described (31). In brief, cells ($5x10^4$ cells per well) were seeded in 96-well microtiter plates in the presence of the designated doses of AMA with or without NAC, BSO, DDC or AT. After exposure to drugs for 72 h, 20 μl of MTT (Sigma) solution (2 mg/ml in PBS) was added to each well of 96-well plates. The plates were incubated for further 4 h at 37˚C. MTT solution in the medium was aspirated off and 200 μl of DMSO were added to each well to solubilize the formazan crystals formed in viable cells. Optical density was measured at 570 nm using a microplate reader (Spectra MAX 340, Molecular Devices Co., Sunnyvale, CA).

Annexin V staining. Apoptosis was determined by staining cells with annexin V-fluorescein isothiocyanate (FITC) (Ex/Em = 488/519 nm) as previously described (32). In brief, $1x10^6$ cells were incubated with AMA with or without NAC, BSO, DDC or AT for 72 h. 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 $1x10^6$ cells/ml. Five microliters of annexin V-FITC (PharMingen, San Diego, CA) was then added to these cells, which were analyzed with a FACStar flow cytometer (Becton-Dickinson).

Measurement of mitochondrial membrane potential (MMP) ($\Delta\Psi_m$). The MMP ($\Delta\Psi_m$) levels were measured by the Rhodamine 123 fluorescent dye (Ex/Em = 485/535 nm), as previously described (33). In brief, $1x10^6$ cells were incubated with AMA with or without NAC, BSO, DDC or AT for 72 h. They were washed twice with PBS and incubated with Rhodamine 123 (0.1 μg/ml; Sigma) at 37˚C for 30 min. Rhodamine 123 staining intensity was determined by flow cytometry.

Detection of intracellular ROS and $O_2^{\bullet-}$ levels. Intracellular ROS such as H_2O_2, $^{\bullet}OH$ and $ONOO^{\bullet}$ 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 (34). H_2DCFDA (Ex/Em = 495/529 nm) is poorly selective for superoxide anion radical ($O_2^{\bullet-}$). In contrast, dihydroethidium (DHE) (Ex/Em = 518/605 nm) (Invitrogen Molecular Probes) is a fluorogenic probe that is highly selective for $O_2^{\bullet-}$ among ROS as previously described (34). In brief, $1x10^6$ cells were incubated with the designated doses of AMA with or without NAC, BSO, DDC or AT for 72 h. Cells were then washed in PBS and incubated with 20 μM H_2DCFDA or DHE at 37˚C for 30 min. DCF and DHE fluorescences were detected using a FACStar flow cytometer (Becton-Dickinson). ROS and $O_2^{\bullet-}$ levels were expressed as mean fluorescence intensity (MFI), which was calculated by CellQuest software.

Detection of the intracellular glutathione (GSH). Cellular GSH levels were analyzed using 5-chloromethylfluorescein diacetate (CMFDA, Molecular Probes) (Ex/Em = 522/595 nm) as previously described (34). In brief, $1x10^6$ cells were incubated with AMA with or without NAC, BSO, DDC or AT for 72 h. They were then washed with PBS and incubated

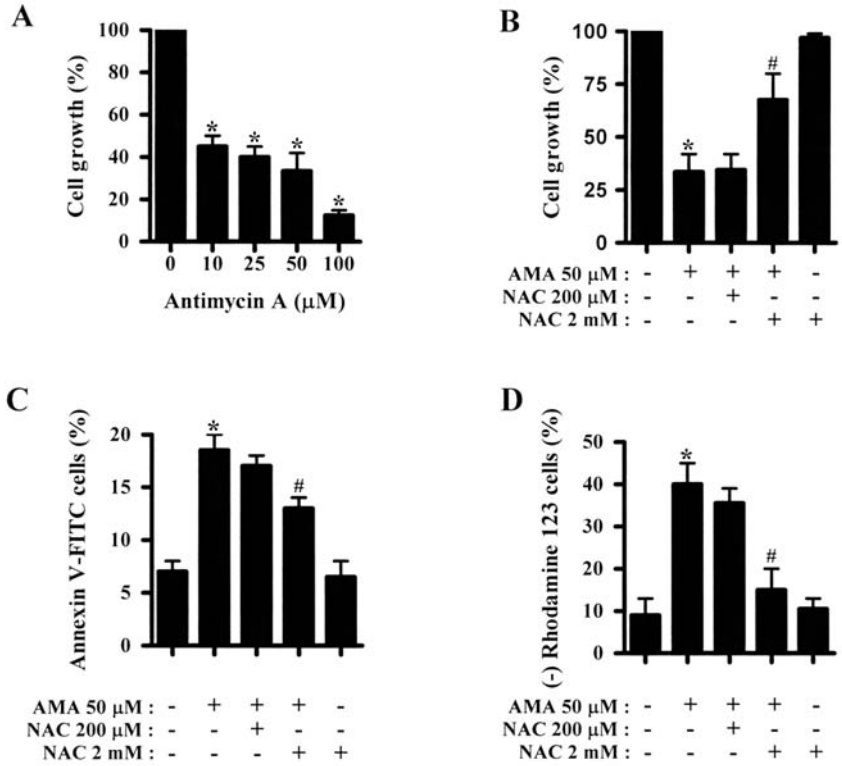

Figure 1. Effect of NAC on cell growth and death in AMA-treated Calu-6 cells. Exponentially-growing cells were treated with the indicated amounts of AMA with or without 200 μM or 2 mM NAC for 72 h. (A and B) The growth of Calu-6 cells was assessed by an MTT assay. Cell growth in AMA-treated cells (A) and AMA and/or NAC-treated cells is shown (B). (C and D) Annexin positive cells and Rhodamine 123 negative cells were measured using flow cytometric analysis as described in Materials and methods. The percents of annexin positive cells (C) and Rhodamine 123 negative [MMP loss ($\Delta\Psi_m$)] cells are shown (D). *P<0.05 as compared with the control group cells. #P<0.05 as compared with cells treated with only AMA.

with 5 μM CMFDA at 37˚C for 30 min. Cytoplasmic esterases convert non-fluorescent CMFDA to fluorescent 5-chloromethylfluorescein, which can then react with the glutathione. CMF fluorescence was measured using a FACStar flow cytometer (Becton-Dickinson) and CellQuest software. Negative CMF staining (GSH depleted) cells were expressed as the percent of (-) CMF cells.

Sub-G1 cell analysis. Sub-G1 DNA content cells were determined by propidium iodide (PI, Sigma-Aldrich; Ex/Em = 488/617 nm) staining as previously described (35). In brief, 1x10^6 cells were incubated with 50 μM AMA with or without BSO for 72 h. Cells were then washed with PBS and fixed in 70% ethanol. Cells were washed again with PBS, then incubated with PI (10 μg/ml) with simultaneous RNase treatment at 37˚C for 30 min. Cell DNA content was measured using a FACStar flow cytometer (Becton-Dickinson) and analyzed using lysis II and CellFIT software (Becton-Dickinson).

Statistical analysis. The results shown represent the mean of at least two independent experiments; bar, SD. The data were analyzed using Instat software (GraphPad Prism4, San Diego, CA). The 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. The statistical significance was defined as $p<0.05$.

Results

Effect of NAC on cell growth, cell death and mitochondrial membrane potential (MMP) ($\Delta\Psi_m$) loss in AMA-treated Calu-6 cells. We first examined the effect of AMA on the growth of Calu-6 cells using an MTT assay. Treatment with AMA reduced the cell growth in a dose-dependent manner at 72 h (Fig. 1A). The cell growth was completely inhibited at the concentration of 100 μM AMA (Fig. 1A). We examined whether NAC (a well-known antioxidant) rescues Calu-6 cells from AMA-induced insults. Treatment with 50 μM AMA, which concentration inhibited the growth of Calu-6 cells about 65% (Fig. 1A) was considered to be suitable dose to differentiate the levels of cell growth inhibition in the presence of AMA vs. in the presence of AMA and NAC. While 2 mM NAC significantly prevented the growth inhibition of AMA-treated Calu-6 cells, 200 μM NAC did not (Fig. 1B). In addition, treatment with 200 μM, but not 2 mM NAC significantly decreased the number of sub-G1 cells induced by 50 μM AMA (data not shown). AMA induced annexin V staining cells in Calu-6 cells, implying that AMA seemed to trigger apoptosis in Calu-6 cells (Fig. 1C). The percents of annexin V positive staining cells in AMA-treated cell were significantly decreased by treatment with 2 mM NAC (Fig. 1C). In relation to MMP ($\Delta\Psi_m$), AMA induced MMP ($\Delta\Psi_m$) loss in Calu-6 cells (Fig. 1D). Treatment with 2 mM NAC prohibited the loss of MMP ($\Delta\Psi_m$) induced by

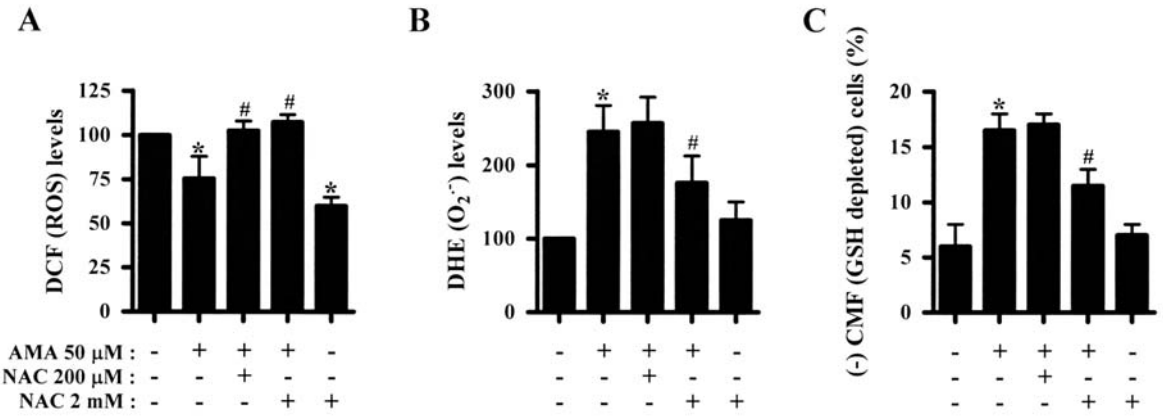

Figure 2. Effect of NAC on ROS and GSH levels in AMA-treated Calu-6 cells. Exponentially-growing cells were treated with 50 μM AMA with or without 200 μM or 2 mM NAC for 72 h. ROS and GSH levels were determined by a FACStar flow cytometer as described in Materials and methods. The levels of mean DCF fluorescence (% of control cells) (A) and mean DHE ($O_2^{\bullet-}$) fluorescence (% of control cells) (B), and the percent of CMF negative fluorescence (GSH depleted) cells are shown (C). *P<0.05 as compared with the control group cells. $^{\#}$P<0.05 as compared with cells treated with only AMA.

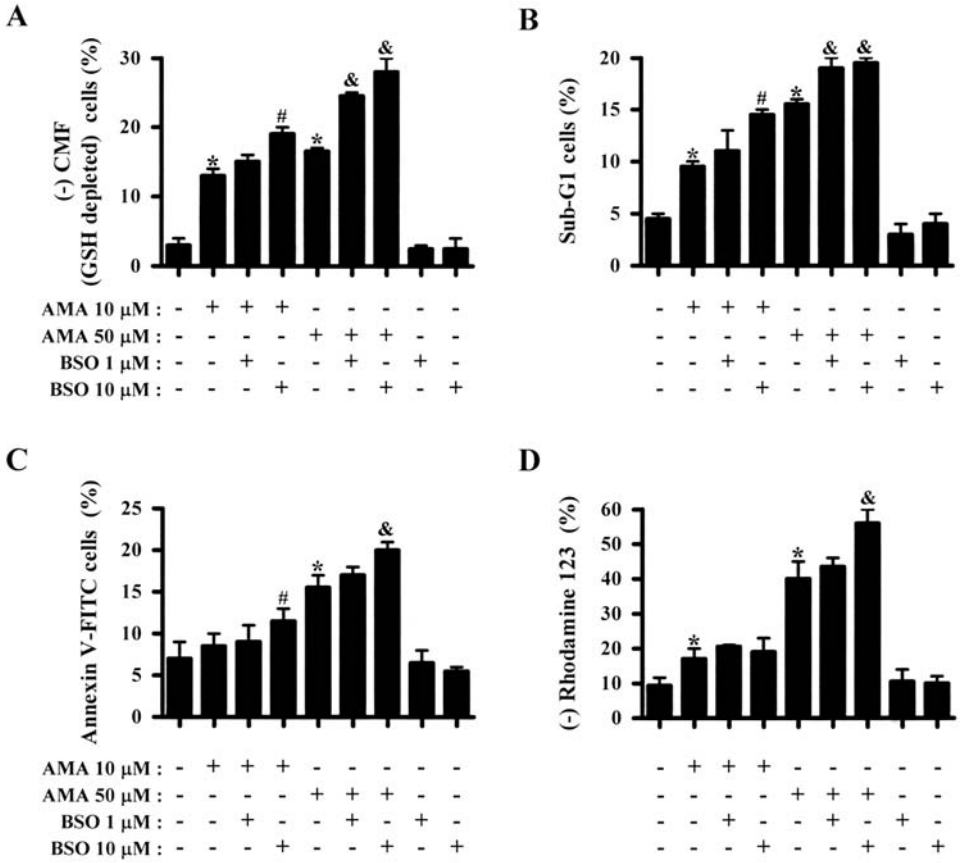

Figure 3. Effect of BSO on GSH levels and cell death in AMA-treated Calu-6 cells. Exponentially-growing cells were treated with 10 or 50 μM AMA with or without 1 or 10 μM BSO for 72 h. The percents of CMF negative fluorescence (GSH depleted) cells (A), sub-G1 cells (B), annexin V staining cells (C) and Rhodamine 123 negative [MMP ($\Delta\Psi_m$) loss] cells are shown (D). *P<0.05 as compared with the AMA-untreated control group cells. $^{\#}$P<0.05 as compared with 10 μM AMA-treated group cells and $^{\&}$P<0.05 as compared with 50 μM AMA-treated group cells.

AMA (Fig. 1D). Treatment with 2 mM NAC alone did not significantly change cell growth and death in Calu-6 control cells (Fig. 1B, C and D).

Effect of NAC on ROS and GSH levels in AMA-treated Calu-6 cells. We determined whether the levels of ROS and GSH in AMA-treated Calu-6 cells were changed by NAC treatment. As shown in Fig. 2A, ROS (DCF) level was decreased in 50 μM AMA-treated Calu-6 cells at 72 h. Treatment with 200 μM or 2 mM NAC recovered the decreased ROS level to the basal level in control cells, and NAC alone decreased ROS level in control cells (Fig. 2A). Intracellular $O_2^{\bullet-}$ level was increased in 50 μM AMA-treated Calu-6 cells (Fig. 2B). While 200 μM NAC did not alter the $O_2^{\bullet-}$ level in AMA-

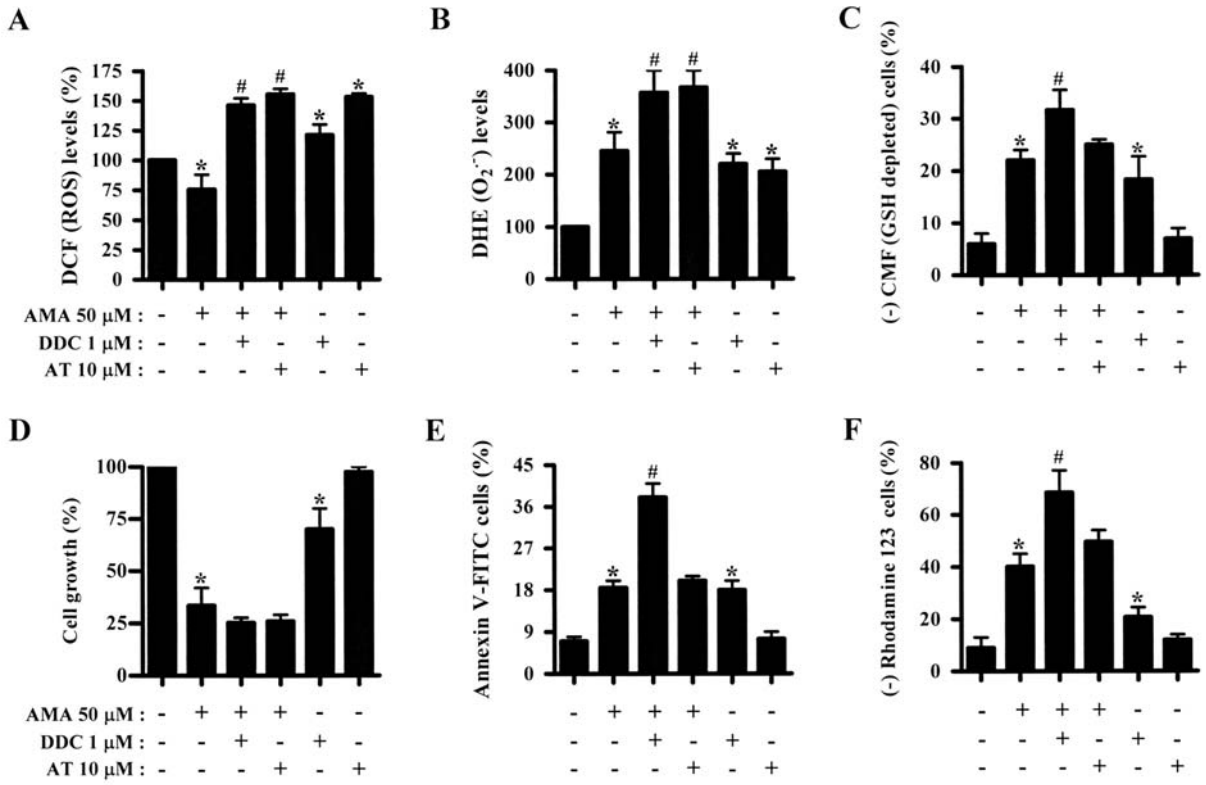

Figure 4. Effect of DDC and AT on ROS and GSH levels, cell growth and cell death in AMA-treated Calu-6 cells. Exponentially-growing cells were treated with 50 μM AMA with or without 1 μM DDC or 10 μM AT for 72 h. The levels of mean DCF fluorescence (% of control cells) (A), mean DHE ($O_2^{\bullet-}$) fluorescence (% of control cells) (B) and the percents of CMF negative fluorescence (GSH depleted) cells (C), sub-G1 cells (D), annexin V staining cells (E) and Rhodamine 123 negative [MMP ($\Delta\Psi_m$) loss] cells are shown (F). *P<0.05 as compared with the control group cells. #P<0.05 as compared with cells treated with only AMA.

treated cells, 2 mM NAC decreased the $O_2^{\bullet-}$ level in these cells (Fig. 2B). Next, we analyzed GSH in Calu-6 cells using CMF fluorescence. AMA significantly elevated the number of GSH depleted cells (Fig. 2C). Treatment with 2 mM NAC significantly prevented GSH depletion in AMA-treated cells (Fig. 2C).

Effects of BSO on GSH depletion, cell death and mitochondrial membrane potential (MMP) ($\Delta\Psi_m$) loss in AMA-treated Calu-6 cells. Treatment of 1 or 10 μM BSO dose-dependently intensified GSH depletion in 50 μM AMA-treated Calu-6 cells (Fig. 3A). Furthermore, BSO increased GSH depletion in Calu-6 cells treated with the lower dose of 10 μM AMA. Treatment with 1 or 10 μM BSO alone did not induce GSH depletion in control cells (Fig. 3A). Treatment with BSO increased the number of sub-G1 cells in 10 or 50 μM AMA-treated Calu-6 cells (Fig. 3B). BSO intensified the percents of annexin V positive cells in 10 or 50 μM AMA-treated cell (Fig. 3C). BSO alone did not induce cell death in Calu-6 cells (Fig. 3B and C). Concerning MMP ($\Delta\Psi_m$), 10 μM BSO increased MMP ($\Delta\Psi_m$) loss in 50 μM AMA-treated Calu-6 cells (Fig. 3D). However, BSO alone did not affect MMP ($\Delta\Psi_m$) loss in Calu-6 control cells (Fig. 3D).

Effects of DDC and AT on ROS, GSH, cell growth and cell death in AMA-treated Calu-6 cells. We investigated whether DDC or AT changes ROS and GSH levels in AMA-treated Calu-6 cells. Treatment with 1 μM DDC or 10 μM AT increased the intracellular ROS level in AMA-treated and untreated Calu-6 cells (Fig. 4A). Treatment with DDC or AT also increased the intracellular $O_2^{\bullet-}$ level in AMA-treated and -untreated Calu-6 cells (Fig. 4B). While DDC increased GSH depletion levels in AMA-treated and untreated Calu-6 cells, AT did not alter this deletion in these cells (Fig. 4C). Next, we investigated whether DDC or AT changes cell growth and cell death in AMA-treated Calu-6 cells. Treatment with DDC or AT slightly intensified the growth inhibition of AMA-treated Calu-6 cells (Fig. 4D). DDC also decreased the growth of Calu-6 control cells (Fig. 4D). The number of annexin V positive staining cells in AMA-treated cells was augmented by DDC (Fig. 4E). DDC increased the number in Calu-6 control cells as well (Fig. 4E). AT did not change the number in AMA-treated and untreated Calu-6 cells (Fig. 4E). In relation to the MMP ($\Delta\Psi_m$), treatment with DDC increased the loss of MMP ($\Delta\Psi_m$) in AMA-treated Calu-6 cells (Fig. 4F). DDC also induced the MMP ($\Delta\Psi_m$) loss in Calu-6 control cells (Fig. 4F). AT did not affect the loss in AMA-treated or untreated cells (Fig. 4F).

Discussion

In the present study, we focused on evaluating the effects of NAC, BSO, DDC or AT on cell growth and death in AMA-treated Calu-6 lung cells in relation to ROS and GSH levels since we observed that AMA decreased cell growth and ROS levels in Calu-6 cells and increased GSH deletion as well.

AMA can disturb the natural oxidation/reduction equilibrium in cells by causing a breakdown in the MMP ($\Delta\Psi_m$) (11,13,36). It is known that the collapse of MMP ($\Delta\Psi_m$) is closely related to apoptosis (37). The increased intracellular H_2O_2 played an important role in AMA-induced cell death in liver cells (38,39) and A549 human lung cancer cells (14). Likewise, AMA induced MMP ($\Delta\Psi_m$) loss in Calu-6 cells. However, ROS (DCF) level was decreased in 50 μM AMA-treated Calu-6 cells AMA at 72 h, but ROS in these cells were increased at the early time phases of 10-300 min (27). In 100 μM AMA-treated Calu-6 cells for 72 h, ROS level was increased and the increase was also observed from the early time point of 10 min (27). These results imply that the change of ROS levels in AMA-treated Calu-6 cells is dependent on the dose and exposure times. When treated with DDC, AMA-treated cells showed increases in ROS, MMP ($\Delta\Psi_m$) loss and apoptosis. While 2 mM, but not 200 μM NAC protected some Calu-6 cells against AMA insults and prevented MMP ($\Delta\Psi_m$) loss in AMA-treated cells, both doses of NAC recovered the decreased ROS level to the basal level in control cells, and 2 mM NAC decreased ROS level in control cells. Treatment with AT, which showed an increased effect on ROS level in AMA-treated cells did not intensify MMP ($\Delta\Psi_m$) loss and apoptosis in these cells. AT increased ROS level in Calu-6 control cells, but did not induce cell death. In addition, BSO showing intensified MMP ($\Delta\Psi_m$) loss and apoptosis in AMA-treated Calu-6 cells also increased ROS levels in these cells (data not shown). However, BSO showing increasing ROS in Calu-6 control cells did not induce apoptosis (data not shown). Correctively, these results suggest that MMP ($\Delta\Psi_m$) loss in AMA-treated Calu-6 cells leads to apoptosis and ROS changes by AMA is not tightly correlated with MMP ($\Delta\Psi_m$) loss or the cell death in Calu-6 cells. Recently, we have reported that the increase of the intracellular ROS levels in AMA-treated As4.1 juxtaglomerular cells is not closely related to apoptosis (17).

AMA inhibits succinate oxidase and NADH oxidase, and also inhibits mitochondrial electron transport between cytochrome b and c (11,12,36,40), which may result in production of $O_2^{\bullet-}$ at the mitochondria. According to our data, the level of intracellular $O_2^{\bullet-}$ was significantly increased in AMA-treated Calu-6 cells at 72 h. The $O_2^{\bullet-}$ levels were also markedly increased within 10 min in an AMA dose-dependent manner (27). It is reported that AMA increased $O_2^{\bullet-}$ levels in human lung epithelial cells (41) and As4.1 juxtaglomerular cells (17). These data suggest the possibility that the apoptotic effects of AMA are comparative to the changes of intracellular $O_2^{\bullet-}$ levels. This notion can be supported by the result that treatment with NAC showing the reduction of intracellular $O_2^{\bullet-}$ levels significantly inhibited AMA-induced cell death. In addition, DDC which showed the increased effect on $O_2^{\bullet-}$ levels in AMA-treated cells intensified apoptosis. BSO increased $O_2^{\bullet-}$ levels in AMA-treated cells (data not shown) and intensified apoptosis as well. In contrast, although AT intensified the increased $O_2^{\bullet-}$ levels in AMA-treated cells, AT did not augment apoptosis. These data suggest that the changes of $O_2^{\bullet-}$ levels by AMA are partially related to the death of Calu-6 cells. The change of $O_2^{\bullet-}$ level rather than ROS (DCF) level in AMA-treated Calu-6 cells seems to be more involved in cell death.

The intracellular GSH content has a decisive effect on anticancer drug-induced apoptosis, which indicates that apoptotic effects are inversely comparative to GSH content (42,43). Likewise, our result clearly indicated that treatment with AMA increased the number of GSH depleted cells in Calu-6 lung cells. It is known that NAC is a direct substitution for GSH in alkylation or oxidation reactions, or of use in providing cysteine for GSH synthesis. Treatment with 2 mM but not 200 μM NAC prevented GSH deletion and apoptosis in AMA-treated Calu-6 cells, which suggests that NAC might be a GSH precursor in AMA-treated Calu-6 cells. DDC treatment increased this depletion in AMA-treated cells along with the enhancement of apoptosis. DDC also induced both GSH depletion and apoptosis in Calu-6 control cells. However, AT did not affect GSH depletion and apoptosis in AMA-treated or untreated Calu-6 cells. In addition, BSO enhanced GSH depletion and apoptosis in AMA-treated cells. However, treatment with 1 or 10 μM BSO induced neither GSH depletion nor apoptosis in Calu-6 control cells, suggesting that the lower dose of 1 μM BSO can reduce the threshold level for triggering apoptosis in AMA-treated cells. Collectively, our data suggest that the intracellular GSH content has a crucial role in anticancer drug-induced cell death.

In conclusion, we demonstrated that GSH depletion rather than ROS level in AMA-treated Calu-6 cells is more tightly related to apoptosis. Our present data could provide useful information for the action of AMA, a mitochondrial transport inhibitor in lung cancer cells in relation to cell growth, ROS and GSH levels.

Acknowledgements

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