# p38 Inhibitor Intensified Cell Death in Antimycin A-treated As4.1 Juxtaglomerular Cells *via* the Enhancement of GSH Depletion

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**Abstract.** *Antimycin A (AMA) inhibits succinate oxidase, NADH oxidase and mitochondrial electron transport chain between cytochrome b and c. Here, we investigated the effects of AMA and/or mitogen-activated protein kinase (MAPK) inhibitors on As4.1 juxtaglomerular cells in relation to cell growth, cell death, reactive oxygen species (ROS) and glutathione (GSH) levels. Treatment with 50 nM AMA inhibited the growth of As4.1 cells at 48 hours and induced apoptosis, which was accompanied by the loss of mitochondrial membrane potential ($\Delta\Psi_m$). AMA increased ROS levels including that of intracellular $O_2^{\bullet-}$. AMA also induced GSH depletion. MEK inhibitor did not affect cell growth, cell death, $\Delta\Psi_m$ loss, ROS level or GSH depletion in AMA-treated As4.1 cells. c-Jun N-terminal kinase (JNK) inhibitor also did not influence cell growth, cell death, ROS level and GSH depletion but did slightly increase $\Delta\Psi_m$ loss. Treatment with p38 inhibitor magnified cell growth inhibition by AMA and increased cell death, $\Delta\Psi_m$ loss and GSH depletion in AMA-treated As4.1 cells. Conclusively, p38 inhibitor intensified cell death in AMA-treated As4.1 cells. The changes of GSH content rather than ROS level by AMA and/or MAPK inhibitors were more closely related to the growth and death of As4.1 cells.*

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Reactive oxygen species (ROS) include hydrogen peroxide ($H_2O_2$), superoxide anion ($O_2^{\bullet-}$) and hydroxyl radical ($^{\bullet}OH$). These 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 oxidase and NADH oxidase and also inhibits the mitochondrial electron transport chain between cytochromes *b* and *c* (11-13). The inhibition of electron transport causes a collapse of the proton gradient across the mitochondrial inner membrane, thereby breaking down the mitochondrial membrane potential ($\Delta\Psi_m$) (11, 13-14). This inhibition also results in the production of ROS (14-15). Evidence indicates that either the presence of ROS or the collapse of $\Delta\Psi_m$ opens the mitochondrial permeability transition pore, which is accompanied by the release of proapoptotic molecules such as cytochrome *c* into the cytoplasm (16-17). Because

AMA acts directly on the mitochondria, AMA-induced apoptosis has been reported in many experiments, including our report on As4.1 juxtaglomerular cells and lung cancer cells (18-24).

The mitogen-activated protein kinases (MAPKs) are serine/threonine kinases, which are major components of signaling pathways in cell proliferation, differentiation, embryogenesis and cell death in respond to activation of receptor tyrosine kinase, protein tyrosine kinases, receptors of cytokines and growth factors and heterotrimeric G protein-coupled receptors (25-26). There are currently four known MAPKs: the extracellular signal regulated kinase (ERK1/2), the c-Jun *N*-terminal kinase/stress-activated protein kinase (JNK/SAPK), the p38 and the big mitogen-activated protein kinase 1 (BMK1) (25). Each MAPK pathway has relatively different upstream activators and specific substrates (27). Numerous evidence demonstrates that JNK and p38 are strongly activated by ROS or by a mild oxidative shift of the intracellular thiol/disulfide redox state, leading to apoptosis (28-31). ROS also are known to induce ERK phosphorylation and activate the ERK pathway (32). In most instances, ERK activation has a pro-survival function rather than pro-apoptotic effects (33-34). Since different ROS levels and diverse functions of MAPKs by ROS may have opposite effects even in the same type of cells, the relationship between ROS and MAPKs in view of cell survival and cell death signaling needs to be clarified in the future.

As4.1 cells have been used as a model for the juxtaglomerular (JG) cell. This cell line was isolated from kidney neoplasm in a transgenic mouse containing a renin SV40 T-antigen transgene (35). However, the relationship between ROS and MAPKs in kidney cell death, especially JG cells, has not been studied. Therefore, understanding the molecular mechanism of kidney cell death by AMA and/or MAPK inhibitors is important. We recently demonstrated that AMA treatment induced apoptosis (22) and increased ROS levels in As4.1 JG cells (19). In the present study, we investigated the effects of AMA and/or MAPK inhibitors on As4.1 cells in relation to cell growth, cell death, ROS and GSH levels.

## Materials and Methods

*Cell culture*. As4.1 cells (American Tissue Culture Collection (ATCC) No. CRL-2193) are a renin-expressing clonal cell line derived from the kidney neoplasm of a transgenic mouse (35) and maintained in a humidified incubator containing 5% $CO_2$ at 37°C. As4.1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (GIBCO BRL, Grand Island, NY, USA). Cells were routinely grown in 100-mm plastic tissue culture dishes (Nunc, Roskilde, Denmark) and harvested with a solution of trypsin-EDTA while in a logarithmic phase of growth.

*Reagent*. AMA was purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). AMA was dissolved in ethanol at 20 mM as a stock solution. JNK inhibitor (SP600125), MEK inhibitor (PD98059) and p38 inhibitor (SB203580) were purchased from Calbiochem (San Diego, CA, USA). These agents were dissolved in dimethyl sulfoxide (DMSO) solution at 10 mM as a stock solution. Cells were pretreated with MAPK inhibitors for 30 min prior to treatment with AMA. Ethanol and DMSO were used as control vehicles. All bottled stock solutions were wrapped in foil and kept at –20°C.

*Cell growth assay*. The effect of drugs on As4.1 cell growth was determined by trypan blue exclusion cell counting or measuring of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye absorbance of living cells as described elsewhere (36). In brief, $1×10^4$ cells per well were seeded in 24-well plates (Nunc, Roskilde, Denmark) for cell counting, and $5×10^3$ cells per well were seeded in 96-well microtiter plates for an MTT assay. After exposure to 50 nM AMA with or without 10 μM MEK, JNK or p38 inhibitor for 48 hours, cells in 24-well plates were collected with trypsin digestion for trypan blue exclusion cell counting and cells in 96-well plates were used for an MTT assay. Twenty μl of MTT (Sigma) solution (2 mg/ml in PBS) were added to each well of 96-well plates. The plates were incubated for 4 additional hours 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, USA).

*Cell cycle analysis*. Sub-$G_1$ DNA content and cell cycle analysis were determined by propidium iodide (PI; Sigma-Aldrich; Ex/Em=488 nm/617 nm) staining as described elsewhere (37). In brief, $1×10^6$ cells were incubated with 50 nM AMA with or without 10 μM MEK, JNK or p38 inhibitor for 48 hours. 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, San Jose, CA, USA) and analyzed using lysis II and CellFIT software (Becton Dickinson).

*Annexin V staining*. Apoptosis was determined by staining cells with annexin V-fluorescein isothiocyanate (FITC) (Ex/Em=488 nm/ 519 nm) as described elsewhere (38). In brief, $1×10^6$ cells were incubated with 50 nM AMA with or without 10 μM MEK, JNK or p38 inhibitor for 48 hours. 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 density of $1×10^6$ cells/ml. Five microliters of annexin V-FITC (PharMingen, San Diego, CA, USA) and 10 μl PI (10 μg/ml) were added to these cells, which were then analyzed with a FACStar flow cytometer (Becton Dickinson).

*Measurement of mitochondrial membrane potential ($\Delta\Psi_m$)*. $\Delta\Psi_m$ levels were measured by rhodamine 123 fluorescent dye (Ex/Em= 485 nm/535 nm), as described elsewhere (39). In brief, $1×10^6$ cells were incubated with 50 nM AMA with or without 10 μM MEK, JNK or p38 inhibitor for 48 hours. Cells 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
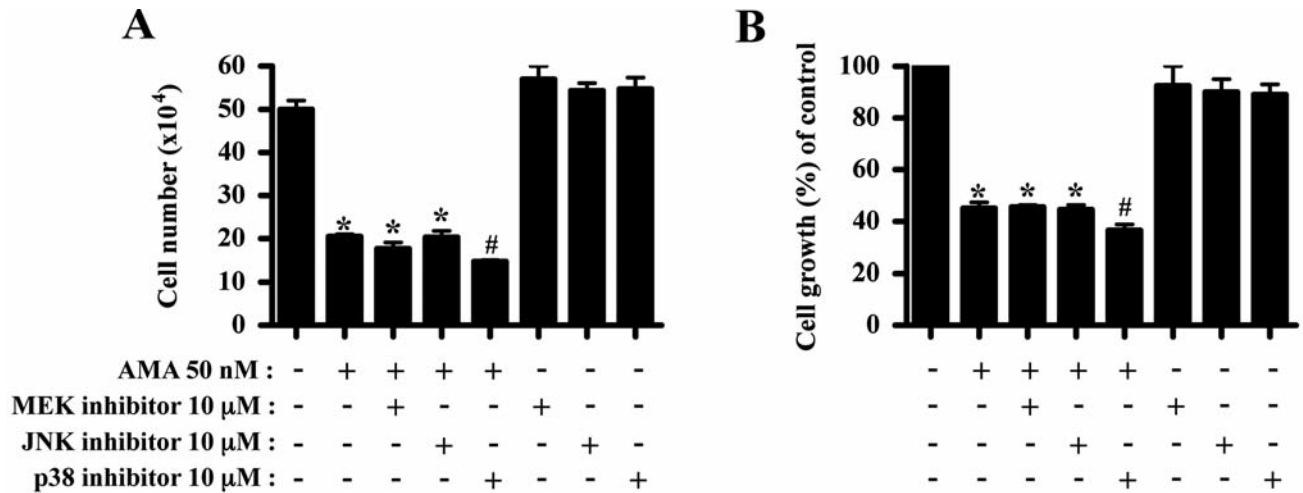
Figure 1. *Effects of MAPK inhibitors on cell growth in AMA-treated As4.1 cells. Exponentially growing cells were treated with 50 nM AMA for 48 hours following 30 min pre-incubation with 10 μM MEK, JNK or p38 inhibitor. Cell growth was assessed by an MTT assay. \*p<0.05 compared with the control group. #p<0.05 compared with cells treated with AMA only.*

determined by flow cytometry. An absence of rhodamine 123 staining indicates the loss of $\Delta\Psi_m$ in As4.1 cells. $\Delta\Psi_m$ levels (positively stained cells) in cells except $\Delta\Psi_m$ loss cells were expressed as mean fluorescence intensity (MFI), which was calculated by CellQuest software.

*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, USA) as described elsewhere (40). $H_2DCFDA$ (Ex/Em=495 nm/ 529 nm) is poorly selective for superoxide anion radical ($O_2^{\bullet-}$). In contrast, dihydroethidium (DHE) (Ex/Em=518 nm/605 nm) (Invitrogen Molecular Probes) is a fluorogenic probe that is highly selective for $O_2^{\bullet-}$ among ROS as previously described (40). In brief, $1\times10^6$ cells were incubated with 50 nM AMA with or without 10 μM MEK, JNK or p38 inhibitor for 48 hours. Cells were then washed in PBS and incubated with 20 μM $H_2DCFDA$ or 20 μM DHE at 37°C for 30 min according to the instructions of the manufacturer. DCF and DHE fluorescence was detected using a FACStar flow cytometer (Becton Dickinson). ROS and $O_2^{\bullet-}$ levels were expressed as 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 nm/595 nm) as described elsewhere (40). In brief, $1\times10^6$ cells were incubated with 50 nM AMA with or without 10 μM MEK, JNK or p38 inhibitor for 48 hours. Cells were then washed with PBS and incubated with 5 μM CMFDA at 37°C for 30 min. CMF fluorescence intensity was determined using a FACStar flow cytometer (Becton Dickinson). An absence of CMF staining indicates GSH depletion in cells. Data were expressed as the percentage of CMF⁻ cells. CMF levels (positively stained cell) in cells except GSH depleted cells were expressed as MFI, which was calculated by CellQuest software.

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

## Results

*Effects of MAPK inhibitors on cell growth in AMA-treated As4.1 cells*. We examined the effect of MAPK inhibitors on the growth of AMA-treated As4.1 cells. For this experiment, we chose 50 nM AMA as a suitable dose to differentiate the levels of cell growth inhibition and death in the presence or absence of each MAPK inhibitor (41). Treatment with 50 nM AMA inhibited cell growth by approximately 60% and 58% using trypan blue cell counting and an MTT assay at 48 hours, respectively (Figures 1A and B). We also determined the optimal dose of MEK inhibitor (PD98059), JNK inhibitor (SP600125) and p38 inhibitor (SB203580) to be 10 μM, a concentration which did not strongly affect the growth of As4.1 control cells (Figure 1). Treatment with 1 μM MAPK inhibitors did not affect the growth of AMA-treated As4.1 cells, while 20 μM MAPK inhibitors significantly reduced control cell growth (data not shown). While MEK and JNK inhibitor did not significantly affect the growth of AMA-treated As4.1 cells, p38 inhibitor intensified the growth inhibition of AMA-treated As4.1 cells (Figure 1).

*Effects of MAPK inhibitors on the cell cycle distribution in AMA-treated As4.1 cells*. We examined the effect of MAPK inhibitors on the cell cycle distribution in AMA-treated As4.1 cells. As shown in Figure 2, DNA flow
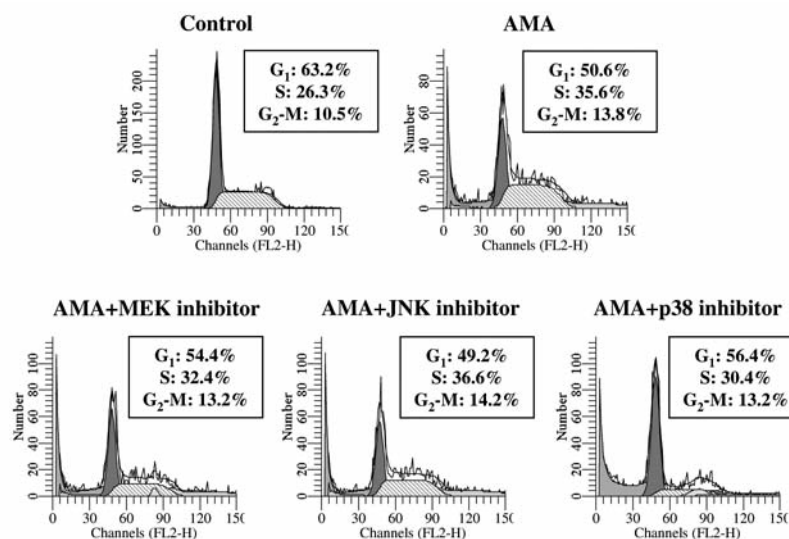
Figure 2. *Effects of MAPK inhibitors on the cell cycle distribution in AMA-treated As4.1 cells. Exponentially growing cells were treated with 50 nM AMA for 48 hours following 30 min pre-incubation of 10 μM MEK, JNK or p38 inhibitor. The changes of cell cycle phase distribution were assessed by DNA flow cytometric analysis.*

cytometric analysis indicated that treatment with AMA induced an S-phase arrest of the cell cycle at 48 hours. None of MAPK inhibitors significantly changed the cell cycle distribution in AMA-treated As4.1 cells (data now shown).

*Effects of MAPK inhibitors on apoptosis and $\Delta\Psi_m$ in AMA-treated As4.1 cells.* In the current study, we observed that AMA seemed to induce apoptosis in As4.1 cells at 48 hours, as evidenced by the properties of sub-$G_1$ cells and annexin V staining (Figures 3A and B). We determined whether MAPK inhibitors changed AMA-induced apoptosis. While MEK and JNK inhibitors did not significantly alter the number of sub-$G_1$ cells (Figures 3A and C) and annexin V-positive cells in AMA-treated As4.1 cells (Figures 3B and D), p38 inhibitor significantly increased the number of both (Figures 3A and B).

It is known that apoptosis is closely related to the collapse of $\Delta\Psi_m$ (42). Therefore, we determined the loss of $\Delta\Psi_m$ in AMA-treated As4.1 cells. As expected, a loss of $\Delta\Psi_m$ was observed in AMA-treated As4.1 cells at 48 hours (Figures 4A and B). All the MAPK inhibitors further increased the $\Delta\Psi_m$ loss in AMA-treated As4.1 cells, and that with p38 inhibitor was significant (Figures 4A and B). In relation to $\Delta\Psi_m$ levels in cells except rhodamine 123-negative cells, AMA reduced the $\Delta\Psi_m$ level in As4.1 cells (Figures 4A and C). MEK and JNK inhibitor further reduced $\Delta\Psi_m$ in AMA-treated As4.1 cells (Figures 4A and C). All the MAPK inhibitors reduced the basal $\Delta\Psi_m$ level in As4.1 control cells (Figures 4A and C).

*Effects of MAPK inhibitors on ROS levels in AMA-treated As4.1 cells.* Next, we determined whether the levels of intracellular ROS in AMA-treated As4.1 cells were changed by each inhibitor of MAPK. The level of intracellular ROS (DCF) such as $H_2O_2$ was increased in AMA-treated As4.1 cells (Figures 5A and C). Addition of the MAPK inhibitors did not significantly alter ROS levels further in AMA-treated As4.1 cells (Figures 5A and C), but did not increase the basal ROS level in As4.1 control cells (Figures 5A and C).

When we assayed the intracellular $O_2^{\bullet-}$ levels in AMA-treated As4.1 cells, the red fluorescence derived from DHE reflecting the increased the intracellular $O_2^{\bullet-}$ level (Figures 5B and D). All the MAPK inhibitors significantly reduced $O_2^{\bullet-}$ levels in AMA-treated As4.1 cells but did not significantly affect the level in As4.1 control cells (Figures 5B and D).

*Effects of MAPK inhibitors on GSH levels in AMA-treated As4.1 cells.* Cellular GSH can regulate cell growth and apoptosis (5-6). Therefore, we analyzed the changes of GSH in As4.1 cells in the presence of AMA and/or each MAPK inhibitor. Treatment with AMA increased the number of GSH depleted As4.1 cells by about 17% as compared with control cells at 48 hours (Figures 6A and B). Only p38 inhibitor significantly intensified GSH depletion in AMA-treated As4.1 cells and also induced significantly GSH depletion in As4.1 control cells (Figures 6A and B). When CMF (GSH) level in As4.1 cells except GSH-depleted cells was assessed, AMA increased GSH level in As4.1 cells (Figures 6A and C). While MEK inhibitor did not change the GSH level in AMA-treated As4.1 cells, JNK and p38 inhibitor significantly augmented
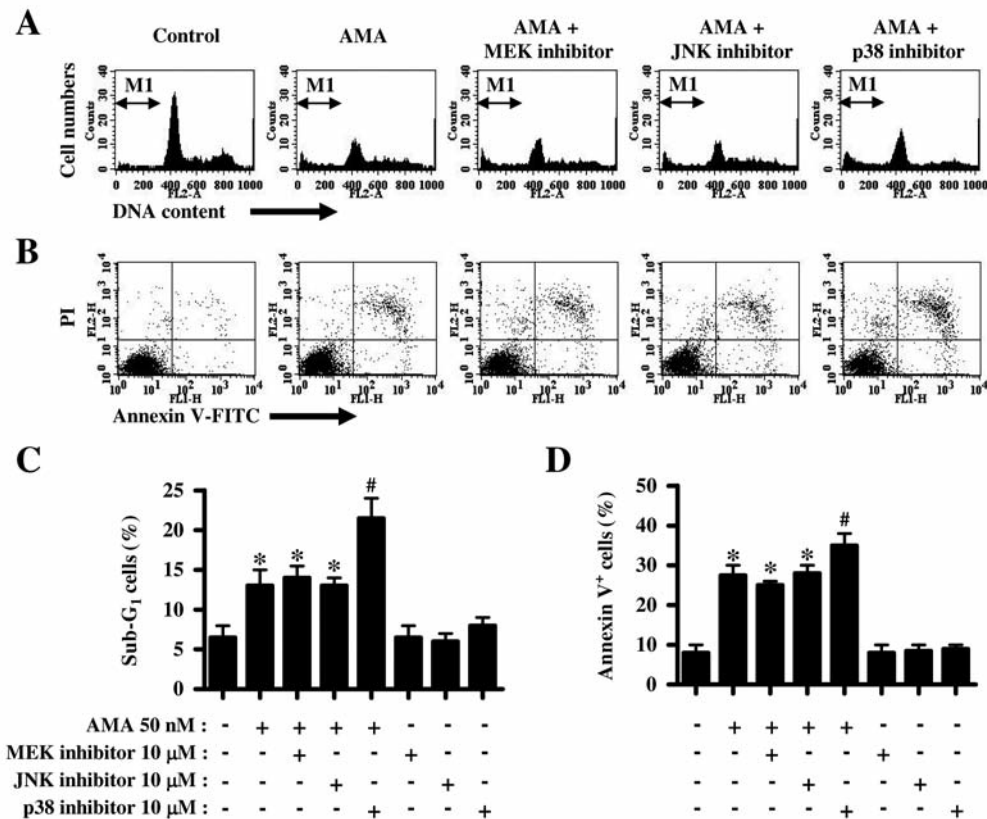
Figure 3. *Effects of MAPK inhibitors on cell death in AMA-treated As4.1 cells. Exponentially growing cells were treated with 50 nM AMA for 48 hours following 30 min pre-incubation of 10 μM MEK, JNK or p38 inhibitor. Sub-G$_1$ and annexin V-positive cells were measured with a FACStar flow cytometer. Graphs show the percentage of sub-G$_1$ cells (M1 region in A) (C) and annexin V-positive cells from B (D). \*p<0.05 compared with the control group. #p<0.05 compared with cells treated with AMA only.*

the level (Figures 6A and C). In addition, all the MAPK inhibitors significantly increased the GSH level in As4.1 control cells (Figures 6A and C).

## Discussion

AMA can disturb the natural oxidation/reduction equilibrium in cells by causing a breakdown in $\Delta\Psi_m$ (11, 13-14). It has been reported that increased intracellular $H_2O_2$ plays an important role in AMA-induced cell death in liver cells (43-44). Our recent and the present results demonstrate that AMA inhibited As4.1 cell growth and induced apoptosis, which was accompanied by the loss of $\Delta\Psi_m$ (22) and increased ROS (DCF) (19). These data suggest that the apoptotic effects of AMA are comparable to those induced by intracellular ROS levels, especially $H_2O_2$. As we already demonstrated that the increase of ROS in AMA-treated As4.1 juxtaglomerular cells is not closely related to apoptosis (19), our current result showed that p38 inhibitor enhanced the death of AMA-treated As4.1 cells without increasing the ROS (DCF) level.

ERK activation has a pro-survival function rather than pro-apoptotic effects (33-34). However, MEK inhibitor did not change cell growth inhibition and cell death in AMA-treated As4.1 cells. In addition, this inhibitor did not alter ROS (DCF) levels. These results suggest that the signaling of MEK in AMA-treated As4.1 cells is not related to cell death and ROS levels, or is not tightly regulated by ROS levels. The activation of JNK and/or p38 generally leads to apoptosis (28-31). According to our current data, JNK inhibitor which presumably inhibited JNK signaling neither affected cell growth inhibition and cell death in AMA-treated As4.1 cells nor changed ROS (DCF) levels. However, p38 inhibitor did intensify cell growth inhibition and cell death in AMA-treated As4.1 cells without changing the ROS (DCF) level. Therefore, our data implied that p38 signaling rather than JNK signaling is more closely related to AMA-induced cell death of As4.1 cells. In addition, none of the MAPK inhibitors affected cell growth or death in As4.1 control cells, whereas they significantly increased ROS (DCF) levels. Collectively, our data suggest that the changes of ROS (DCF)
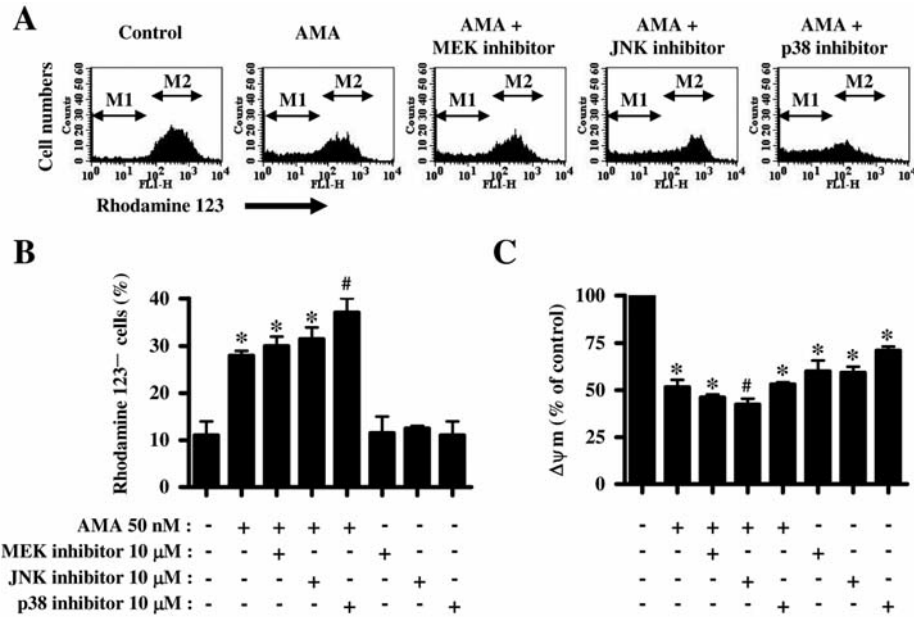
Figure 4. *Effects of MAPK inhibitors on $\Delta\Psi_m$ in AMA-treated As4.1 cells. Exponentially growing cells were treated with 50 nM AMA for 48 hours following 30 min pre-incubation of 10 μM MEK, JNK or p38 inhibitor. $\Delta\Psi_m$ in As4.1 cells was measured with a FACStar flow cytometer. Graphs show the percentage cells which were rhodamine 123 negative ($\Delta\Psi_m$ loss) (M1 region in A) (B) and $\Delta\Psi_m$ levels (%) compared with As4.1 control cells (M2 region in A) (C). \*p<0.05 compared with the control group. #p<0.05 compared with cells treated with AMA only.*
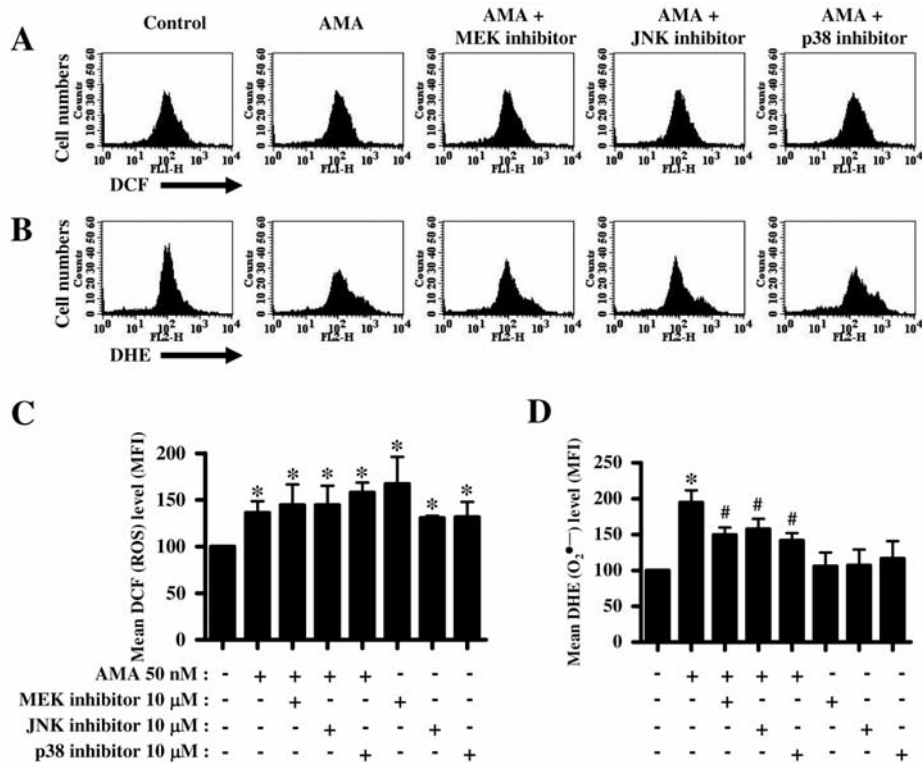


Figure 5. *Effects of MAPK inhibitors on ROS levels in AMA-treated As4.1 cells. Exponentially growing cells were treated with 50 nM AMA for 48 hours following 30 min pre-incubation of 10 μM MEK, JNK or p38 inhibitor. ROS levels in As4.1 cells were measured using a FACStar flow cytometer. A and B indicate histograms for DCF and DHE intensity in As4.1 cells, respectively. Graphs indicate DCF (ROS) levels (%) compared with control cells (C) and DHE ($O_2^{\bullet-}$) levels (%) compared with control cells (D). \*p<0.05 compared with the control group. #p<0.05 compared with cells treated with AMA only.*
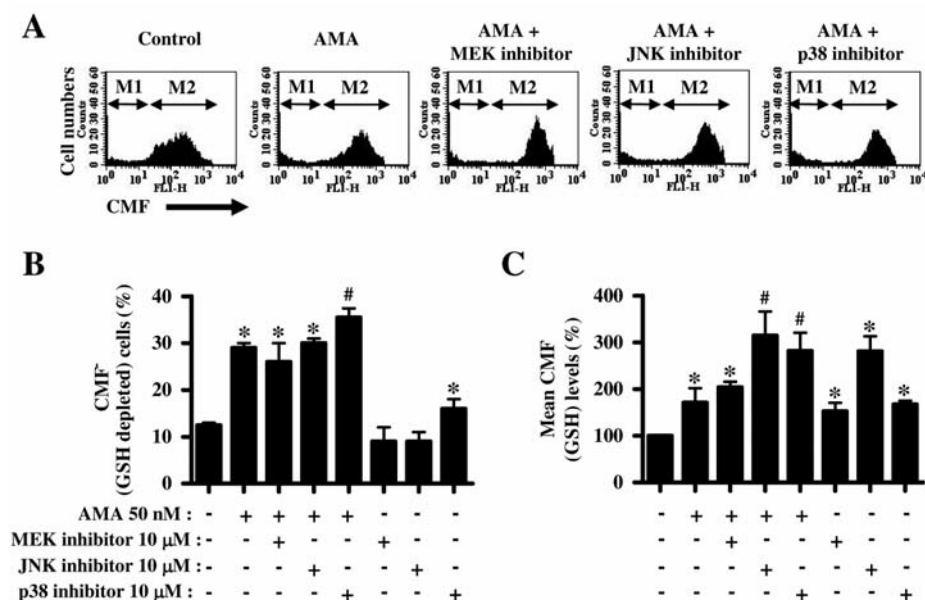
Figure 6. *Effects of MAPK inhibitors on GSH levels in AMA-treated As4.1 cells. Exponentially-growing cells were treated with 50 nM AMA for 48 hours following 30 min pre-incubation of 10 μM MEK, JNK or p38 inhibitor. GSH levels in As4.1 cells were measured using a FACStar flow cytometer. Graphs show the percent of (–) CMF (GSH depleted) cells (M1 region in A) (B) and mean CMF (GSH) levels compared with control cells (M2 region in A) (C). \*p<0.05 compared with the control group. #p<0.05 compared with cells treated with AMA only.*

levels by AMA and/or MAPK inhibitors are not tightly correlated with cell death in As4.1 cells. In relation to the cell cycle distributions in As4.1 cells treated with AMA and/or each MAPK inhibitor, AMA induced an S-phase arrest of the cell cycle. None of the MAPK inhibitors strongly changed the cell cycle distribution in AMA-treated As4.1 cells, which implies that the signaling of MAPKs is not tightly related to the regulation of the cell cycle in these cells.

AMA inhibits succinate oxidase and NADH oxidase and also inhibits mitochondrial electron transport between cytochrome *b* and *c* (11-13), which may result in production of $O_2^{\bullet-}$ at the mitochondria. As increases in $O_2^{\bullet-}$ levels following exposure to AMA were reported in human lung epithelial cells (45), our recent and current data demonstrate that the $O_2^{\bullet-}$ level was significantly increased in AMA-treated As4.1 cells (19). It is reported that ROS formation may cause mitochondrial dysfunction and subsequent cytochrome *c* release, which leads to cell viability loss (46-47). The collapse of $\Delta\Psi_m$ occurs during apoptosis (42). Correspondingly, AMA induced the loss of $\Delta\Psi_m$ and reduced $\Delta\Psi_m$ levels in As4.1 cells. All the MAPK inhibitors increased $\Delta\Psi_m$ loss in AMA-treated As4.1 cells but only that brought about by p38 inhibitor was significant. These results suggest that the loss of $\Delta\Psi_m$ following treatment with AMA and/or MAPK inhibitors leads to apoptosis in As4.1 cells and the signaling of p38 increases $\Delta\Psi_m$ loss in AMA-treated As4.1 cells. Interestingly, all the MAPK inhibitors reduced the $O_2^{\bullet-}$ level in AMA-treated As4.1 cells.

This result implies that the changes of $O_2^{\bullet-}$ level in As4.1 cells treated with AMA and MAPK inhibitors did not simply result from mitochondrial damage and were not correlated to cell death. The activities of MAPKs in As4.1 cells seem to be involved in the maintenance of MMP ($\Delta\Psi_m$).

In relation to GSH content, AMA increased the number of GSH-depleted As4.1 cells. Only p38 inhibitor intensified GSH depletion in AMA-treated As4.1 cells. Therefore, our previous (19) and current results support the notion that the intracellular GSH content has a decisive effect on anticancer drug-induced apoptosis (48-49) but it is not sufficient to predict cell death accurately, since p38 inhibitor significantly induced GSH depletion without significant cell death in As4.1 control cells. It is of note that the CMF (GSH) level in AMA-treated As4.1 cells increased. It is likely that the increased GSH level was able to counter the increasing ROS level induced by AMA treatment. Thus, As4.1 cells beyond their normal capacity to resist ROS stress would be dead. However, JNK and p38 inhibitors, while showing no effect on ROS levels increased the GSH level in AMA-treated As4.1 cells. In addition, all the MAPK inhibitors increased the GSH level in As4.1 control cells. Therefore, the ROS level cannot be explained wholly by the GSH level changes alone. Increased ROS by certain drugs are not all considered as causing sufficient oxidative stress to trigger cell death. The signaling of each MAPK seems to regulate GSH levels differently depending on other agents used and the intracellular ROS level.

Conclusively, p38 inhibitor intensified cell death in AMA-treated As4.1 cells. The changes of GSH content rather than the ROS level by AMA and/or MAPK inhibitors were more closely related to the growth and death of As4.1 cells.

## Acknowledgements

## Conflict of Interest Statement

None declared.

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