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Treatment with a JNK inhibitor increases, whereas treatment with a p38 inhibitor decreases, H_2O_2-induced calf pulmonary arterial endothelial cell death

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Abstract. Oxidative stress induces apoptosis in endothelial cells (ECs). Reactive oxygen species (ROS) promote cell death by regulating the activity of various mitogen-activated protein kinases (MAPKs) in ECs. The present study investigated the effects of MAPK inhibitors on cell survival and glutathione (GSH) levels upon H_2O_2 treatment in calf pulmonary artery ECs (CPAECs). H_2O_2 treatment inhibited the growth and induced the death of CPAECs, as well as causing GSH depletion and the loss of mitochondrial membrane potential (MMP). While treatment with the MEK or JNK inhibitor impaired the growth of H_2O_2-treated CPAECs, treatment with the p38 inhibitor attenuated this inhibition of growth. Additionally, JNK inhibitor treatment increased the proportion of sub-G_1 phase cells in H_2O_2-treated CPAECs and further decreased the MMP. However, treatment with a p38 inhibitor reversed the effects of H_2O_2 treatment on cell growth and the MMP. Similarly, JNK inhibitor treatment further increased, whereas p38 inhibitor treatment decreased, the proportion of GSH-depleted cells in H_2O_2-treated CPAECs. Each of the MAPK inhibitors affected cell survival, and ROS or GSH levels differently in H_2O_2-untreated, control CPAECs. The data suggest that the exposure of CPAECs to H_2O_2 caused the cell growth inhibition and cell death through GSH depletion. Furthermore, JNK inhibitor treatment further enhanced, whereas p38 inhibitors attenuated, these effects. Thus, the results of the present study suggest a specific protective role for the p38 inhibitor, and not the JNK inhibitor, against H_2O_2-induced cell growth inhibition and cell death.

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Abbreviations: ECs, endothelial cells; CPAECs, calf pulmonary arterial endothelial cells; ROS, reactive oxygen species; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase 1; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MMP, mitochondrial membrane potential; FITC, fluorescein isothiocyanate; DCF, 2',7'-dichlorodihydrofluorescein diacetate; DHE, dihydroethidium; GSH, glutathione; CMF, 5-chloromethylfluorescein diacetate

Key words: H_2O_2, cell death, calf pulmonary arterial endothelial cells, MAPK inhibitors, reactive oxygen species, glutathione

Introduction

Vascular cells, particularly endothelial cells (ECs), generate reactive oxygen species (ROS) including superoxide anions ($O_2^{\cdot-}$), hydroxyl radicals ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2). ROS are considered harmful to the vasculature and may initiate pathological processes that contribute to atherosclerosis, restenosis, hypertension and diabetic vascular complications (1,2). However, there is also an apparent role for ROS in the maintenance of vascular homeostasis via the regulation of cellular events that govern cell death, differentiation and proliferation (1,3,4). Due to its solubility in lipid and aqueous environments, H_2O_2 can freely diffuse through the cell membrane to reach remote cells prior to reacting with particular molecular targets (5).

The modulation of ROS levels by oxygen concentrations in lung tissue is important for control of the pulmonary vascular system (6). Vascular ECs are implicated in the control of blood pressure, blood coagulation, inflammation and angiogenesis (7). H_2O_2 influences the function of ECs via intricate mechanisms; for example, the ambient production of $O_2^{\cdot-}$ in vasculature and the subsequent low level generation of H_2O_2 affects EC growth and proliferation (2), and enhanced oxidative stress owing to the high level of H_2O_2 may lead to the apoptotic death of ECs, causing endothelial dysfunction in vascular system (1).

Mitogen-activated protein kinases (MAPKs) are evolutionarily preserved signaling proteins in eukaryotes that arbitrate responses to various stimuli (8). Extracellular signal regulated kinases (ERK1/2), the c-Jun N-terminal kinase/stress-activated protein kinases (JNK/SAPK) and the p38 kinases are the three major MAPK groups identified in mammals (9). The activation of multiple MAPKs is the primary constituent of the various signaling pathways that control cell proliferation,

survival, differentiation and cell death (10). MAPKs in ECs and smooth muscle cells are activated by a variety of growth factors, including platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and angiotensin II (Ang II) (11-13). MAPKs can discern the cellular redox status and they are, in turn, targets for ROS; for example, JNK and p38 are generally activated by mild oxidative stress, and their activation then leads to apoptosis (14,15). However, these two kinases differentially affect apoptosis in pyrogallol-treated ECs; JNK promotes survival in these cells, whereas p38 is associated with cell death (16). In addition, ROS can stimulate the ERK pathway via ERK phosphorylation (17). ERK activation typically produces a pro-survival effect rather than a pro-apoptotic effect (18). Furthermore, the activity of MAPKs is sustained by the activity of MAPK phosphatases, which are directly regulated by H_2O_2 (19).

H_2O_2 inhibits the phosphorylation of ERK1/2 in human umbilical vein ECs (HUVEC) (20), whereas other studies have demonstrated that H_2O_2 enhances the phosphorylation of ERK1/2 in HUVEC (21) and bovine aortic ECs (BAEC), which is associated with their apoptosis (22). Treatment with H_2O_2 promotes p38 phosphorylation in HUVEC (20,21) and BAEC (23). JNKs and their downstream target, c-Jun, have been demonstrated to be involved in the apoptosis of ECs induced by H_2O_2 and other stresses (12,21,24). Thus, the effects of H_2O_2 on the activities of MAPKs, particularly mitogen-activated protein kinase kinase 1 (MEK)-ERK signaling, may differ depending on EC types and experimental conditions, resulting in diverse cellular responses. The action of H_2O_2 in aggravating endothelial dysfunction and cell death has been extensively investigated (25,26). However, the mechanisms underlying the varied outcomes with respect to MAPKs remain obscure.

Using MAPK-specific inhibitors (including the SP600125 JNK inhibitor, the PD98059 MEK inhibitor and the SB203580 p38 inhibitor), the present study addressed the function of various MAPKs in H_2O_2-induced cell death and the attenuation of cell growth. H_2O_2 exposure to well-established calf pulmonary arterial ECs (CPAECs), as performed in our previous studies (27,28), was used to analyze the effect of MAPK inhibitors on cell growth, death, mitochondrial membrane potential (MMP) and glutathione (GSH) levels.

Materials and methods

Cell culture. CPAECs were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea) and were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and 1% penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). CPAECs were harvested using a solution of trypsin-EDTA (Gibco; Thermo Fisher Scientific, Inc.) during the exponential phase of growth. CPAECs were maintained in 100-mm plastic tissue culture dishes (Nalge Nunc International, Penfield, NY, USA) in humidified incubator containing 5% CO_2, at 37˚C.

Reagents. H_2O_2 was purchased from Sigma-Aldrich (Merck KGaA). The JNK inhibitor (SP600125), MEK inhibitor (PD98059) and p38 inhibitor (SB203580) were obtained from Calbiochem (Merck KGaA). All reagents were dissolved in dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA) to 10 mM. Cells were pretreated with each MAPK inhibitor for 30 min prior to treatment with H_2O_2 in the conditions previously described. A dose of 10 μM of each MAPK inhibitor was applied in all experiments.

Cell growth assay. The effect of drugs on the growth of CPAECs was determined by evaluating the MTT (Sigma-Aldrich; Merck KGaA) dye absorbance, according to a previously described method (29). Cells were exposed to 30 μM H_2O_2 with or without 10 μM JNK inhibitor, MEK inhibitor or p38 inhibitor for 24 h in the conditions previously described.

Cell cycle analysis. Sub-G_1 cells were assessed using propidium iodide (Sigma-Aldrich; Merck KGaA) staining, as per a previously described method (30). Cells were exposed to 30 μM H_2O_2 in the presence or absence of 10 μM JNK inhibitor, MEK inhibitor or p38 inhibitor for 24 h in the conditions previously described. Cell DNA content was assessed using a BD FACStar™ flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and CellQuest Pro software (version 5.1; BD Biosciences).

Annexin V staining for the detection of apoptosis. Apoptotic cell death was verified by measuring cells stained with Annexin V-fluorescein isothiocyanate (FITC; Invitrogen; Thermo Fisher Scientific, Inc.), as per a previously described method (31). Cells were exposed to 30 μM H_2O_2 with or without 10 μM JNK inhibitor, MEK inhibitor or p38 inhibitor for 24 h in the conditions previously described. Annexin V staining was analyzed with a BD FACStar flow cytometer, as aforementioned.

Measurement of MMP. MMP was measured using a rhodamine 123 fluorescent dye (Sigma-Aldrich; Merck KGaA), as previously described (32). Cells were exposed to 30 μM H_2O_2 in the presence or absence of 10 μM JNK inhibitor, MEK inhibitor or p38 inhibitor for 24 h in the conditions previously described. Rhodamine 123 staining intensity was assessed by a BD FACStar flow cytometer as aforementioned. The absence of rhodamine 123 in cells designated the loss of MMP in CPAECs. MMP levels in the cells were expressed as mean fluorescence intensity (MFI), which was calculated using CellQuest™ Pro software, as aforementioned.

Measurement of intracellular ROS levels. Intracellular ROS levels were measured with 2',7'-dichlorodihydrofluorescein diacetate (DCF; Invitrogen; Thermo Fisher Scientific, Inc.), and $O_2^{\cdot-}$ levels were evaluated using dihydroethidium (DHE, Invitrogen; Thermo Fisher Scientific, Inc.) fluorescent dyes. Cells were exposed to 30 μM H_2O_2 with or without 10 μM JNK inhibitor, MEK inhibitor or p38 inhibitor for 24 h in the previously described conditions. Cells were then incubated with 20 μM H_2DCFDA or DHE at 37˚C for a further 30 min. DCF and DHE fluorescence levels were measured using the BD FACStar flow cytometer. ROS and $O_2^{\cdot-}$ levels were stated as MFI.

Detection of the intracellular glutathione (GSH). The GSH level was analyzed with a 5-chloromethylfluorescein diacetate

dye (CMF; Invitrogen; Thermo Fisher Scientific, Inc.). Cells were incubated with 30 μM H_2O_2 in the presence or absence of 10 μM JNK inhibitor, MEK inhibitor or p38 inhibitor for 24 h in the previously described conditions. Cells were then incubated with 5 μM CMF at 37˚C for a further 30 min. CMF fluorescence intensity was measured using the BD FACStar flow cytometer as previously described. GSH depletion was indicated with negative CMF staining. CMF levels in cells were expressed as MFI.

Statistical analysis. The data are presented as the mean ± standard deviation of ≥2 independent experiments. The data were analyzed using GraphPad Prism software version 5 (GraphPad Software, Inc., La Jolla, CA, USA). The Student's t-test and one-way analysis of variance followed by Tukey's multiple comparison test were utilized for parametric data. P<0.05 was considered to indicate a statistically significance difference.

Results

MAPK inhibitors affect cell growth and death in H_2O_2-treated CPAECs. The effects of MAPK inhibitors (including a JNK inhibitor, MEK inhibitor and p38 inhibitor) on the growth of H_2O_2-treated CPAECs were examined using MTT assays. The inhibitors were selected based on those used in our prior studies (33-36). According to another previous study (25), the IC_{50} of H_2O_2 in CPAECs is ~20 μM at 24 h. Therefore, 30 μM of H_2O_2 was selected for use in the present study.

H_2O_2 treatment caused ~70% growth inhibition of CPAECs within 24 h (Fig. 1; P<0.05, compared with no treatment). The addition of the MEK or JNK inhibitors further stalled cell growth (P<0.05, compared with the H_2O_2-only group). The JNK inhibitor was the most potent in augmenting the negative effect of H_2O_2 on cell growth, although it was not statistically different from the MEK inhibitor (P=0.168; Fig. 1). On the other hand, treatment with the p38 inhibitor partially attenuated the effect of H_2O_2 on CPAEC growth (P<0.05; Fig. 1). All inhibitors also diminished the growth of the CPAECs when administered without H_2O_2 (P<0.05; Fig. 1).

The percentages of the sub-G_1 cells in CPAECs were measured in a similar manner to a number of previous studies (37-39). Treatment with H_2O_2 alone increased the percentage of the sub-G_1 cells by ~20% compared with the H_2O_2-untreated CPAEC control group (Fig. 2A and B). MEK inhibitor treatment exhibited a trend towards increasing the number of sub-G_1 cells in H_2O_2-treated CPAECs (Fig. 2A and B). Treatment with the JNK inhibitor significantly increased, whereas the p38 inhibitor significantly decreased the number of sub-G_1 cells in H_2O_2-treated CPAECs (both P<0.05; Fig. 2A and B). In addition, H_2O_2 treatment also increased the percentage of Annexin V-FITC stained CPAECs, indicating that the death of CPAECs subsequent to H_2O_2 treatment may occur via apoptosis (P<0.05; Fig. 2C). None of the MAPK inhibitors significantly affected the Annexin V-FITC positive cell number in H_2O_2-treated CPAECs (Fig. 2C); however, treatment with the JNK inhibitor alone increased the number of Annexin V-FITC positive cells in CPAECs in the absence of H_2O_2 (P<0.05; Fig. 2C).

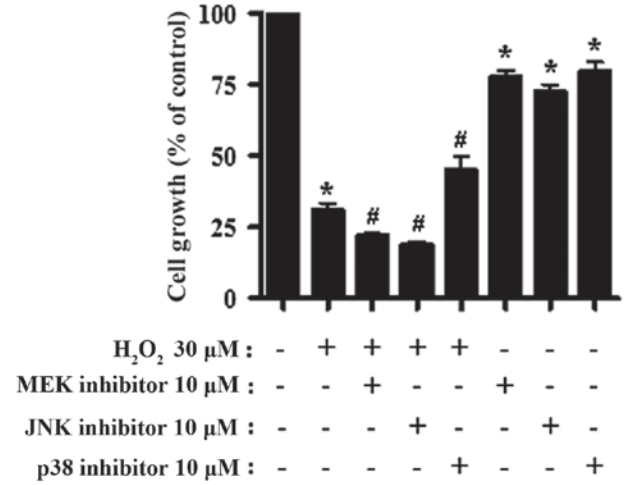

Figure 1. Effect of MAPK inhibitors on cell growth in H_2O_2-treated CPAECs. CPAECs in the exponential growth phase were treated with H_2O_2 for 24 h. Prior to H_2O_2 treatment, cells were pre-incubated with each MAPK inhibitor for 30 min. The graph depicts the change in CPAEC viability, as assessed using an MTT assay. *P<0.05, compared with the control (no treatment) group. #P<0.05, compared with cells treated with H_2O_2 only. MAPK, mitogen-activated protein kinase; CPAEC, calf pulmonary arterial endothelial cell; MEK, mitogen-activated protein kinase kinase 1; JNK, c-Jun N-terminal kinase.

MAPK inhibitors influence MMP in H_2O_2-treated CPAECs. Cell death is closely associated with the loss of MMP (40). Thus, MMP in H_2O_2-treated CPAECs was determined using a rhodamine 123 dye at 24 h of treatment. There was a significant loss of MMP in H_2O_2-treated cells (P<0.05; Fig. 3A and B). Treatment with the MEK inhibitor did not influence the MMP level in H_2O_2-treated CPAECs (Fig. 3A and B). JNK inhibitor boosted, whereas p38 inhibitor decreased, the loss of MMP in H_2O_2-treated CPAECs (both P<0.05; Fig. 3A and B). Treatment with JNK inhibitor alone triggered a significant loss of MMP in the control CPAECs (Fig. 3A and B). When disregarding rhodamine 123-negative cells, treatment with H_2O_2 non-significantly increased the MMP level in CPAECs (Fig. 3A and C). Treatment with the MEK or JNK inhibitor reduced the MMP level in H_2O_2-treated CPAECs (P<0.05; Fig. 3A and C), whereas treatment with the p38 inhibitor did not alter the level (Fig. 3A and C). Whilst treatment with the MEK or JNK inhibitor reduced the MMP level in H_2O_2-untreated control CPAECs, treatment with the p38 inhibitor augmented the level (P<0.05; Fig. 3A and C).

MAPK inhibitors alter ROS, including $O_2^{\cdot-}$, levels in H_2O_2-treated CPAECs. Alterations to ROS levels were assessed in H_2O_2- and MAPK inhibitor-treated CPAECs. As presented in Fig. 4A, the ROS levels (including H_2O_2) significantly decreased in CPAECs treated with H_2O_2 at 24 h (P<0.05). None of the MAPK inhibitors significantly altered ROS levels in the H_2O_2-treated CPAECs (Fig. 4A). By contrast, all MAPK inhibitors, particularly the p38 inhibitor, increased the ROS levels in the control CPAECs (P<0.05; Fig. 4A). When $O_2^{\cdot-}$ levels in H_2O_2-treated CPAECs were measured, the DHE MFI, reflecting intracellular $O_2^{\cdot-}$, decreased (P<0.05; Fig. 4B). None of the MAPK inhibitors significantly altered the DHE MFI level of H_2O_2-treated CPAECs (Fig. 4B). MEK and JNK

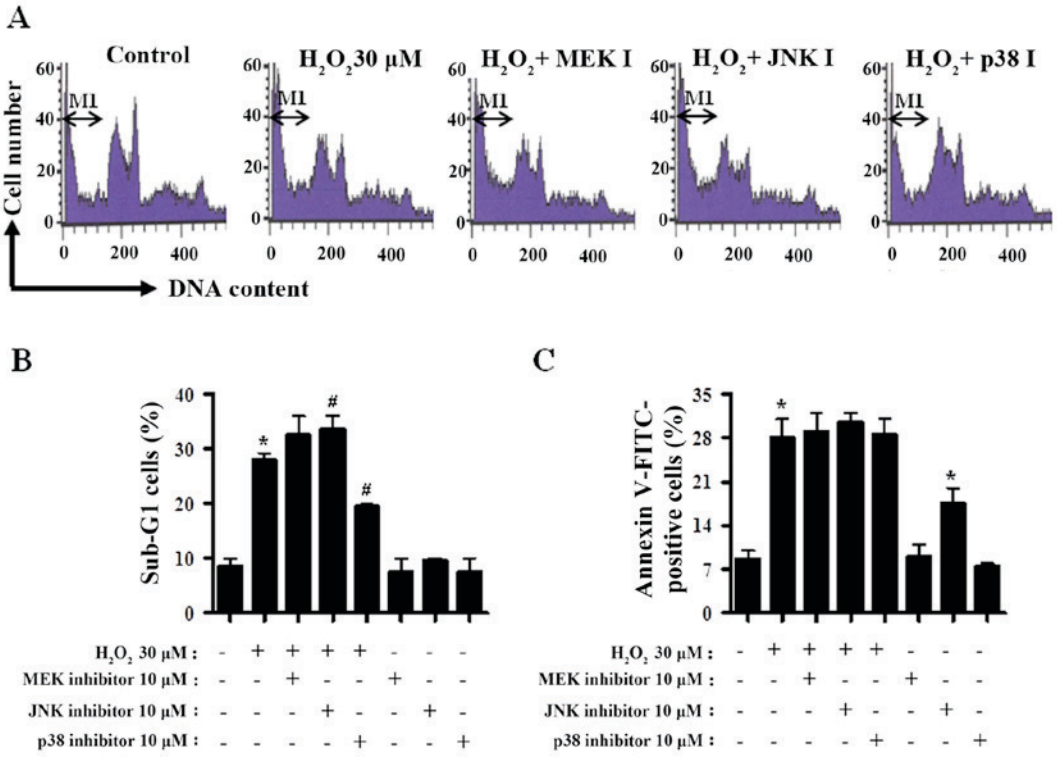

Figure 2. Analysis of apoptosis in MAPK inhibitor- and H_2O_2-treated CPAECs. Following a 30-min pre-incubation with each MAPK inhibitor, CPAECs in the exponential growth phase were treated with H_2O_2 for 24 h, and subsequently analyzed for the sub-G_1 and apoptotic population using flow cytometry. (A) Representative of DNA content histograms of cells. M1 regions indicate the portion of sub-G_1 cells. (B) Percentage of sub-G_1 cells (M1 regions in A). (C) Percentage of Annexin V-FITC positive cells, indicative of apoptosis. *P<0.05, compared with the control (no treatment) group. $^\#$P<0.05, compared with cells treated with H_2O_2 only. MAPK, mitogen-activated protein kinase; CPAEC, calf pulmonary arterial endothelial cell; MEK, mitogen-activated protein kinase kinase 1; JNK, c-Jun N-terminal kinase; FITC, fluorescein isothiocyanate.

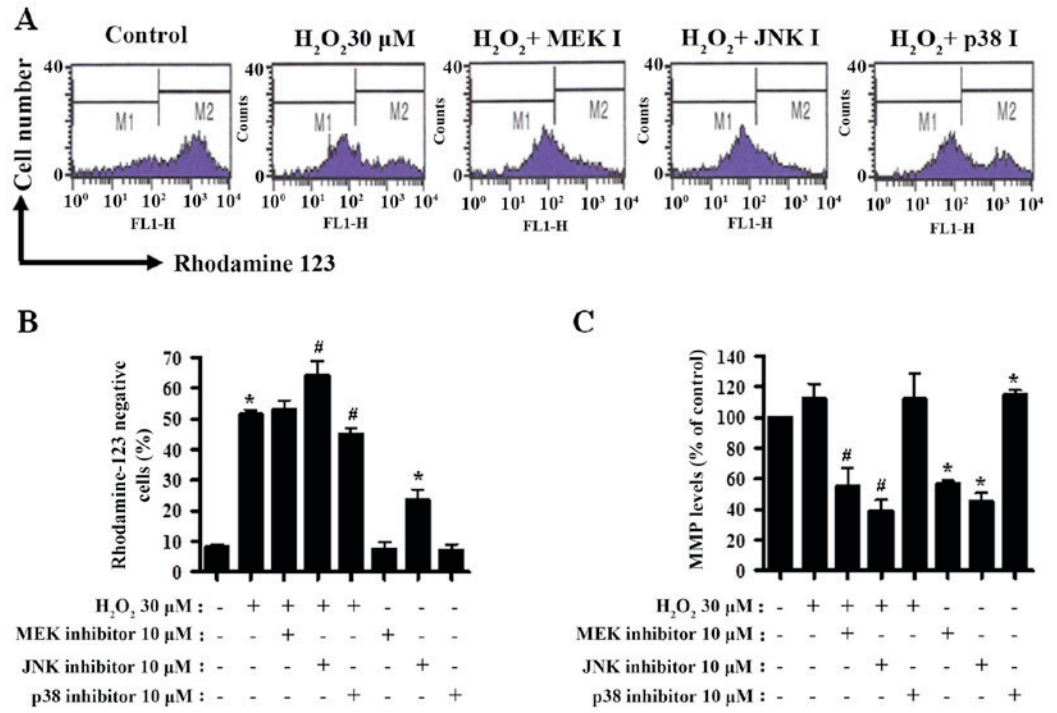

Figure 3. Assessment of MMP in H_2O_2-treated CPAECs in the presence and absence of MAPK inhibitors. Following a 30-min pre-incubation with each MAPK inhibitor, CPAECs in the exponential growth phase were treated with H_2O_2 for 24 h. MMP in CPAECs was measured using rhodamine 123 intensity with flow cytometry. (A) Representative rhodamine 123 stained cell histograms. M1 regions contain rhodamine 123-negative cells, i.e., cells where MMP is reduced; M2 regions contain rhodamine 123-positive cells. (B) Percentage of rhodamine 123-negative cells (M1 regions in A). (C) Percentage of rhodamine 123-positive cells (M2 regions in A). *P<0.05, compared with the control (no treatment) group. $^\#$P<0.05, compared with cells treated with H_2O_2 only. MMP, mitochondrial membrane potential; CPAEC, calf pulmonary arterial endothelial cell; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase 1; JNK, c-Jun N-terminal kinase.

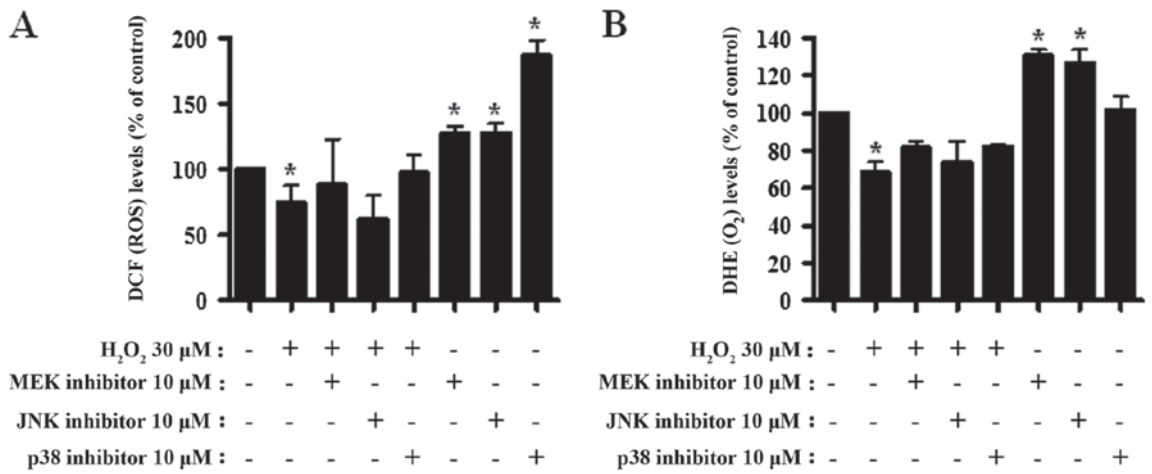

Figure 4. Assessment of ROS levels following treatment with MAPK inhibitors and H_2O_2 in CPAECs. CPAECs in the exponential growth phase were treated with H_2O_2 for 24 h following a 30-min MAPK inhibitor pre-treatment. ROS levels in CPAECs were assessed using DCF and DHE dyes in flow cytometry. Graphs indicate the percentage of (A) DCF (ROS) and (B) DHE ($O_2^{\cdot-}$) levels compared with the control (no treatment) CPAECs. *P<0.05, compared with the control (no treatment) group. $^\#$P<0.05, compared with cells treated with H_2O_2 only. ROS, reactive oxygen species; MAPK, mitogen-activated protein kinase; CPAEC, calf pulmonary arterial endothelial cell; DCF, 2',7'-dichlorodihydrofluorescein diacetate; DHE, dihydroethidium; MEK, mitogen-activated protein kinase kinase 1; JNK, c-Jun N-terminal kinase.

inhibitors enhanced $O_2^{\cdot-}$ levels in the control CPAECs (P<0.05; Fig. 4B).

MAPK inhibitors change GSH levels in H_2O_2-treated CPAECs. GSH levels in CPAECs were analyzed by CMF fluorescence. In Fig. 5A, the M1 regions indicate CMF-positive cells, whereas the M2 regions indicate CMF-negative (GSH-depleted) cells. H_2O_2 treatment resulted in an ~40% increase in the number of GSH-depleted CPAECs (M2 region), compared with in non-treated control cells (P<0.05; Fig. 5A and B). MEK and JNK inhibitors appeared to increase the number of GSH-depleted cells in H_2O_2-treated CPAECs; this increase was significant for JNK inhibitor treatment (P<0.05; Fig. 5A and B). Unlike with the MEK and JNK inhibitors, treatment with the p38 inhibitor decreased the number of GSH-depleted cells in H_2O_2-treated CPAECs (P<0.05; Fig. 5A and B). JNK inhibitor treatment alone increased the number of GSH-depleted cells in H_2O_2-untreated CPAECs (Fig. 5A and B). Furthermore, when the GSH levels in CPAECs, without considering CMF-negative cells, were measured, the GSH level increased in H_2O_2-treated CPAECs (P<0.05; Fig. 5A and C). While treatment with the MEK inhibitor did not alter the level of GSH in H_2O_2-treated CPAECs, treatment with the JNK or p38 inhibitors increased the levels in these cells (P<0.05; Fig. 5A and C). All MAPK inhibitors promoted an increase in GSH levels in the control CPAECs; the effect was more pronounced following treatment with JNK or p38 (Fig. 5A and C).

Discussion

A variety of MAPKs occur in the vasculature, activated by diverse growth factors, including Ang II, PDGF and VEGF (11-13). ROS regulate MAPKs in ECs (12,20-24). Since H_2O_2 inhibits the growth of CPAECs and induces their death, the present study focused on evaluating the effects of MAPK inhibitors on cell growth and death, and GSH in H_2O_2-treated CPAECs. ERK activation typically has a pro-survival role rather than a pro-apoptotic role (18). Treatment with the MEK inhibitor enhanced growth inhibition in H_2O_2-treated CPAECs and slightly increased the proportion of the sub-G_1 cell population. Thus, H_2O_2 treatment may have inactivated ERK proteins in CPAECs, resulting in growth inhibition and cell death.

The activity of JNK and p38 can be stimulated by ROS or an oxidative alteration to the intracellular thiol/disulfide redox state, leading to apoptosis (14,15). H_2O_2 promotes p38 phosphorylation in HUVEC (20,21) and BAEC (23). JNKs and their downstream target, c-Jun, have been demonstrated to be involved the apoptosis of ECs triggered by H_2O_2 and other stresses (12,21,24). According to data from the present study, treatment with the JNK inhibitor augmented growth inhibition and death in H_2O_2-treated CPAECs, whereas treatment with the p38 inhibitor decreased the relative extent of growth inhibition and death in these cells. Therefore, JNK has pro-growth and survival effects, and p38 has anti-growth and pro-death effects in H_2O_2-treated CPAECs. In addition, our previous study demonstrated that JNK inhibitor treatment increased the rate of apoptosis in pyrogallol-treated CPAECs, whereas p38 inhibitor treatment decreased the level of apoptosis (16). This suggests that the JNK and p38 signal transduction pathways differentially affect the growth and death of CPAECs treated with H_2O_2 or pyrogallol. However, Machino *et al* (41) previously identified that H_2O_2 promoted the phosphorylation of JNK and p38 in human pulmonary vascular ECs. Thus, the effect of H_2O_2 on JNK activity appears to be EC-type specific; for example, it may differ in artery vs. vein, large vessels vs. small vessels, coronary vs. pulmonary, human vs. other species. Additionally, all the MAPK inhibitors used in the present study reduced the growth of the control CPAECs, indicating that individual MAPK signaling pathways may differentially affect the growth of CPAECs in the presence or absence of H_2O_2.

Treatment with 30 μM H_2O_2 increased the proportion of Annexin V-FITC positive cells in CPAECs. Our prior study demonstrated that treatment with the pan-caspase inhibitor

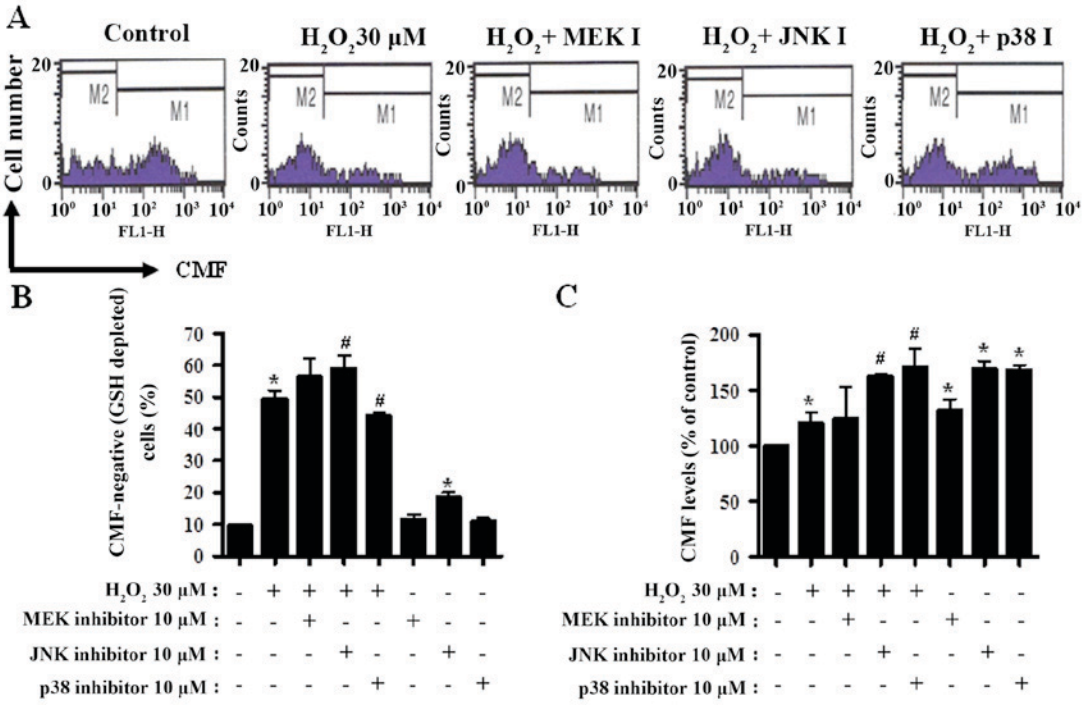

Figure 5. Analysis of GSH levels in CPAECs treated with H_2O_2 and MAPK inhibitors. CPAECs in the exponential growth phase were treated with H_2O_2 for 24 h following a 30-min pre-incubation with MAPK inhibitors. GSH levels in CPAECs were then assessed based on the CMF intensity values obtained from flow cytometry. (A) Representative histograms of CMF cell staining. M1 regions contain CMF-positive cells; M2 regions contain CMF-negative cells (GSH-depleted). (B) Percentage of CMF-negative (GSH-depleted) cells from M2 regions in A. (C) Comparison between mean relative GSH levels from the M1 regions in A. *P<0.05, compared with the control (no treatment) group. #P<0.05, compared with cells treated with H_2O_2 only. GSH, glutathione; CPAEC, calf pulmonary arterial endothelial cell; MAPK, mitogen-activated protein kinase; CMF, 5-chloromethylfluorescein diacetate; MEK, mitogen-activated protein kinase kinase 1; JNK, c-Jun N-terminal kinase.

Z-VAD significantly prohibited cell death in H_2O_2-treated CPAECs (25). Thus, the H_2O_2-induced death of CPAECs predominantly occurs via apoptosis. However, MAPK inhibitors that affect the sub-G_1 cell proportion in H_2O_2-treated CPAECs did not alter the levels of Annexin V-FITC positive cells. Therefore, MAPK inhibitors may promote the death of CPAECs via necrosis rather than apoptosis. In addition, treatment with the JNK inhibitor alone increased the number of Annexin V-FITC positive cells in the control CPAECs, suggesting that the inhibition of JNK signaling increases the susceptibility of CPAECs to exogenous H_2O_2.

ROS can disturb the natural oxidation/reduction equilibrium in cells by triggering a reduction in MMP (42). Accordingly, H_2O_2 treatment induced a loss of MMP in CPAECs in the present study. Similar to the effect on sub-G_1 cells, treatment with the JNK inhibitor increased the loss of MMP in H_2O_2-treated CPAECs, whereas treatment with the p38 inhibitor reduced the MMP loss in the cells. In addition, treatment with the JNK inhibitor alone increased the loss of MMP in CPAECs without H_2O_2 treatment, suggesting that JNK signaling may be involved in the maintenance of MMP in CPAECs. Treatment with H_2O_2 slightly increased the MMP level of CPAECs; treatment with the MEK and JNK inhibitors decreased the MMP levels of H_2O_2-treated and -untreated CPAECs, whereas treatment with the p38 inhibitor slightly increased the MMP level in H_2O_2-treated and -untreated CPAECs. These results indicate that each MAPK signaling pathway has distinct and specific effects on MMP in CPAECs.

The primary ROS associated with cell signaling pathways are $O_2^{\cdot-}$ and H_2O_2. ROS toxicity is generally mediated by $^{\cdot}OH$ (6). As treatment with 30 μM H_2O_2 significantly induced the death of CPAECs, it is possible that exogenous H_2O_2 was converted into the more cytotoxic $^{\cdot}OH$ through the Fenton reaction to eliminate CPAECs (43). Notably ROS levels, including the levels of $O_2^{\cdot-}$, decreased in H_2O_2-treated CPAECs after 24 h. It is possible that the actual ROS level of the H_2O_2-treated CPAECs was distorted, as dead cells have a reduced capacity for the uptake of DCF and DHE. Our previous study also reported a decrease in $O_2^{\cdot-}$ levels following 24 h of treatment with 5-50 μM H_2O_2 in CPAECs (25). As ROS have a short half-life in the cell (44), further study on H_2O_2-treated CPAECs is required to assess ROS levels at an earlier time point, such as 30 min or 1 h. None of the MAPK inhibitors significantly altered the levels of ROS, including $O_2^{\cdot-}$, in H_2O_2-treated CPAECs. However, MEK or p38 inhibitor treatments non-significantly increased ROS levels, including $O_2^{\cdot-}$, in the control CPAECs without a corresponding induction of cell death. Treatment with the JNK inhibitor, as induced cell death and the loss of MMP in the control CPAECs, also increased the levels of ROS, including $O_2^{\cdot-}$. The results suggest that the death of CPAECs subsequent to H_2O_2 and/or individual MAPK inhibitor treatment could only weakly be attributed to an increase in ROS levels, and that treatment with each MAPK inhibitor changed the ROS levels in CPAECs via dissimilar mechanisms. The molecular mechanisms underlying these effects require further study, ideally with small interfering RNA knockdown of the MAPKs.

The extent of the induction of apoptosis is inversely proportional to the GSH content of cells (37,45,46). In the present study, H_2O_2 treatment increased the number of GSH-depleted cells in CPAECs. Furthermore, JNK inhibitor treatment increased the number of GSH-depleted cells in H_2O_2-treated CPAECs, whereas treatment with the p38 inhibitor decreased it. The results appear to reflect the proportion of sub-G_1 cells. In our previous study, treatment with the JNK inhibitor, as is associated with a pro-apoptotic effect on pyrogallol-treated CPAECs, enhances GSH depletion, whereas treatment with the p38 inhibitor had the opposite effect on pyrogallol-induced GSH depletion (16). Only JNK inhibitor treatment was associated with cell death in control CPAECs while also inducing GSH depletion in the present and previous studies. These results support the hypothesis that intracellular GSH content has a decisive role in cell death (45-47). Notably, GSH levels in the viable cells among H_2O_2-treated CPAECs increased, which may be a defense mechanism in response to exogenous H_2O_2. While MEK inhibitor treatment did not alter the GSH level in H_2O_2-treated CPAECs, treatment with JNK or p38 inhibitors did increase the GSH levels. Each MAPK inhibitor influenced the GSH levels in H_2O_2-treated CPAECs in different ways when considering the GSH levels of the non-GSH-depleted cells. The increased GSH levels in the control cells following MAPK inhibitor treatment may be a direct response to ROS generated by these inhibitors. GSH levels are high in typical cells (≤10 mM) and GSH transferase is ubiquitously present (48). Thus, measuring CMF fluorescence, which is produced upon reacting with thiol groups via a glutathione S-transferase-mediated reaction, is useful to evaluate GSH levels (49). However, CMF dye has limitations in accurately determining whole GSH content and GSH:glutathione disulfide (GSSG, the oxidized form of GSH) ratios in cells, as this dye may also detect other thiol groups (48). The determination of exact GSH levels and GSH:GSSG ratios in H_2O_2-treated CPAECs with or without each MAPK inhibitor are further required in order to understand the precise role of GSH in the regulation of CPAEC redox status.

In conclusion, treatment with H_2O_2 induced cell growth inhibition and death in CPAECs through GSH depletion. Treatment with the JNK inhibitor boosted cell growth inhibition and death, whereas the p38 inhibitor diminished the growth inhibition and death of H_2O_2-treated CPAECs.

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