

Enhancement of propyl gallate-induced calf pulmonary arterial endothelial cell death by MEK and JNK inhibitors

YONG HWAN HAN, SUNG ZOO KIM, SUHN HEE KIM and WOO HYUN PARK

Department of Physiology, Medical School, Institute for Medical Sciences,
Chonbuk National University, Jeonju 561-180, Republic of Korea

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Abstract. Propyl gallate (PG), a synthetic antioxidant, exerts a variety of effects on tissue and cell functions. Here, we investigated the effect of mitogen-activated protein kinase (MAPK) inhibitors on PG-treated calf pulmonary artery endothelial cells (CPAECs) in relation to changes in cell death, reactive oxygen species (ROS) and glutathione (GSH). PG inhibited CPAEC growth at 24 h and induced cell death, which was accompanied by the loss of mitochondrial membrane potential. PG also increased ROS levels in the CPAECs, while GSH depleted cell number. Treatment with MAPK (MEK, JNK and p38) inhibitors resulted in the slight enhancement of cell growth inhibition by PG. MEK and JNK inhibitors increased cell death and GSH depletion in PG-treated CPAECs without affecting ROS levels. In conclusion, PG inhibited the growth of CPAECs by regulating GSH levels. The pro-apoptotic effect of MEK and JNK inhibitors on PG-induced CPAEC death was related to a decrease in GSH levels.

Introduction

Propyl gallate (3,4,5-trihydroxybenzoic acid propyl ester) (PG) is used as a synthetic antioxidant in processed food, cosmetics

and food packaging materials to prevent rancidity and spoilage. PG is also used to preserve and stabilize medicinal preparations on the US Food and Drug Administration list (1). Due to this prevalent usage, the potential toxicity of PG has been investigated *in vivo* (2,3) and *in vitro* to assess its toxicological properties, including its mutagenicity (4) and cytogenetic effects (5). Despite the assumed low toxicity of PG, it exerts a variety of effects on tissue and cell functions. Several studies have demonstrated the benefits of PG as an antioxidant (6,7) and a chemopreventive agent (8). For instance, PG efficiently protects liver cells from lipid peroxidation by oxygen radicals (3). PG also has protective effects against oxidative DNA damage using 8-oxoguanine formation as a marker (7). In contrast, it has been reported that PG has pro-oxidant properties (9,10). In isolated rat hepatocytes, PG is cytotoxic since it impairs mitochondria, leading to ATP depletion (11). PG inhibits the growth of microorganisms by inhibiting respiration and nucleic acid synthesis (12). Contradictorily, the effects of PG have been reported to enhance and suppress carcinogenesis and mutagenesis (4,13). The antioxidative and cytoprotective properties of PG may change to pro-oxidative, cytotoxic and genotoxic properties in the presence of Cu(II) (14). In order to clarify the discrepancies between the varying effects of PG, further studies are needed to re-evaluate its function in cells and tissues and the safety of its use.

The mitogen-activated protein kinases (MAPKs) are a large family of proline-directed serine/threonine kinases that are major components of the signaling pathways involved in cell proliferation, differentiation, embryogenesis and cell death, in response to the activation of receptor tyrosine kinases, protein tyrosine kinases, cytokine and growth factor receptors, and heterotrimeric G protein-coupled receptors (15,16). There are currently four known MAPKs: extracellular signal-regulated kinase (ERK1/2), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), p38 kinase, and big mitogen-activated protein kinase 1 (BMK1) (15). Each MAP kinase pathway has relatively different upstream activators and specific substrates (17). Ample evidence demonstrates that JNK and p38 are strongly activated by reactive oxygen species (ROS) or by a mild oxidative shift in the intracellular thiol/disulfide redox state, leading to apoptosis (18,19). ROS are also known to induce ERK phosphorylation and activate the ERK pathway (20). In most instances, ERK activation has a pro-survival function rather than a pro-apoptotic effect

Correspondence to: Dr Woo Hyun Park, Department of Physiology, Medical School, Institute for Medical Sciences, Chonbuk National University, Jeonju 561-180, Republic of Korea
E-mail: parkwh71@chonbuk.ac.kr

Abbreviations: CMFDA, 5-chloromethylfluorescein diacetate; CPAEC, calf pulmonary arterial endothelial cells; DHE, dihydroethidium; EC, endothelial cells; ERK, extracellular signal-regulated kinase; FITC, fluorescein isothiocyanate; GSH, glutathione; H₂DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, MAP kinase or ERK kinase; MFI, mean fluorescence intensity; MMP ($\Delta\Psi_m$), mitochondrial membrane potential; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PG, propyl gallate; ROS, reactive oxygen species

Key words: propyl gallate, apoptosis, calf pulmonary arterial endothelial cells, mitogen-activated protein kinase inhibitor, reactive oxygen species, glutathione

(21,22). Since different ROS levels and diverse functions of MAPKs affected by ROS may have opposite effects in the same cell type, the relationship between ROS and MAPKs in terms of cell survival or cell death signaling requires further elucidation.

Vascular endothelial cells (ECs) are involved in various regulatory functions, such as vascular permeability for gases and metabolites, vascular smooth muscle tone, blood pressure, blood coagulation, inflammation and angiogenesis (23). The vascular endothelium can experience extensive degrees of oxidative stress, ultimately leading to endothelial dysfunction. Endothelial dysfunction has been implicated in the initiation and propagation of cardiovascular disease, including atherosclerosis, hypertension and congestive heart failure (24). Thus, enhanced oxidative stress may contribute to endothelial dysfunction in cardiovascular disease by the induction of EC apoptosis (25). Nonetheless, the relationship between EC apoptosis and antioxidants such as PG is poorly understood.

In the present study, we demonstrated that PG inhibited the growth of calf pulmonary artery endothelial cells (CPAECs). Furthermore, we investigated the effects of MAPK inhibitors on PG-treated CPAECs in relation to changes in cell death, ROS and glutathione (GSH).

Materials and methods

Cell culture. CPAECs were obtained from the Korean Cell Line Bank (KCLB) and were maintained in a humidified incubator containing 5% CO₂ at 37°C. The cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Gibco BRL, Grand Island, NY, USA), and were routinely grown in 100-mm plastic tissue culture dishes (Nunc, Roskilde, Denmark) and harvested with a trypsin-EDTA solution (Gibco BRL) while in a logarithmic phase of growth. The CPAECs were maintained in these culture conditions for all experiments.

Reagents. PG was purchased from the Sigma-Aldrich Chemical Company (St. Louis, MO, USA) and was dissolved in ethanol at 200 mM as a stock solution. JNK inhibitor (SP600125), MEK inhibitor (PD98059) and p38 inhibitor (SB203580) were purchased from Calbiochem (San Diego, CA, USA). These were dissolved in DMSO solution buffer at 10 mM as a stock solution. Cells were pre-treated with each MAPK inhibitor for 30 min prior to treatment with PG. Ethanol (0.2%) and DMSO (0.2%) were used as a control vehicle. All stock solutions were wrapped in foil and maintained at -20°C.

Cell growth assay. The effect of drugs on CPAEC growth was determined by measuring the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) dye absorbance of living cells as previously described (26). In brief, 3x10⁴ cells/well were seeded in 96-well microtiter plates. After exposure to 400 μM PG with or without 10 μM JNK inhibitor, MEK inhibitor or p38 inhibitor for 24 h, 20 μl of MTT solution (2 mg/ml in PBS) was added to each well of 96-well plates. The plates were incubated for an additional 4 h at 37°C. MTT solution in medium was aspirated off, and 200 μl of DMSO was 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).

Sub-G1 cell analysis. Sub-G1 cells were identified by propidium iodide (Ex/Em=488 nm/617 nm) (Sigma-Aldrich) staining as previously described (27). In brief, 1x10⁶ cells were incubated with 400 μM PG with or without 10 μM JNK inhibitor, MEK inhibitor or p38 inhibitor for 24 h. Cells were then washed with PBS and fixed in 70% ethanol. Subsequently, cells were washed again with PBS, then incubated with propidium iodide (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) or ModFit software (Verity Software House Inc., ME, USA).

Annexin V staining. Apoptosis was determined by staining cells with Annexin V-fluorescein isothiocyanate (FITC) (Ex/Em=488 nm/519 nm) as previously described (27). In brief, 1x10⁶ cells were incubated with 400 μM PG with or without 10 μM JNK inhibitor, MEK inhibitor or p38 inhibitor for 24 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 CaCl₂) at a concentration of 1x10⁶ cells/ml. Annexin V-FITC (5 μl) (Pharmingen, San Diego, CA, USA) was then added to the cells, which were analyzed with a FACStar flow cytometer.

Measurement of MMP ($\Delta\Psi_m$). Mitochondrial membrane potential [MMP ($\Delta\Psi_m$)] levels were measured using Rhodamine 123 fluorescent dye (Ex/Em=485 nm/535 nm) (Sigma) as previously described (28). In brief, 1x10⁶ cells were incubated with 400 μM PG with or without 10 μM JNK inhibitor, MEK inhibitor or p38 inhibitor for 24 h. Cells were washed twice with PBS and incubated with Rhodamine 123 (0.1 μg/ml) at 37°C for 30 min. Rhodamine 123 staining intensity was determined by flow cytometry. CPAECs negative for rhodamine 123 were identified as having experienced the loss of MMP ($\Delta\Psi_m$). MMP ($\Delta\Psi_m$) levels in cells, excluding the MMP ($\Delta\Psi_m$) loss cells, were expressed as the mean fluorescence intensity (MFI), calculated by CellQuest software.

Detection of intracellular ROS and O₂^{•-} levels. Intracellular ROS such as H₂O₂, •OH and ONOO• were detected by means of an oxidation-sensitive fluorescent probe dye, 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA; Ex/Em=495 nm/529 nm) (Invitrogen Molecular Probes, Eugene, OR, USA) (29). H₂DCFDA is poorly selective for O₂^{•-}. In contrast, dihydroethidium (DHE) (Ex/Em=518 nm/605 nm) (Invitrogen Molecular Probes) is highly selective for O₂^{•-} among the ROS. DHE is cell permeable and reacts with superoxide anion to form ethidium, which in turn intercalates in deoxyribonucleic acid, thereby exhibiting a red fluorescence. In brief, 1x10⁶ cells were incubated with 400 μM PG with or without 10 μM JNK inhibitor, MEK inhibitor or p38 inhibitor for 24 h. Cells were then washed in PBS and incubated with 20 μM H₂DCFDA or DHE at 37°C for 30 min, according to the manufacturer's instructions. DCF and DHE fluorescence was detected using a FACStar flow cytometer. ROS and O₂^{•-} levels were expressed as the MFI.

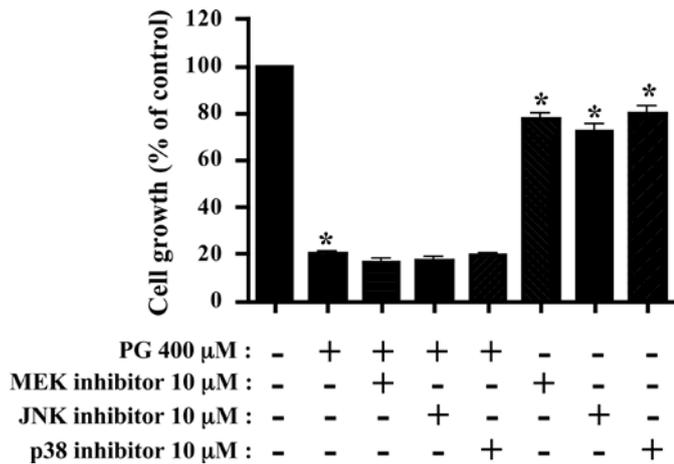


Figure 1. Effect of MAPK inhibitors on cell growth in PG-treated CPAECs. Exponentially growing CPAECs were treated with PG for 24 h following a 30-min pre-incubation with each MAPK inhibitor. The graph shows CPAEC growth as assessed by the MTT assay. * $P < 0.05$ compared with the control group. Bar, SD.

Detection of intracellular glutathione. Cellular GSH levels were analyzed using 5-chloromethylfluorescein diacetate (CMFDA, Molecular Probes) (Ex/Em=522 nm/595 nm) as previously described (29). In brief, 1×10^6 cells were incubated with 400 μ M PG with or without 10 μ M JNK inhibitor, MEK inhibitor or p38 inhibitor for 24 h. Cells were then washed with PBS and incubated with 5 μ M CMFDA at 37°C for 30 min. Cytoplasmic esterases convert non-fluorescent CMFDA to fluorescent 5-chloromethylfluorescein, which can then react with GSH. CMF fluorescence intensity was determined using a FACStar flow cytometer. Cells negative for CMF staining (GSH-depleted) cells expressed as the percent of (-) CMF cells. CMF levels in cells, excluding GSH-depleted cells, were expressed as the MFI.

Statistical analysis. The results shown in the figures represent the mean of at least three independent experiments. Data were analyzed using InStat software (GraphPad Prism4, San Diego, CA, USA). 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. Statistical significance was defined as $P < 0.05$.

Results

Effect of MAPK inhibitors on cell growth, cell death and MMP ($\Delta\Psi_m$) in PG-treated CPAECs. The effect of MAPK inhibitors on the growth of PG-treated CPAECs was examined using the MTT assay. For this experiment, 400 μ M PG was selected as a suitable dose to differentiate the levels of cell growth inhibition and cell death in the presence or absence of each MAPK inhibitor. Treatment with 400 μ M PG inhibited the growth of CPAECs by ~80% at 24 h (Fig. 1). The optimal dose of MEK inhibitor (PD98059), JNK inhibitor (SP600125) and p38 inhibitor (SB203580) was determined to be 10 μ M. The 20 μ M concentration of each MAPK inhibitor strongly reduced the growth of CPAECs (data not shown). Treatment with each MAPK inhibitor slightly intensified the growth inhibition of

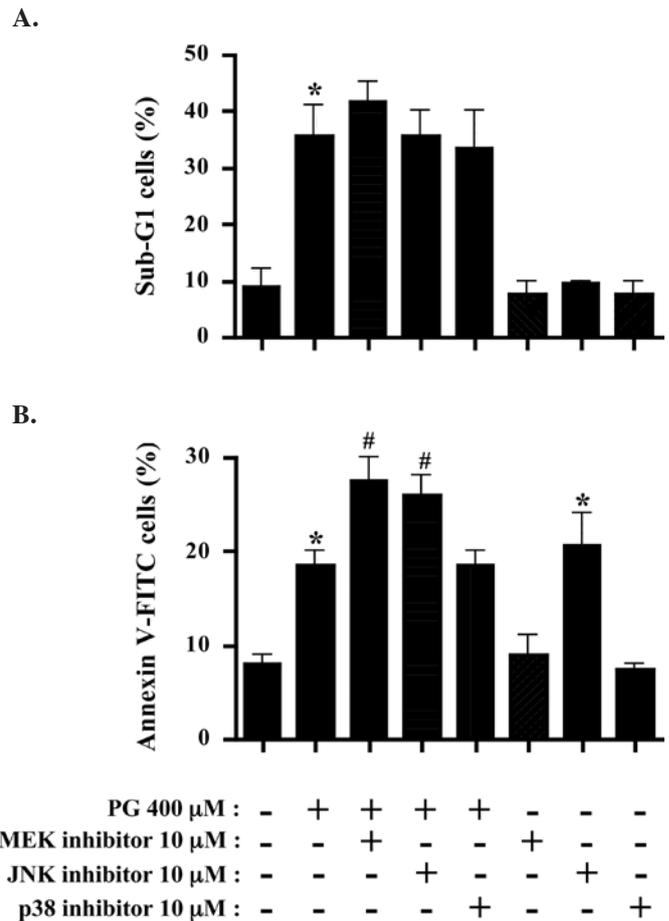


Figure 2. Effect of MAPK inhibitors on cell death in PG-treated CPAECs. Exponentially growing CPAECs were treated with PG for 24 h following a 30-min pre-incubation with each MAPK inhibitor. The percentage of sub-G1 cells (A) and Annexin V stained cells (B) was measured using a FACStar flow cytometer. * $P < 0.05$ compared with the control group; # $P < 0.05$ compared with cells treated with PG only. Data are presented as the means \pm SD of three replicates. Bar, SD.

PG-treated CPAECs and also inhibited the growth of control CPAECs (Fig. 1).

Treatment with 400 μ M PG induced an increase in CPAEC death of 20-30% compared to control CPAECs not treated with PG, as evidenced by sub-G1 cells and cells stained by Annexin V (Fig. 2A and B). To determine whether MAPK inhibitors affect PG-induced cell death, CPAECs were treated with PG and/or each MAPK inhibitor for 24 h. As shown in Fig. 2, only MEK inhibitor slightly increased the number of sub-G1 cells in PG-treated cells (Fig. 2A), while MEK and JNK inhibitors significantly increased Annexin V-FITC-positive cells in the PG-treated cells (Fig. 2B). JNK inhibitor also increased the number of Annexin V-FITC-positive cells in control CPAECs (Fig. 2B).

It is known that apoptosis is closely related to the collapse of MMP ($\Delta\Psi_m$) (30). Therefore, we determined the loss of MMP ($\Delta\Psi_m$) in PG-treated CPAECs using Rhodamine 123 dye at 24 h. As expected, the loss of MMP ($\Delta\Psi_m$) was observed in PG-treated cells (Fig. 3A). None of the MAPK inhibitors affected the degree of loss in PG-treated CPAECs (Fig. 3A). JNK inhibitor alone triggered loss in control CPAECs (Fig. 3A).

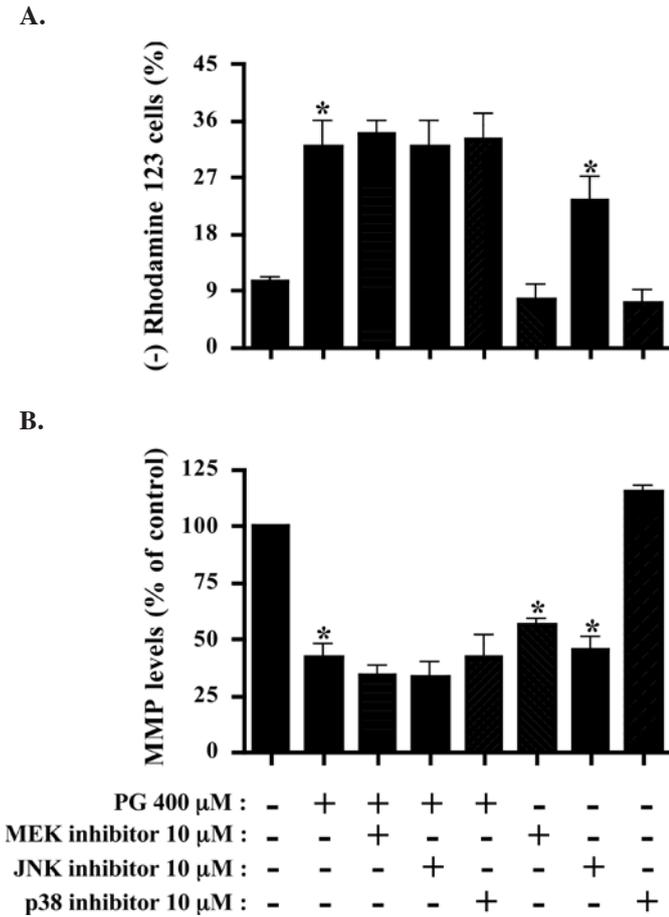


Figure 3. Effect of MAPK inhibitors on MMP ($\Delta\Psi_m$) in PG-treated CPAECs. Exponentially growing CPAECs were treated with PG for 24 h following a 30-min pre-incubation with each MAPK inhibitor. MMP ($\Delta\Psi_m$) in CPAECs was measured using a FACStar flow cytometer. The percentage of Rhodamine 123-negative [MMP ($\Delta\Psi_m$) loss] cells (A) and MMP ($\Delta\Psi_m$) levels (%) compared with control CPAECs (B) is shown. *P<0.05 compared with the control group. Data are presented as the means \pm SD of three replicates. Bar, SD.

In relation to MMP ($\Delta\Psi_m$) levels in CPAECs, treatment with PG reduced the MMP ($\Delta\Psi_m$) level by ~60% (Fig. 3B). MEK and JNK slightly decreased the MMP ($\Delta\Psi_m$) level in PG-treated cells (Fig. 3B). In addition, MEK and JNK inhibitors reduced the MMP ($\Delta\Psi_m$) level in control CPAECs (Fig. 3B).

Effect of MAPK inhibitors on ROS and O₂⁻ levels in PG-treated CPAECs. Next, to determine whether the levels of intracellular ROS in PG-treated CPAECs were affected by each MAPK inhibitor, at 24 h we assessed ROS levels in CPAECs using various fluorescence dyes (Fig. 4). Intracellular ROS (DCF) levels, such as those of H₂O₂, were increased in PG-treated cells (Fig. 4A). While ROS levels in PG-treated CPAECs were not affected by MEK and JNK inhibitors, they were increased by p38 inhibitor (Fig. 4A). All the MAPK inhibitors increased ROS levels in control CPAECs, with p38 inhibitor exhibiting a strong effect (Fig. 4A).

When intracellular O₂⁻ levels in PG-treated CPAECs were detected, red fluorescence derived from DHE reflecting intracellular O₂⁻ levels was not altered at 24 h (Fig. 4B). JNK and p38 inhibitors slightly increased the O₂⁻ level, but without significance (Fig. 4B). MEK and JNK inhibitors increased O₂⁻ levels in control CPAECs (Fig. 4B).

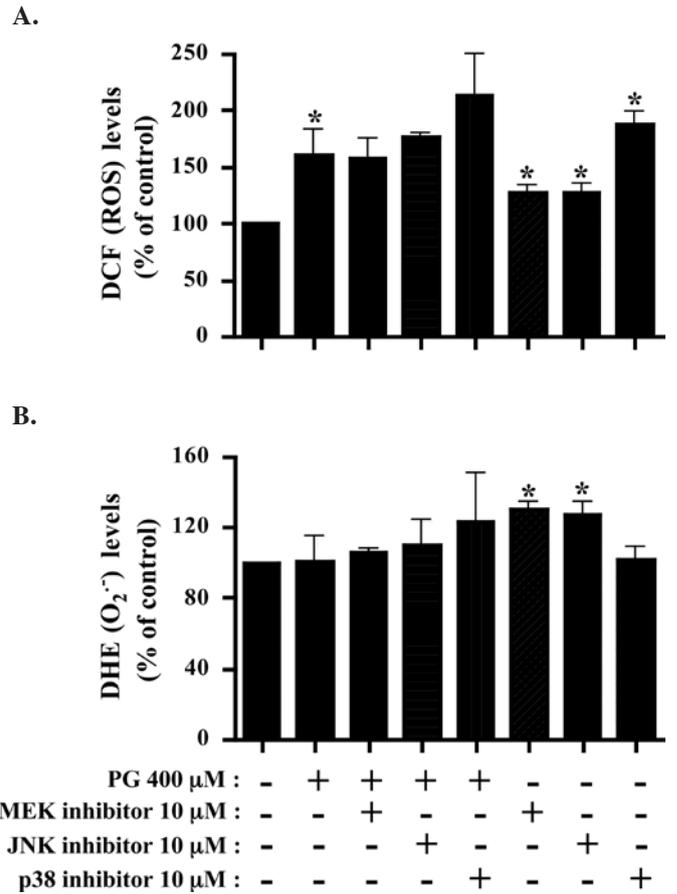


Figure 4. Effect of MAPK inhibitors on ROS levels in PG-treated CPAECs. Exponentially growing CPAECs were treated with PG for 24 h following a 30-min pre-incubation with each MAPK inhibitor. ROS levels in CPAECs were measured using a FACStar flow cytometer. DCF (ROS) levels (%) compared with control CPAECs (A) and DHE (O₂⁻) levels (%) compared with control CPAECs (B) are shown. *P<0.05 compared with the control group. Data are presented as the means \pm SD of three replicates. Bar, SD.

Effect of MAPK inhibitors on GSH levels in PG-treated CPAECs. Cellular GSH can regulate cell growth and apoptosis (31,32). Therefore, we analyzed changes in GSH levels in CPAECs using CMF fluorescence dye at 24 h (Fig. 5). Treatment with PG increased the number of GSH-depleted cells in CPAECs (Fig. 5A). MEK and JNK inhibitors mildly magnified GSH depletion in PG-treated CPAECs (Fig. 5A). JNK inhibitor also increased GSH-depleted cell number in control CPAECs (Fig. 5A). When CMF (GSH) levels in CPAECs, excluding cells negative for CMF staining, were assessed, the GSH level was decreased by PG treatment (Fig. 5B). MEK and JNK inhibitors enhanced the reduced GSH level (Fig. 5B). All the MAPK inhibitors increased GSH levels in control CPAECs (Fig. 5B).

Discussion

In the present study, we focused on evaluating the effect of MAPK inhibitors on PG-treated CPAECs in relation to changes in cell death, ROS and GSH, since PG induced CPAEC growth inhibition and death. ERK activation has a pro-survival function rather than pro-apoptotic effects (21,22). Similarly, MEK inhibitor, which presumably inactivates ERK, increased the number of sub-G1 and Annexin V-FITC positive cells in

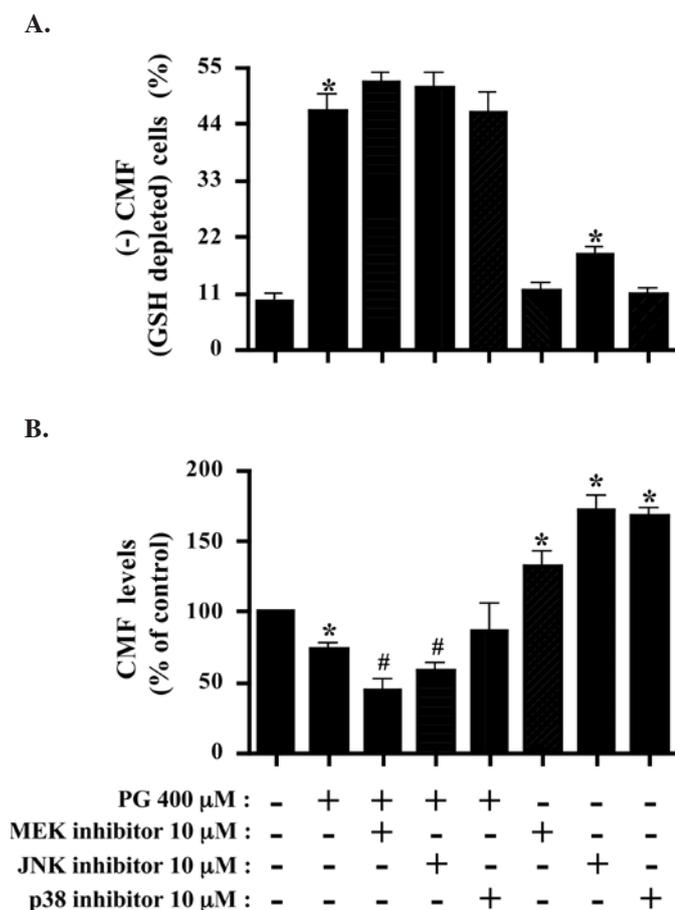


Figure 5. Effect of MAPK inhibitors on GSH levels in PG-treated CPAECs. Exponentially growing CPAECs were treated with PG for 24 h following a 30-min pre-incubation with each MAPK inhibitor. GSH levels in CPAECs were measured using a FACStar flow cytometer. The percentage of (-) CMF (GSH-depleted) cells (A) and mean CMF (GSH) levels compared with control CPAEC (B) is shown. * $P < 0.05$ compared with the control group; # $P < 0.05$ compared with cells treated with PG only. Data are presented as the means \pm SD of three replicates. Bar, SD.

PG-treated CPAECs. Thus, inactivation of ERK by MEK inhibitor appeared to be an enhancing factor on PG-induced CPAEC death. It is known that the activation of JNK or p38 generally leads to apoptosis (18,19). According to our data, JNK inhibitor increased the number of Annexin V-FITC positive cells in PG-treated CPAECs, but p38 inhibitor did not. This implies that JNK and p38 affect CPAEC death differently, and that JNK signaling in PG-treated CPAECs is involved in a pro-survival rather than a pro-apoptotic function. In addition, all the MAPK inhibitors slightly increased CPAEC growth inhibition by PG. Taken together, these data suggest that the MAPK signaling pathways have different involvement in cell growth and death in PG-treated CPAECs. In relation to MMP ($\Delta\Psi_m$), PG induced the loss of MMP ($\Delta\Psi_m$) in CPAECs and reduced MMP ($\Delta\Psi_m$) levels. None of the MAPK inhibitors altered the loss of MMP ($\Delta\Psi_m$). However, MEK and JNK inhibitors decreased MMP ($\Delta\Psi_m$) levels in PG-treated CPAECs. These results suggest that the loss of MMP ($\Delta\Psi_m$) following treatment with PG leads to apoptosis, and that MAPK signaling is not involved in the loss of MMP ($\Delta\Psi_m$) by PG, but is to some extent involved in MMP ($\Delta\Psi_m$) levels. Moreover, all the MAPK inhibitors significantly reduced the growth of control

CPAECs. JNK inhibitor also increased Annexin V-FITC-positive cells in control CPAECs and triggered the loss of MMP ($\Delta\Psi_m$). In addition, MEK and JNK inhibitors reduced MMP ($\Delta\Psi_m$) levels. Therefore, the MAPK signaling pathways appear to have different involvement in cell growth and death in CPAECs. In particular, the prevention of JNK signaling by its inhibitor is related to CPAEC death.

PG acts as an antioxidant (3,6,7) or a pro-oxidant (9,10). Increasing evidence suggests that oxidative stress regulates the apoptosis of ECs. In fact, H_2O_2 , tumor necrosis factor- α (TNF- α) and angiotensin II induce EC apoptosis and stimulate the generation of ROS (33,34). According to our results, PG as a pro-oxidant increased DCF (ROS) levels in CPAECs. However, the $\text{O}_2^{\cdot-}$ level was not increased by 400 μM PG, though it was strongly increased by 800 μM PG (data not shown). These results suggest that the PG effect on the levels of different ROS in the same cell type depends on the incubation dose. Treatment with MEK inhibitor, which exhibited the slight enhancement of cell death in PG-treated CPAECs, did not alter ROS levels, including $\text{O}_2^{\cdot-}$. In addition, MEK inhibitor alone increased ROS levels in CPAECs without triggering cell death. These results suggest that CPAEC death by PG and/or MEK inhibitor is independent of changes in the level of ROS. JNK inhibitor exhibiting a slight enhancement of cell death in PG-treated CPAECs did not increase ROS levels, but did increase the levels in control CPAECs along with cell death and the loss of MMP ($\Delta\Psi_m$). JNK inhibitor seemed to affect cell death and ROS levels in PG-treated and -untreated CPAECs differently. Treatment with p38 inhibitor increased ROS levels in PG-treated and -untreated CPAECs, regardless of cell death. Therefore, changes in ROS levels caused by p38 inhibitor are not related to CPAEC death. Taken together, CPAEC death by PG and/or MAPK inhibitors might not be strongly correlated to changes in the levels of ROS.

GSH is a main non-protein antioxidant in cells. It is capable of eliminating $\text{O}_2^{\cdot-}$ and provides electrons for enzymes such as GSH peroxidase, which reduces H_2O_2 to H_2O . It has been reported that intracellular GSH content has a decisive effect on anticancer drug-induced apoptosis, indicating that apoptotic effects are inversely comparable to GSH content (31,32). Likewise, PG increased GSH-depleted cell numbers and decreased GSH levels in CPAECs. In addition, MEK and JNK inhibitors mildly magnified GSH depletion in PG-treated CPAECs. JNK inhibitor also increased GSH-depleted cell numbers in control CPAECs. These results are probably correlated with the Annexin V-FITC results from CPAECs treated with PG and/or MAPK inhibitors. MEK and JNK inhibitors enhanced reduced GSH levels in PG-treated CPAECs. However, p38 inhibitor did not affect these levels. These results suggest that MAPK inhibitors regulate GSH levels in CPAECs differently depending on the co-treatment drug. Interestingly, all the MAPK inhibitors increased GSH levels in control CPAECs. It is likely that the increased GSH levels reduced increases in ROS by the MAPK inhibitors.

In conclusion, PG inhibited the growth of CPAECs by regulating the level of GSH. The pro-apoptotic effect of the MEK and JNK inhibitors on PG-induced CPAEC death was related to the decrease in GSH levels. Our preliminary results provide important information on the anti-growth mechanisms of PG in ECs based on MAPK signaling.

Acknowledgements

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