

p38 inhibitor enhances growth inhibition and death in gallic acid-treated endothelial cells

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Abstract. Gallic acid (GA) found in various plants, fruits and foods has various biological effects including apoptosis. However, little is known about the relationship between reactive oxygen species (ROS) and p38 signaling in GA-treated endothelial cells (ECs). In this study, we investigated the effects of p38 inhibitor on GA-induced calf pulmonary artery endothelial cells (CPAEC) and human umbilical vein endothelial cell (HUVEC) death in view of ROS and glutathione (GSH). GA inhibited the growth of both ECs and induced apoptosis, which was accompanied by the loss of mitochondrial membrane potential (MMP; $\Delta\Psi_m$). The susceptibility of CPAEC to GA is higher than that of HUVEC. GA differently increased or decreased ROS levels in ECs. GA increased GSH depleted cell numbers in ECs. p38 inhibitor seemed to enhance cell growth inhibition and cell death in GA-treated ECs. This inhibitor increased or decreased ROS levels in GA-treated ECs. p38 inhibitor to some extent enhanced GSH depletion in GA-treated CPAEC but it clearly increased GSH depletion cell numbers in GA-treated and -untreated HUVEC. In conclusion, p38 inhibitor appeared to enhance growth inhibition and death in GA-treated ECs, which were partially influenced by the changes of ROS and GSH depletion levels.

Introduction

Gallic acid (GA; 3,4,5-trihydroxyl-benzoic acid) as a poly-hydroxylphenolic compound is widely distributed in various plants, fruits and foods (1) and is very well absorbed in humans (2). Various biological activities of GA have been reported, including anti-bacterial (3), anti-viral (4) and anti-inflammatory (5). The major interest in GA is related to its antitumoral activity. Anticancer activity of GA has been reported in various cancer cells, such as prostate cancer (6), lung cancer (7,8), gastric, colon, breast, cervical and esophageal cancer (9). Apoptosis induced by GA is associated with oxidative stresses derived from reactive oxygen species (ROS), mitochondrial dysfunction and an increase in intracellular Ca^{2+} level (10,11). Controversially, GA has been suggested to have both pro-oxidant and antioxidant properties depending on iron or H_2O_2 in medium and plasma (12,13).

The mitogen-activated protein kinases (MAPKs) are a large family of serine/threonine kinases, which are major components of signaling pathways in cell proliferation, differentiation and cell death (14). Substantial evidence demonstrates that p38 signaling among MAPKs is involved in drug-induced apoptosis and redox state changes (15-17). In addition, p38 inhibitor prevented anisomycin-induced macrophage death (18) and it decreased the death of pyrogallol-induced calf pulmonary artery endothelial cells (19). Vascular endothelium is involved in various regulatory processes such as blood pressure, inflammation and angiogenesis (20). Angiogenesis involving formation of new blood vessels from pre-existing vasculature is a crucial part of the transition of tumors from a latent to malignant state.

Despite critical roles for vascular endothelial cells (ECs) in tumor biogenesis and progression, the effects of GA on ECs remain relatively poorly understood. In addition, little is known about the relationships between ROS and p38 signaling in GA-treated ECs. Therefore, in the present study we investigated the effects of p38 inhibitor on cell growth, cell death, ROS and GSH levels in GA-treated CPAEC and HUVEC.

Materials and methods

Cell culture. CPAEC obtained from KCLB (Korean Cell Line Bank, Seoul, Korea) were maintained in humidified incubator containing 5% CO_2 at 37°C. CPAEC were cultured

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Abbreviations: GA, gallic acid; ECs, endothelial cells; CPAEC, calf pulmonary arterial endothelial cells; HUVEC, human umbilical vein endothelial cells; ROS, reactive oxygen species; MAPK, mitogen-activated protein kinase; MMP ($\Delta\Psi_m$), mitochondrial membrane potential; FBS, fetal bovine serum; 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-diphenyl-tetrazolium bromide; PI, propidium iodide

Key words: gallic acid, endothelial cells, cell death, p38, reactive oxygen species

in RPMI-1640 supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich Chemical Co., St. Louis, MO) and 1% penicillin-streptomycin (Gibco BRL, Grand Island, NY). The primary HUVEC from PromoCell GmbH (Heidelberg, Germany) were maintained in humidified incubator containing 5% CO₂ at 37°C. HUVEC were cultured in complete endothelial cell growth medium (ECGM, PromoCell) with 2% FBS. CPAEC and HUVEC were grown in 100-mm plastic tissue culture dishes (Nunc). HUVEC were washed and detached with HEPES BSS (30 mM HEPES), trypsin-EDTA and trypsin neutralization solution (PromoCell). For experiments, HUVEC were used among passages four and eight.

Reagents. GA purchased from the Sigma-Aldrich Chemical Co. was dissolved in ethanol at 200 mM. p38 inhibitor (SB203580) obtained from Calbiochem (San Diego, CA) was dissolved in DMSO at 10 mM. Cells were pretreated with p38 inhibitor for 1 h before GA treatment. Based on previous experiments (19), 10 μM of p38 inhibitor was used as an optimal dose in this experiment. Ethanol (0.2%) and DMSO (0.3%) were used as a control vehicle. All stock solutions were wrapped in foil and kept at -20°C.

Cell growth assay. The effect of GA and/or p38 inhibitor on EC growth was determined by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye absorbance of living cells as previously described (21). In brief, cells 3x10⁴ cells per well were seeded in 96-well microtiter plates (Nunc) for MTT assays. After exposure to the indicated amounts of GA with or without p38 inhibitor for 24 h, 20 μl of MTT solution (2 mg/ml in PBS) were added to each well of 96-well plates (Nunc). The plates were incubated for 4 additional hours at 37°C. Medium in plates was withdrawn using pipetting and 200 μl DMSO was added to each well to solubilize the formazan crystals. Optical density was measured at 570 nm using a microplate reader (Spectra MAX 340, Molecular Devices Co., Sunnyvale, CA).

Annexin V staining for cell death detection. Apoptosis was determined by staining cells with Annexin V-fluorescein isothiocyanate (FITC, PharMingen, San Diego, CA; Ex/Em = 488 nm/519 nm) and propidium iodide (PI; Sigma-Aldrich; Ex/Em = 488 nm/617 nm). In brief, 1x10⁶ cells in 60 mm culture dish (Nunc) were incubated with the indicated amounts of GA with or without 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. Five microliters of Annexin V-FITC and PI (1 μg/ml) were then added to these cells, which were analyzed with a FACStar flow cytometer (Becton-Dickinson).

Measurement of MMP ($\Delta\Psi_m$). MMP ($\Delta\Psi_m$) levels were measured using rhodamine 123 fluorescent dye (Sigma-Aldrich Chemical Co.; Ex/Em = 485 nm/535 nm) as previously described (22). In brief, 1x10⁶ cells in 60 mm culture dish (Nunc) were incubated with the indicated amounts of GA with or without 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 (Becton-Dickinson). An

absence of rhodamine 123 from cells indicated the loss of MMP ($\Delta\Psi_m$) in HUVEC.

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, Invitrogen Molecular Probes, OR; Ex/Em = 495 nm/529 nm) (23). As H₂DCFDA is poorly selective for O₂^{•-}, dihydroethidium (DHE, Invitrogen Molecular Probes; Ex/Em = 518 nm/605 nm), which is highly selective for O₂^{•-}, was used for its detection. In brief, 1x10⁶ cells in 60 mm culture dish (Nunc) were incubated with the indicated amounts of GA with or without 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. DCF and DHE fluorescence intensities were detected using a FACStar flow cytometer (Becton-Dickinson).

Detection of the intracellular glutathione (GSH). Cellular GSH levels were analyzed using 5-chloromethylfluorescein diacetate (CMFDA, Invitrogen Molecular Probes; Ex/Em = 522 nm/595 nm) as previously described (23). In brief, 1x10⁶ cells in 60 mm culture dish (Nunc) were incubated with the indicated amounts of GA with or without p38 inhibitor for 24 h. 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). Negative CMF staining (GSH depleted) cells were expressed as the percent of (-) CMF cells.

Results

Effects of p38 inhibitor on cell growth in GA-treated ECs. We examined the effect of p38 inhibitor on the growth of GA-treated CPAEC and HUVEC. GA dose-dependently inhibited both ECs at 24 h (Fig. 1). The IC₅₀ of it in CPAEC ranged between 25 and 50 μM (Fig. 1A) and that in HUVEC ranged between 200 and 400 μM (Fig. 1B). p38 inhibitor slightly enhanced the growth inhibition in 5 μM GA-treated CPAEC but not in 25 μM GA-treated cells (Fig. 1A). In addition, p38 inhibitor mildly intensified that in GA-treated HUVEC (Fig. 1B). p38 inhibitor alone did not significantly affect the growth of either EC (Fig. 1).

Effects of p38 inhibitor on cell death and MMP ($\Delta\Psi_m$) in GA-treated ECs. GA dose-dependently induced cell death in CPAEC and HUVEC, as evidenced by sub-G1 cells (data not shown) and Annexin V staining cells (Fig. 2). p38 inhibitor increased the number of Annexin V-FITC positive cells in 5 μM GA-treated CPAEC. Especially, the number of Annexin V positive and PI negative (apoptotic) cells was especially increased about 10% by this inhibitor (Fig. 2A). p38 inhibitor also increased the numbers of Annexin V positive cells or Annexin V positive and PI negative (apoptotic) cells in GA-treated HUVEC (Fig. 2B). p38 inhibitor alone increased the number of Annexin V positive and PI negative (apoptotic) cells in control ECs (Fig. 2).

In addition, GA significantly triggered the loss of MMP ($\Delta\Psi_m$) in ECs (Fig. 3). GA did not dose-dependently increase

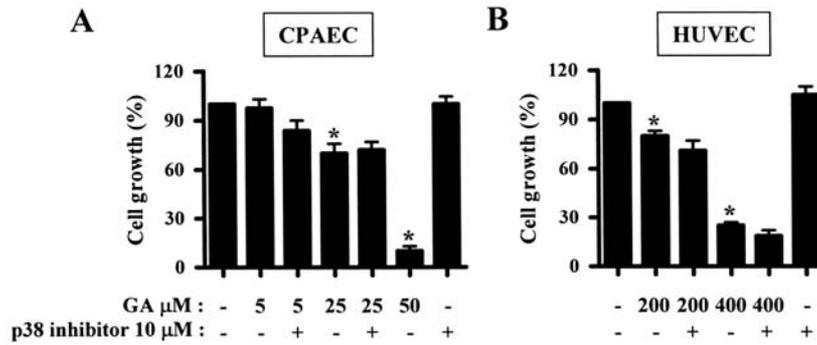


Figure 1. Effects of p38 inhibitor on cell growth in GA-treated ECs. Exponentially growing cells were treated with the indicated doses of GA for 24 h following 1 h pre-incubation of $10 \mu\text{M}$ p38 inhibitor. Cell growth changes in CPAEC (A) and HUVEC (B) were assessed by MTT assays. * $P < 0.05$ compared with GA-untreated control cells. The Student's *t*-test was used for parametric data. Statistical significance was defined as $P < 0.05$.

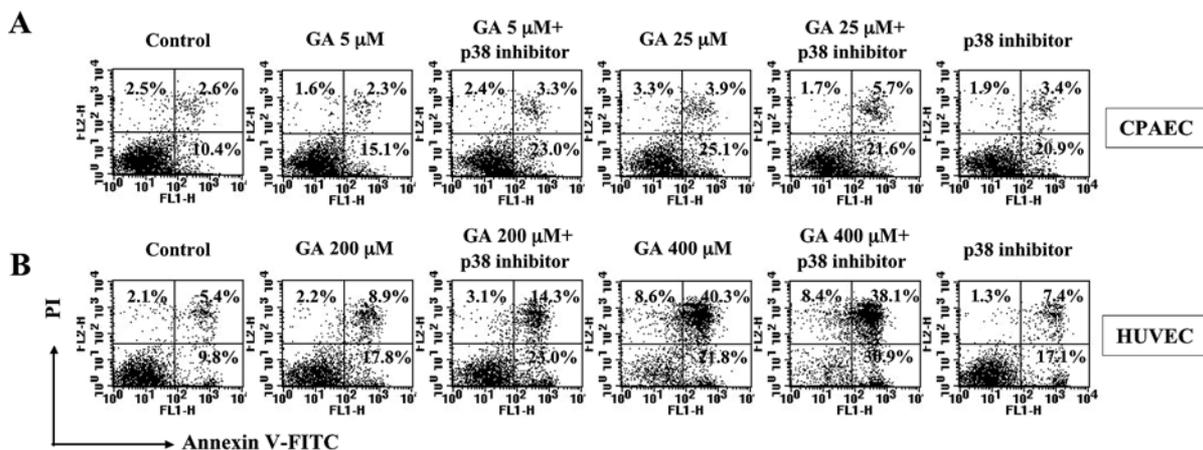


Figure 2. Effects of p38 inhibitor on cell death in GA-treated ECs. Exponentially growing cells were treated with the indicated doses of GA for 24 h following 1 h pre-incubation of $10 \mu\text{M}$ p38 inhibitor. Annexin V-FITC/PI staining cells were measured with a FACStar flow cytometer. Each histogram indicates Annexin V-FITC/PI staining cells in CPAEC (A) and HUVEC (B), respectively. Viable cells were negative for both PI and Annexin V; apoptotic cells were positive for Annexin V and negative for PI, whereas late apoptotic dead cells displayed both high Annexin V and PI labeling. Non-viable cells, which underwent necrosis, were positive for PI and negative for Annexin V.

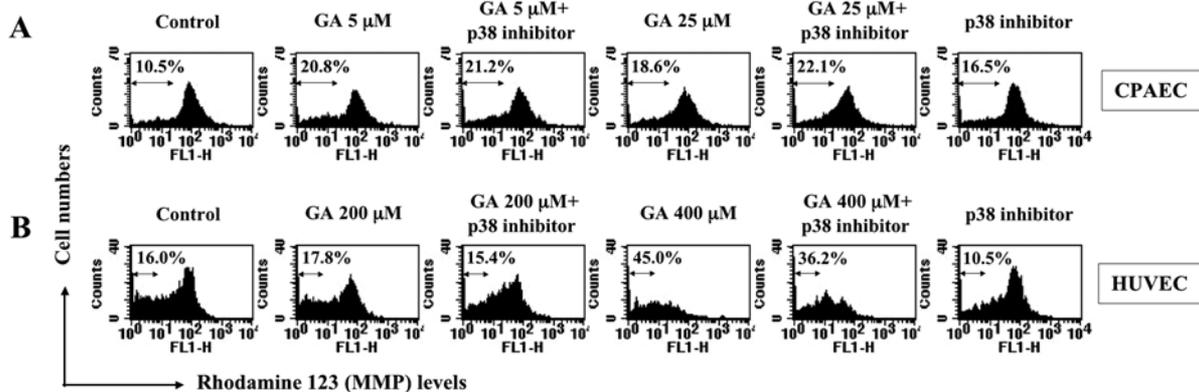


Figure 3. Effects of p38 inhibitor on MMP ($\Delta\Psi_m$) in GA-treated ECs. Exponentially growing cells were treated with the indicated doses of GA for 24 h following 1 h pre-incubation of $10 \mu\text{M}$ p38 inhibitor. MMP ($\Delta\Psi_m$) in cells was measured with a FACStar flow cytometer. Each histogram indicates MMP ($\Delta\Psi_m$) in CPAEC (A) and HUVEC (B), respectively. The arrow marks (or percent numbers) in each histogram indicate the percent of rhodamine 123 negative [MMP ($\Delta\Psi_m$) loss] cells.

the loss of MMP ($\Delta\Psi_m$) in CPAEC but in HUVEC (Fig. 3). p38 inhibitor did not seem to affect the MMP ($\Delta\Psi_m$) loss in GA-treated CPAEC but slightly induced the loss of MMP

($\Delta\Psi_m$) in control CPAEC (Fig. 3A). However, it slightly attenuated the MMP ($\Delta\Psi_m$) loss in GA-treated or -untreated HUVEC (Fig. 3B).

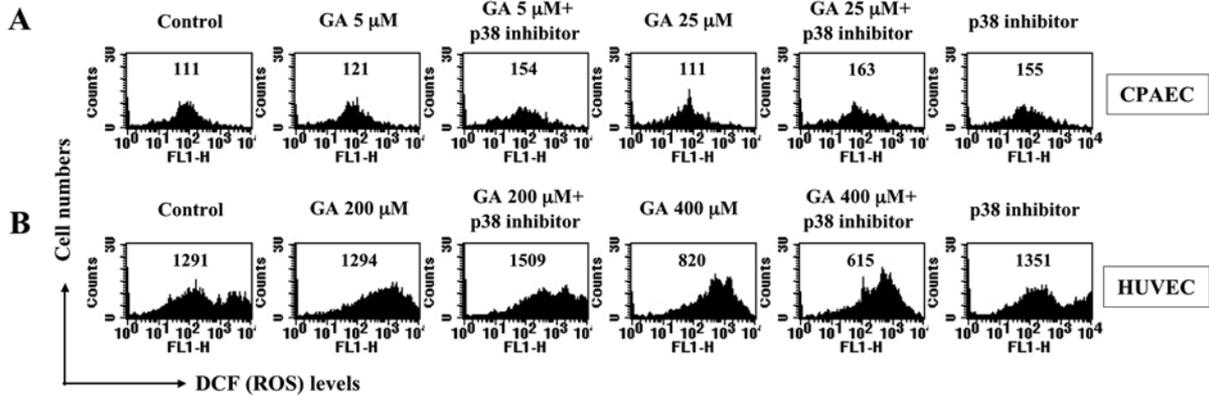


Figure 4. Effects of p38 inhibitor on DCF (ROS) levels in GA-treated ECs. Exponentially growing cells were treated with the indicated doses of GA for 24 h following 1 h pre-incubation of 10 μM p38 inhibitor. Each histogram indicates DCF (ROS) levels in CPAEC (A) and HUVEC (B), respectively. The numbers in each histogram indicate mean fluorescence intensity for DCF (ROS) levels.

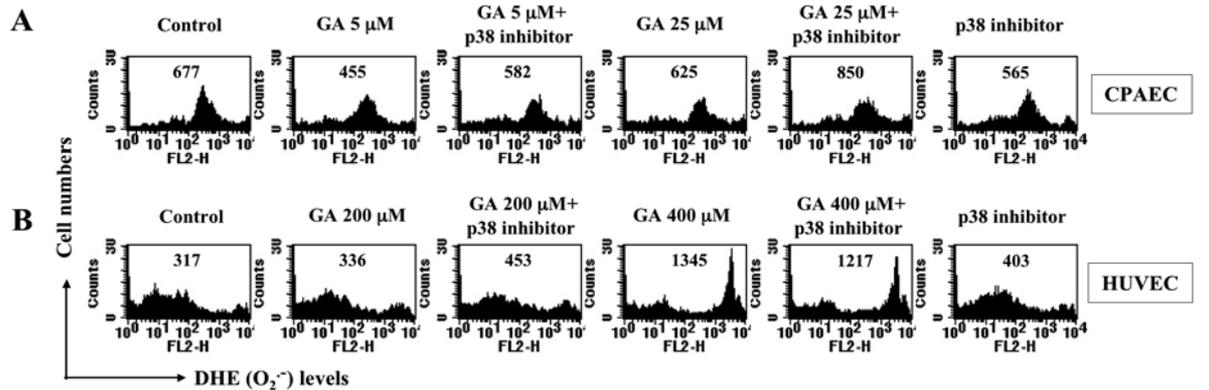


Figure 5. Effects of p38 inhibitor on DHE (O₂^{•-}) levels in GA-treated ECs. Exponentially growing cells were treated with the indicated doses of GA for 24 h following 1 h pre-incubation of 10 μM p38 inhibitor. Each histogram indicates DHE (O₂^{•-}) levels in CPAEC (A) and HUVEC (B), respectively. The numbers in each histogram indicate mean fluorescence intensity for DHE (O₂^{•-}) levels.

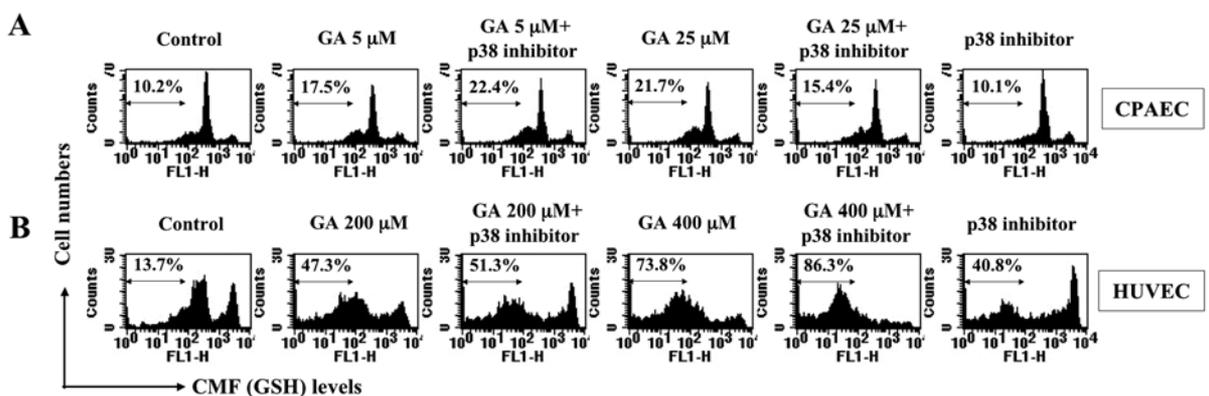


Figure 6. Effects of p38 inhibitor on GSH levels in GA-treated ECs. Exponentially growing cells were treated with the indicated doses of GA for 24 h following 1 h pre-incubation of 10 μM p38 inhibitor. Each histogram indicates CMF (GSH) levels in CPAEC (A) and HUVEC (B), respectively. The arrow mark (or percent numbers) in each histogram indicate the percents of (-) CMF (GSH depleted) cells.

Effects of p38 inhibitor on ROS and GSH levels in GA-treated ECs. Next, we determined whether intracellular ROS and GSH levels in GA-treated ECs were changed by p38 inhibitor. ROS (DCF) level such as H₂O₂ was not altered in GA-treated CPAEC but was decreased in 400 μM GA-treated

HUVEC (Fig. 4). p38 inhibitor seemed to increase ROS (DCF) levels in GA-treated or -untreated CPAEC (Fig. 4A). However, it increased the ROS (DCF) level of 200 μM GA-treated HUVEC but decreased that of 400 μM GA (Fig. 4B). Red fluorescence derived from DHE reflecting intracellular O₂^{•-}

SPANDIDOS PUBLICATIONS decreased in CPAEC treated with 5 μM GA but not in HeLa (Fig. 5A). p38 inhibitor seemed to increase $\text{O}_2^{\cdot-}$ levels in GA-treated CPAEC (Fig. 5A). GA strongly increased $\text{O}_2^{\cdot-}$ level in HUVEC treated with 400 μM GA but not 200 μM GA (Fig. 5B). p38 inhibitor increased $\text{O}_2^{\cdot-}$ level in 200 μM GA-treated HUVEC and decreased the level in 400 μM GA-treated cells (Fig. 5B). This inhibitor alone decreased $\text{O}_2^{\cdot-}$ level in control CPAEC but increased that in control HUVEC (Fig. 5).

Treatment with 5-25 μM GA increased the number of GSH depleted cells about 10% in CPAEC compared with GA-untreated control cells (Fig. 6A). GA also increased GSH depleted cell number in HUVEC (Fig. 6B) and the depletion level was relatively high compared with that in GA-treated CPAEC (Fig. 6). p38 inhibitor mildly increased GSH depleted cell number in 5 μM GA-treated CPAEC but decreased that in 25 μM GA-treated cells (Fig. 6A). This inhibitor also increased GSH depleted cell number in GA-treated or -untreated HUVEC (Fig. 6B).

Discussion

Treatment with GA dose-dependently inhibited CPAEC and primary normal HUVEC. The IC_{50} in CPAEC ranged between 25 and 50 μM and that in HUVEC ranged between 200 and 400 μM . According to our unpublished data, an IC_{50} of GA in human pulmonary fibroblast cells was approximately 400 μM and the doses of IC_{50} in HeLa cervical cancer cells, Calu-6 and A549 lung cancer cells were between 30-150 μM . Therefore, the susceptibility of CPAEC to GA was higher than that of normal cells including HUVEC and was similar to that of cancer cells. It has been reported that GA shows lower cytotoxicity against normal fibroblast and ECs than that against cancer cells (24,25). In addition, GA dose-dependently increased Annexin V-FITC positive cells in CPAEC and HUVEC. The percents of Annexin V positive and PI negative (apoptotic) cells were 25.1 and 17.8% in 25 μM GA-treated CPAEC and 200 μM GA-treated HUVEC, respectively. These results suggest that GA-induced ECs death occurred via apoptosis. Although we do not explain the reason of these diverse susceptibilities to GA, Isuzugawa *et al* suggested that the different susceptibilities to GA can depend on the catalase contents of the cell (26).

It has been reported that p38 signaling among MAPKs is involved in drug-induced apoptosis and redox state changes (15-17). Furthermore, p38 inhibitor prevents anisomycin-induced macrophage death (18) and it attenuates pyrogallol-induced CPAEC death (19). However, p38 inhibitor, which presumably inhibited p38 signaling, slightly enhanced growth inhibitions in GA-treated ECs. This inhibitor also increased the number of Annexin V-positive cells in these cells. Moreover, p38 inhibitor alone increased the number of Annexin V positive and PI negative (apoptotic) cells in control ECs. Therefore, the inhibition of p38 signaling by its inhibitor seemed to be a pro-apoptotic function in GA-treated or -untreated ECs.

Cell death induced by GA is associated with mitochondrial dysfunction (10). Correspondingly, GA significantly triggered the loss of MMP ($\Delta\Psi_m$) in ECs. However, GA did not dose-

dependently increase the loss of MMP ($\Delta\Psi_m$) in CPAEC. The levels of MMP ($\Delta\Psi_m$) loss in GA-treated ECs were relatively lower than those of Annexin V-positive cells. In addition, p38 inhibitor did not affect MMP ($\Delta\Psi_m$) loss in GA-treated CPAEC and it reduced the loss in GA-treated HUVEC. Furthermore, p38 inhibitor alone induced MMP ($\Delta\Psi_m$) loss in control CPAEC but it reduced the loss in control HUVEC. Therefore, these results suggest that ECs death by GA and/or p38 inhibitor is not tightly correlated with the loss of MMP ($\Delta\Psi_m$). In particular, the inhibition of p38 seemed to be involved in mitochondrial protection in HUVEC.

GA has been reported to have both pro-oxidant and anti-oxidant properties (12,13). Increasing evidence suggests that apoptosis induced by GA is associated with oxidative stresses derived from ROS (11,27). According to our results, ROS (DCF) level such as H_2O_2 was not altered in GA-treated CPAEC and was decreased in 400 μM GA-treated HUVEC. In addition, intracellular $\text{O}_2^{\cdot-}$ level was decreased in CPAEC treated with 5 μM GA. The $\text{O}_2^{\cdot-}$ level was not changed in 200 μM GA-treated HUVEC and was strongly increased in 400 μM GA-treated HUVEC. These results suggest that GA-induced ECs death is not associated with oxidative stresses derived from ROS. However, we cannot exclude the possibility that GA-induced HUVEC death is related to a strong increase in $\text{O}_2^{\cdot-}$ level. p38 inhibitor seemed to increase ROS (DCF) levels in GA-treated or -untreated CPAEC. It also increased $\text{O}_2^{\cdot-}$ levels in GA-treated CPAEC. These results suggest the possibility that the enhancement of GA-induced CPAEC death by p38 inhibitor is related to an increase in ROS levels. p38 inhibitor also increased ROS levels including $\text{O}_2^{\cdot-}$ in 200 μM GA-treated HUVEC. However, this inhibitor decreased the levels of 400 μM GA in HUVEC, implying that the changes of ROS levels by p38 inhibitor in GA-treated HUVEC are not closely related to cell death. Taken together, GA and/or p38 inhibitor differently influence the changes of ROS levels depending on cell types.

GSH as a main non-protein antioxidant eliminates the $\text{O}_2^{\cdot-}$ and provide electrons for enzymes such as GSH peroxidase, which reduce H_2O_2 to H_2O . It has been reported that the intracellular GSH content has a decisive effect on anticancer drug-induced apoptosis, indicating that apoptotic effects are inversely comparative to GSH content (28,29). Likewise, GA increased the number of GSH depleted cells in ECs. In addition, p38 inhibitor increased GSH depleted cell numbers in 5 μM GA-treated CPAEC and GA-treated HUVEC. These results might be correlated with the results derived from Annexin V assays. However, the levels of GSH depletion in HUVEC were relatively higher than that of CPAEC (especially, in 25 μM GA-treated CPAEC vs. 200 μM GA-treated HUVEC). In addition, p38 inhibitor alone strongly increased GSH depleted cell number in control HUVEC. Our data suggest that intracellular GSH content is not sufficient to correctly predict cell death. The inhibition of p38 signaling seemed to be involved in GSH depletion in GA-treated or -untreated HUVEC.

In conclusion, p38 inhibitor appeared to enhance growth inhibition and death in GA-treated ECs, which were partially related to the changes of ROS and GSH depletion levels.

Acknowledgements

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