SPANDIDOS PUBLICATIONS

Propyl gallate inhibits the growth of endothelial cells, especially calf pulmonary arterial endothelial cells via caspase-independent apoptosis

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Abstract. Propyl gallate (PG) as a synthetic antioxidant exerting a variety of effects on tissue and cell functions. We evaluated the effects of PG on the growth of endothelial cells, especially calf pulmonary artery endothelial cells (CPAEC) in relation to apoptosis. PG dose-dependently inhibited the growth of CPAEC and human umbilical vein endothelial cells (HUVEC) at 24 h. The susceptibility of CPAEC to PG was higher than that of HUVEC. PG induced apoptosis in CPAEC, which was accompanied by the loss of mitochondrial membrane potential (MMP; $\Delta\Psi_m$). The tested caspase inhibitors (pan-caspase, caspase-3, -8 or -9 inhibitor) did not rescue CPAEC from PG-induced cell death but instead slightly enhanced the cell death. PG increased reactive oxygen species (ROS) level in CPAEC. The caspase inhibitors did not significantly change the ROS level. Furthermore, PG increased the GSH depleted cell number and decreased GSH level in CPAEC. The tested caspase inhibitors did not significantly change the number in PG-treated CPAEC. Each caspase inhibitor differently alters GSH levels in CPAEC. In conclusion, PG inhibited the growth of endothelial cells, especially CPAEC via caspase-independent apoptosis. PG-induced CPAEC death was accompanied by ROS increase and GSH depletion.

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Abbreviations: PG, propyl gallate; EC, endothelial cells; CPAEC, calf pulmonary arterial endothelial cells; HUVEC, human umbilical vein endothelial cells; ROS, reactive oxygen species; MMP ($\Delta\Psi_m$), mitochondrial membrane potential; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI, propidium iodide; FITC, fluorescein isothiocyanate; Z-VAD-FMK, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; Z-DEVD-FMK, benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone; Z-IETD-FMK, benzyloxycarbonyl-Ile-Glu-Thr-Asp-fluoro-methylketone; Z-LEHD-FMK, benzyloxycarbonyl-Leu-Glu-His-Asp-fluoro-methylketone; H_2DCFDA, 2',7'-dichlorodihydro-fluorescein diacetate; DHE, dihydroethidium; GSH, glutathione; CMFDA, 5-chloromethyl-fluorescein diacetate

Key words: propyl gallate, apoptosis, endothelial cells, caspase, reactive oxygen species, glutathione

Introduction

Propyl gallate (PG, 3,4,5-trihydroxybenzoic acid propyl ester) is used as a synthetic antioxidant in processed food, cosmetics and food packing materials as maximum concentrations of 0.1%, to prevent rancidity and spoilage (1). PG is also used to preserve and stabilize medicinal preparations on the US Food and Drug Administration list (2). Because of its prevalent usage, the potential toxicity of PG has been investigated *in vivo* (3,4) and *in vitro*, to assess various toxicological properties, i.e., mutagenicity (5) and cytogenetic effects (6). Despite the assumed low toxicity of PG, it exerts a variety of effects on tissue and cell functions. Several studies demonstrate the benefits of PG as an antioxidant (7,8) and a chemopreventive agent (9). For instance, PG is an efficient protector of liver cells from lipid peroxidation by oxygen radicals (4). PG also has protective effects against oxidative DNA damage using 8-oxoguanine formation as a marker (8). In contrast, it is reported that PG exerted prooxidant properties (10,11). PG is cytotoxic to isolated rat hepatocytes because it impairs mitochondria, leading to ATP depletion (12). PG inhibits growth of microorganisms by inhibiting respiration and nucleic acid synthesis (13). Controversially, the effects of PG can be enhancing or suppressing on carcinogenesis and mutagenesis (5,14). Antioxidative and cytoprotective properties of PG may change to prooxidative, cytotoxic and genotoxic properties in the presence of Cu(II) (15). Therefore, in order to clarify the discrepancy between the different effects of PG, further studies need to be performed to re-evaluate its function and safety on cells and tissues.

Vascular endothelium is involved in various regulatory functions such as blood pressure, inflammation and angio-genesis (16). Fundamental to the transition of tumors from a

latent to malignant state, angiogenesis involving formation of new blood vessels from pre-existing vasculature is a crucial part. The proliferation of endothelium cells (ECs; sprouting) is the early step of angiogenesis. Despite critical roles for vascular ECs in tumor biogenesis and progression, the effects of antioxidant compounds, especially PG on ECs remain relatively poorly understood.

In the present study we evaluated the effects of PG on ECs, especially calf pulmonary artery endothelial cells (CPAEC) in relation to cell growth, death, reactive oxygen species (ROS) and glutathione (GSH).

Materials and methods

Cell culture. Calf normal pulmonary artery endothelial cells (CPAEC) from KCLB (Korean Cell Line Bank) and primary human umbilical vein endothelial cells (HUVEC) from PromoCell GmbH (Heidelberg, Germany) were maintained in humidified incubator containing 5% CO_2 at 37˚C. CPAEC were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco BRL, Grand Island, NY). CPAEC were routinely grown in 100-mm plastic tissue culture dishes (Nunc, Roskilde, Denmark) and harvested with a solution of trypsin-EDTA (Gibco BRL) while in a logarithmic phase of growth. CPAEC were maintained in these culture conditions for all experiments. HUVEC were cultured in complete endothelial cell growth medium (ECGM, PromoCell) with 2% FBS. HUVEC were washed and detached with HepesBSS (30 mM Hepes), trypsin-EDTA and trypsin neutralization solution (Promocell). HUVEC were used between passages four and six.

Reagents. PG was purchased from the Sigma-Aldrich Chemical Company (St. Louis, MO). PG was dissolved in ethanol at 200 mM as a stock solution. The pan-caspase inhibitor (Z-VAD-FMK, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone), caspase-3 inhibitor (Z-DEVD-FMK, benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone), caspase-8 inhibitor (Z-IETD-FMK, benzyloxycarbonyl-Ile-Glu-Thr-Asp-fluoromethylketone) and caspase-9 inhibitor (Z-LEHD-FMK, benzyloxycarbonyl-Leu-Glu-His-Asp-fluoromethylketone) were obtained from R&D Systems, Inc. (Minneapolis, MN) and were dissolved in DMSO (Sigma) at 10 mM as a stock solution. Cells were pretreated with caspase inhibitor for 1 h prior to treatment with PG. 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 drugs on endothelial cell numbers and growth was determined by trypan blue cell counting or measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye absorbance of living cells as previously described (17). In brief, $1x10^5$ cells per well were seeded in 24-well plates (Nunc) for cell counting, and cells $3x10^4$ cells per well were seeded in 96-well microtiter plates for an MTT assay. After exposure to doses of 50-800 μM PG for 24 h, cells in 24-well plates or 96-well plates were collected with trypsin digestion for trypan blue exclusion cell counting or for an MTT assay. MTT (20 μl) (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). Each plate contained multiple wells at a given experimental condition and multiple control wells. This procedure was replicated for 2 to 4 plates per condition.

Sub-G1 cell analysis. Sub-G1 cells were determined by propidium iodide (PI, Sigma-Aldrich; Ex/Em=488/617 nm) staining as previously described (18). PI is a fluorescent biomolecule that can be used to stain DNA. In brief, $1x10^6$ cells in 60-mm culture dish (Nunc) were incubated with the indicated amounts of PG with or without 15 μM each caspase inhibitor for 24 h. Cells were then washed with PBS and fixed in 70% ethanol. Cells were washed again with PBS, then incubated with PI (10 μg/ml) with simultaneous RNase treatment at 37˚C for 30 min. Cell DNA content was measured using a FACStar flow cytometer (Becton Dickinson, San Jose, CA) and analyzed using Lysis II and CellFIT software (Becton Dickinson) or ModFit software (Verity Software House, Inc., ME).

Annexin V staining. Apoptosis was determined by staining cells with annexin V-fluorescein isothiocyanate (FITC) (Ex/Em=488/519 nm) as previously described (19). In brief, $1x10^6$ cells in 60-mm culture dish (Nunc) were incubated with the indicated amounts of PG with or without 15 μM each caspase 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 $CaCl_2$) at a concentration of $1x10^6$ cells/ml. Annexin V-FITC (5 μl) (PharMingen, San Diego, CA) was then added to these cells, which were analyzed with a FACStar flow cytometer (Becton Dickinson).

DNA extraction and agarose gel electrophoresis. DNA fragmentation was determined using a commercial kit (Quick Apoptotic DNA ladder detection kit, BioVision, CA). In brief, $1x10^6$ cells in 60-mm culture dish (Nunc) were incubated with or without 800 μM PG for 24 h. Cells were lysed in 35 μl TE Lysis butter, incubated with 5 μl Enzyme A solution at 37˚C for 10 min, and then incubated with 5 μl Enzyme B solution at 50˚C for 30 min. DNA was then precipitated with isopropanol and resuspended in 30 μl DNA suspension buffer. DNA were electrophoresed in Tris-borate buffer on 1.2% agarose gel and stained with ethidium bromide.

Measurement of mitochondrial membrane potential (MMP) ($\Delta\Psi_m$). MMP ($\Delta\Psi_m$) levels were measured by the Rhodamine 123 fluorescent dye (Ex/Em=485/535 nm), as previously described (20). In brief, $1x10^6$ cells in 60-mm culture dish (Nunc) were incubated with the indicated amounts of PG with or without 15 μM each caspase inhibitor for 24 h. 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 determined by flow cytometry. Rhodamine 123 negative cells indicate the loss of MMP ($\Delta\Psi_m$) in CPAEC. MMP ($\Delta\Psi_m$) levels in cells except MMP

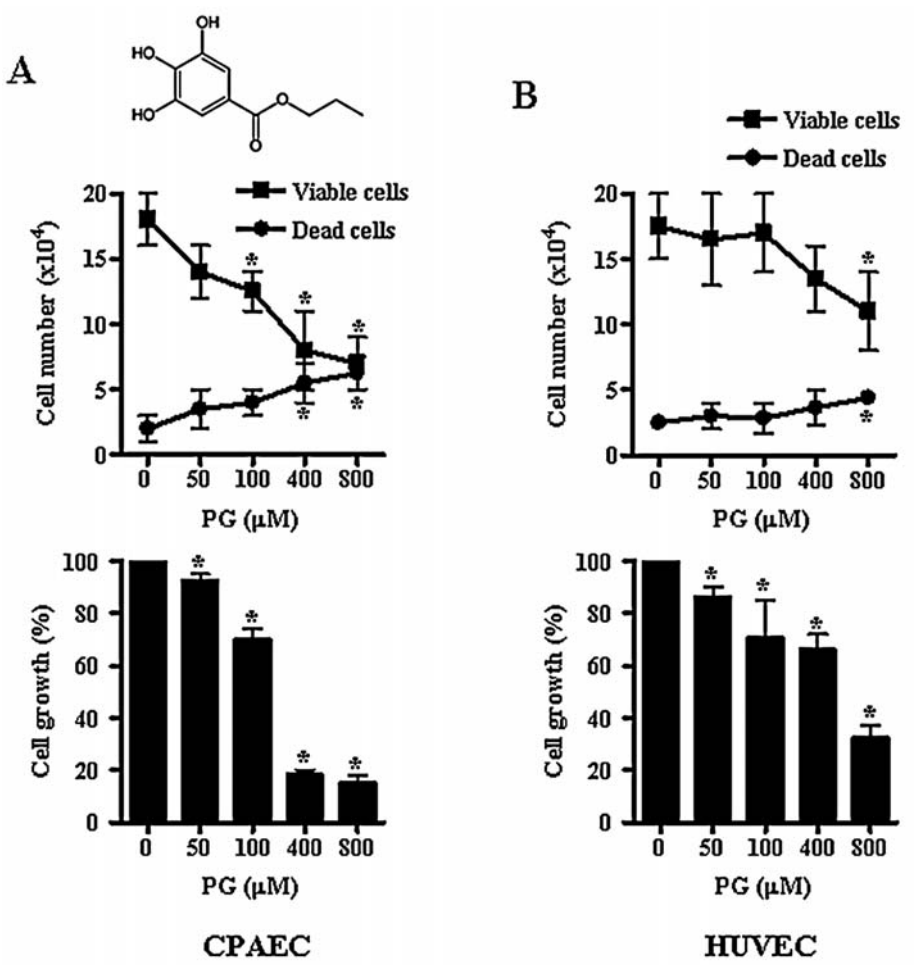

Figure 1. Effects of PG on the growth of CPAEC and HUVEC *in vitro*. Exponentially-growing cells were treated with the indicated concentrations of PG for 24 h. Cell number and cell growth were assessed by trypan blue exclusion cell counting and an MTT assay, respectively. (A) Structure of propyl gallate (PG) and CPAEC. (B) HUVEC. *P<0.05 compared with the PG-untreated control cell group.

($\Delta\Psi_m$) 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; Ex/Em=495/529 nm) (21) (Invitrogen Molecular Probes, Eugene, OR). H_2DCFDA is poorly selective for $O_2^{\bullet-}$. In contrast, dihydroethidium (DHE) (Ex/Em=518/605 nm) (Invitrogen Molecular Probes) is highly selective for $O_2^{\bullet-}$ among ROS. In brief, 1x10^6 cells in 60-mm culture dish (Nunc) were incubated with 400 μM PG with or without 15 μM each caspase inhibitor for 24 h. Cells were then washed in PBS and incubated with 20 μM H_2DCFDA or DHE at 37˚C for 30 min according to the manufacturers instructions. DCF and DHE fluorescences were detected using a FACStar flow cytometer (Becton Dickinson). ROS and $O_2^{\bullet-}$ levels were expressed as mean fluorescence intensity (MFI), which was calculated by CellQuest software.

Detection of the intracellular glutathione (GSH). Cellular GSH levels were analyzed using 5-chloromethylfluorescein diacetate (CMFDA, Molecular Probes) (Ex/Em=522/595 nm) as previously described (21). In brief, 1x10^6 cells in 60-mm culture dish (Nunc) were incubated with 400 μM PG with or without 15 μM each caspase 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). Negatively CMF staining (GSH depleted) cells were expressed as the percent of (-) CMF cells. CMF levels in cells except GSH depleted cells were expressed as mean fluorescence intensity (MFI), which was calculated by CellQuest software.

Statistical analysis. The results shown in the figures represent the mean of at least three independent experiments; bar, SD. The data were analyzed using Instat software (GraphPad Prism4, San Diego, CA). The Student's t-test or one-way analysis of variance (ANOVA) with post hoc analysis using Tukey's multiple comparison test was used for parametric data. The statistical significance was defined as P<0.05.

Results

Effects of PG on the growth of CPAEC and HUVEC. We examined the effect of PG on the growth of CPAEC and HUVEC by trypan blue cell counting. Treatment with 50-800 μM PG significantly decreased the population of

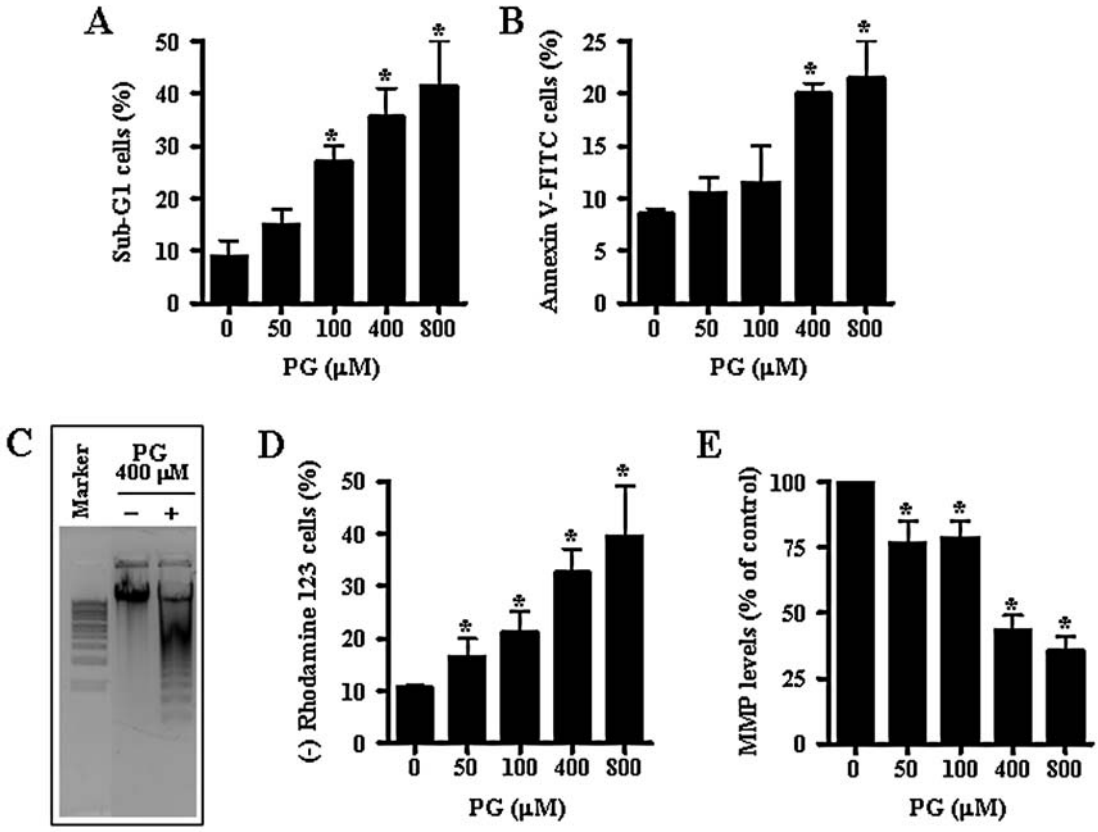

Figure 2. Effects of PG on apoptosis and MMP ($\Delta\Psi_m$) in CPAEC. Exponentially-growing cells were treated with the indicated concentrations of PG for 24 h. (A and B) Graphs show the percents of sub-G1 cells (A) and annexin V-positive cells (B). (C) Analysis of DNA fragmentation patterns by 1.2% agarose gel electrophoresis. (D and E) The graphs show the percent of Rhodamine 123-negative [MMP ($\Delta\Psi_m$) loss] cells (D) and MMP ($\Delta\Psi_m$) levels (%) compared with control CPAEC (E). *P<0.05 compared with the PG-untreated control group.

viable (trypan blue negative) CPAEC at 24 h in a dose-dependent manner (Fig. 1A). However, only the dose of 800 μM PG reduced viable cell numbers of HUVEC at the same time (Fig. 1B). The dead (trypan blue positive) cell numbers of CPAEC and HUVEC dose-dependently increased (Fig. 1A and B). The dead cell number was higher in CPAEC. Collectively, the ratio of dead cells to viable cells was increased in both cell types. When the growth of CPAEC and HUVEC after treatment with PG was also assessed by an MTT assay, dose-dependent reduction of cell growth was observed in both cells with an IC_{50} of ~100-400 μM and 400-800 μM PG at 24 h, respectively (Fig. 1A and B).

Effects of PG on apoptosis and MMP ($\Delta\Psi_m$) in CPAEC. We determined whether PG induces apoptosis in CPAEC. As shown in Fig. 2A, the proportion of sub-G1 DNA content cells in PG-treated CPAEC was increased in a dose-dependent manner. The number of annexin V-staining cells was also increased (Fig. 2B). Furthermore, we observed that PG induced nucleosomal DNA fragmentation, which is a hallmark of apoptosis in mammalian cells, in CPAEC (Fig. 2C). These data imply that PG-induced CPAEC death occurred via apoptosis.

Next, we elucidated the effect of PG on MMP ($\Delta\Psi_m$) using Rhodamine 123. Treatment with PG induced the loss of MMP ($\Delta\Psi_m$) in CPAEC in a dose-dependent manner (Fig. 2D). Following exposure to 400 μM PG for 24 h, the percent of MMP ($\Delta\Psi_m$) loss cells was increased ~20% compared with that of PG-untreated control cells (Fig. 2D). In relation to MMP ($\Delta\Psi_m$) levels in cells except Rhodamine 123 negative cells, PG reduced MMP ($\Delta\Psi_m$) level in CPAEC (Fig. 2E). Treatment with 400 μM PG decreased the level ~60% (Fig. 2E). The dramatic decrease in MMP ($\Delta\Psi_m$) levels was observed at doses >100 μM PG-treated cells (Fig. 2E).

Effects of caspase inhibitors on cell growth and PG-treated CPAEC. To determine which caspase is involved in the growth inhibition and apoptosis of CPAEC by PG, cells were pre-treated with pan-caspase inhibitor (Z-VAD-FMK), caspase-3 inhibitor (Z-DEVD-FMK), caspase-8 inhibitor (Z-IETD-FMK) or caspase-9 inhibitor (Z-LEHD-FMK) at a concentration of 15 μM before 400 PG μM treatment, which concentration was considered as a suitable dose to differentiate the levels of cell growth inhibition and death in the presence or absence of each caspase inhibitor. None of the caspase inhibitors including pan-caspase inhibitor significantly affected the growth of PG-treated CPAEC at 24 h, as measured by an MTT assay (Fig. 3A). All of them slightly reduced the growth of PG-treated CPAEC (Fig. 3A). They also slightly increased the population of sub-G1 cells in PG-treated CPAEC but mildly reduced the numbers of PG-untreated control CPAEC (Fig. 3B). Treatment with pan-caspase, caspase-3 and

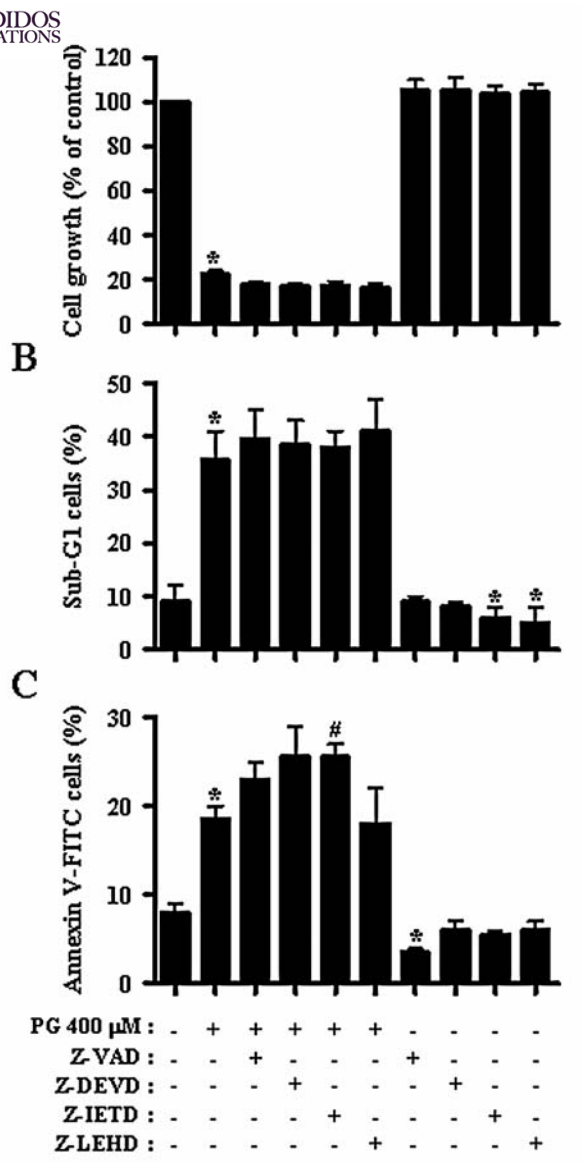

Figure 3. Effects of caspase inhibitors on the cell growth and apoptosis in PG-treated CPAEC. Exponentially-growing cells were treated with 400 μM PG and/or each caspase inhibitor (15 μM) for 24 h. (A) Graph shows the cell growth, as assessed by an MTT assay. (B and C) Graphs show the percent of sub-G1 cells (B) and annexin V staining cells (C). *P<0.05 compared with the control group. #P<0.05 compared with cells treated with PG only. Z-VAD, pan-caspase inhibitor; Z-DEVD, caspase-3 inhibitor; Z-IETD, caspase-8 inhibitor; Z-LEHD, caspase-9 inhibitor.

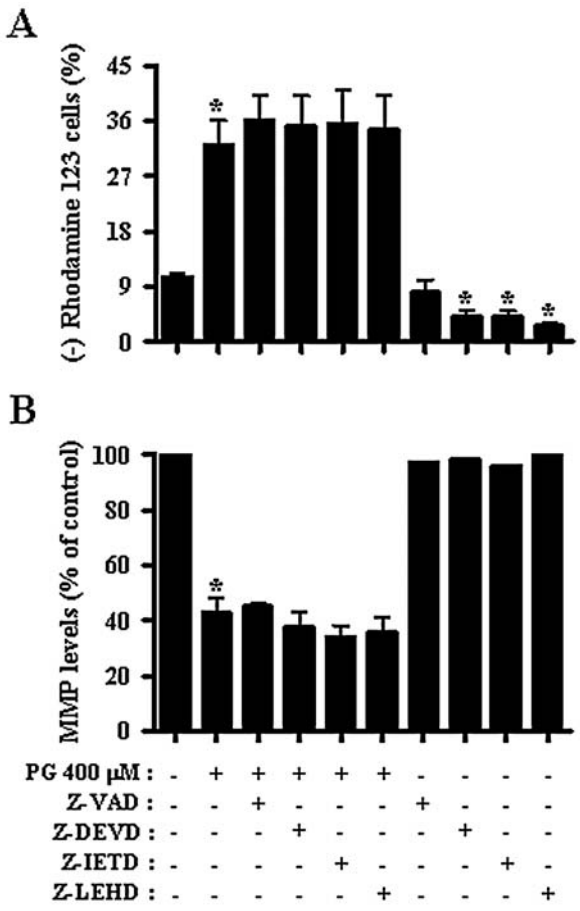

Figure 4. Effects of caspase inhibitors on MMP ($\Delta\Psi_m$) in PG-treated CPAEC. Exponentially-growing cells were treated with 400 μM PG and/or each caspase inhibitor (15 μM) for 24 h. (A and B) The graphs show the percent of Rhodamine 123 negative [MMP ($\Delta\Psi_m$) loss] cells (A) and MMP ($\Delta\Psi_m$) levels (B) in CPAEC, as measured with a FACStar flow cytometer. *P<0.05 compared with the control group.

caspase-8 inhibitors increased annexin V-FITC positive cell numbers in PG-treated CPAEC. All the inhibitors reduced basal annexin V staining cells in control CPAEC (Fig. 3C).

In relation to MMP ($\Delta\Psi_m$) in CPAEC, all the inhibitors slightly enhanced the loss of MMP ($\Delta\Psi_m$) induced by PG (Fig. 4A). However, they reduced the percent of basal MMP ($\Delta\Psi_m$) loss in control CPAEC (Fig. 4A). All the caspase inhibitors except Z-VAD inhibitor slightly reduced MMP ($\Delta\Psi_m$) levels in PG-treated CPAEC (Fig. 4B).

Effects of caspase inhibitors on ROS and GSH levels in PG-treated CPAEC. We assessed the changes of the intracellular ROS levels in PG and/or each caspase inhibitor-treated CPAEC. As shown in Fig. 5A, the intracellular ROS (DCF) levels were significantly increased in CPAEC treated with PG at 24 h. The caspase inhibitors did not significantly change the ROS level in PG-treated CPAEC (Fig. 5A). Only pan-caspase inhibitor slightly increased the level (Fig. 5A). All the caspase inhibitors except caspase-3 inhibitor seemed to increase ROS level in control CPAEC (Fig. 5A). The level of red fluorescence derived from DHE, which reflected $O_2^{\bullet-}$ accumulation, was not altered in PG-treated CPAEC at 24 h (Fig. 5B). All the caspase inhibitors except caspase-9 inhibitor increased $O_2^{\bullet-}$ level in PG-treated CPAEC (Fig. 5B). Treatment with Z-VAD and caspase-9 inhibitor decreased $O_2^{\bullet-}$ level in control CPAEC (Fig. 5B).

Next, we analyzed the changes of GSH levels in CPAEC in the presence of PG and/or each caspase inhibitor. Treatment with PG increased the number of GSH depleted cells in CPAEC cells ~36% compared with PG-untreated control cells (Fig. 5C). The tested caspase inhibitors did not significantly affected GSH depletion in PG-treated CPAEC (Fig. 5C). Furthermore, when CMF (GSH) levels in CPAEC except negative CMF staining cells were assessed, GSH level was decreased in PG-treated CPAEC. While caspase-3

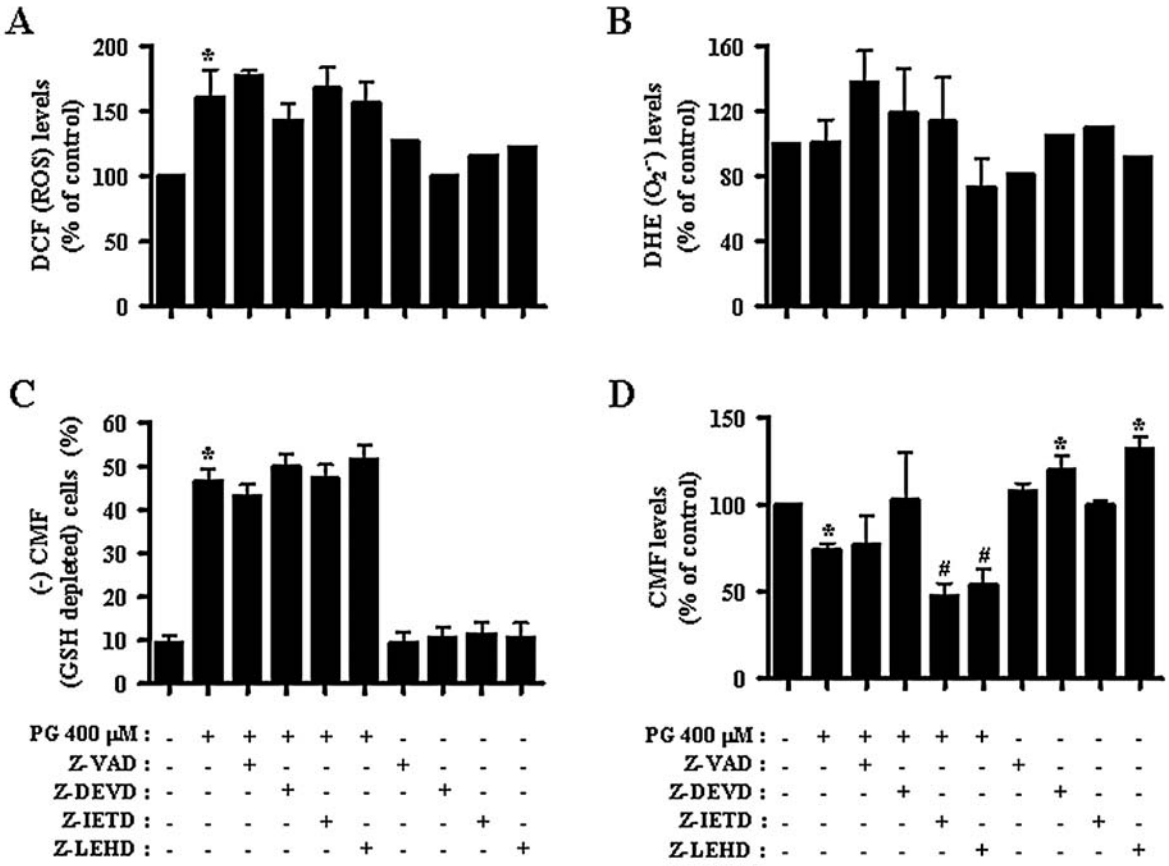

Figure 5. Effects of caspase inhibitors on ROS and GSH levels in PG-treated CPAEC. Exponentially-growing cells were treated with 400 μM PG and/or each caspase inhibitor (15 μM) for 24 h. ROS and GSH levels in CPAEC were measured using a FACStar flow cytometer. (A and B) Graphs indicate DCF (ROS) levels (%) compared with control CPAEC at 24 h (A) and DHE ($O_2^{\bullet-}$) levels (%) compared with control CPAEC at 24 h (B). (C and D) Graphs show the percent of (-) CMF (GSH depleted) cells (C) and mean CMF (GSH) levels compared with control cells (D). *P<0.05 compared with the control group. #P<0.05 compared with cells treated with PG only.

inhibitor increased GSH level in PG-treated CPAEC, caspase-8 and -9 inhibitors significantly intensified the reduced GSH level (Fig. 5D). All the caspase inhibitors except caspase-8 inhibitor increased basal GSH level in control CPAEC (Fig. 5D).

Discussion

In the present study, we focused on evaluating the effects of PG on the growth of endothelial cells, especially CPAEC in relation to apoptosis. PG decreased the population of CPAEC and HUVEC. The ratio of dead cells to viable cells was increased after treatment with PG compared with PG-untreated cells. PG also inhibited the growth of CPAEC and HUVEC in view of an MTT assay. The susceptibility of CPAEC to PG was higher than that of HUVEC. In addition, PG induced apoptosis in CPAEC as evidenced by sub-G1 cells, annexin V staining cells and DNA fragmentation. However, treatment with 400 μM PG, which significantly triggered apoptosis in CPAEC, did not increase annexin V staining cell number in HUVEC (data not shown).

Apoptosis is closely related to the collapse of MMP ($\Delta\Psi_m$) (22). It was reported that PG is cytotoxic to isolated rat hepatocytes because it impairs mitochondria, leading to ATP depletion (12). Correspondingly, PG dose-dependently induced the loss of MMP ($\Delta\Psi_m$) and reduced MMP ($\Delta\Psi_m$) level in CPAEC. However, PG did not strongly trigger MMP ($\Delta\Psi_m$) loss but reduced MMP ($\Delta\Psi_m$) level in HUVEC (data not shown). The difference of susceptibility to PG between these endothelial cells is probably due to the different basal activities of mitochondria and antioxidant enzymes in each cell line. In fact, a number of studies have demonstrated that the activities of mitochondria and antioxidant enzymes are quite differentially regulated depending on cell type, tissue origin and species (23,24), and are associated with a susceptibility to apoptosis (25,26). Collectively, our results demonstrate that PG inhibits the growth of endothelial cells, especially CPAEC via apoptosis and MMP ($\Delta\Psi_m$) loss.

To determine which caspases are required for the induction of apoptosis, PG-treated CPAEC were incubated with various caspase inhibitors. Unexpectedly, all the tested caspase inhibitors slightly reduced the growth of PG-treated CPAEC, and some of caspase inhibitors mildly enhanced apoptosis in these cells. All the inhibitors also increased MMP ($\Delta\Psi_m$) loss. These data suggest that CPAEC death induced by PG is not dependent on the activation of caspase and can be triggered to some extent via necrosis. In particular, inhibitor of caspase-8, which is related to cell death receptor pathway in apoptosis (27,28), significantly increased annexin-V staining cell numbers in PG-treated CPAEC. The slight

ment of cell death in PG-treated CPAEC by caspase probably resulted from the increased necrotic cell death after the hindrance of apoptotic pathway by them. Since we recently observed that all the caspase inhibitors tested in this experiment significantly prevented PG-induced apoptosis in HeLa cells (unpublished data), the requirement of caspase activation for PG-induced apoptosis is dependent on cell types.

PG can play a role as an antioxidant (4,7,8) or a pro-oxidant (10,11). Increasing evidence suggests that oxidative stress regulates 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 (29,30). According to our results, the intracellular ROS (DCF) levels were significantly increased in CPAEC treated with PG. In contrast, PG did not increase $O_2^{\bullet-}$ (DHE) levels. These results suggest that PG can affect the levels of different ROS even in the same cell type and also suggest a possibility that ROS changes by PG is involved to some extent in CPAEC death. The caspase inhibitors did not significantly change ROS (DCF) level in PG-treated CPAEC. However, pan-caspase, caspase-3 and -8 inhibitors increased $O_2^{\bullet-}$ level in PG-treated CPAEC whereas caspase-9 inhibitor decreased $O_2^{\bullet-}$ level without preventing apoptosis. Therefore, these data suggest that the changes of ROS levels by each caspase inhibitor are not tightly related to CPAEC death by PG. The exact role of ROS in PG-induced cell death of CPAEC needs to be defined further.

GSH is a main non-protein antioxidant in cells. It is able to eliminate the $O_2^{\bullet-}$ 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 (31,32). Likewise, PG increased GSH depleted cell number and decreased GSH level in CPAEC. All the tested caspase inhibitors did not affect the number in PG-treated CPAEC. This result is probably correlated to the fact that these inhibitors could not prevent CPAEC death. In addition, caspase-3 inhibitor increased GSH level in PG-treated CPAEC whereas caspase-8 and -9 inhibitors significantly intensified the reduced GSH level. All the caspase inhibitors except caspase-8 inhibitor increased basal GSH level in control CPAEC. These results suggest that each caspase inhibitor differently regulate intracellular GSH levels.

In conclusion, PG inhibited the growth of endothelial cells, especially CPAEC via caspase-independent apoptosis. PG-induced CPAEC death was accompanied by ROS increase and GSH depletion. Since little is known about the relationship between PG and endothelial cells, our preliminary results provide the first information on the molecular anti-growth mechanisms of PG in ECs.

Acknowledgements

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