

Exogenous H₂O₂ induces growth inhibition and cell death of human pulmonary artery smooth muscle cells via glutathione depletion

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Received July 8, 2015; Accepted May 10, 2016

DOI: 10.3892/mmr.2016.5307

Abstract. Reactive oxygen species (ROS) are associated with various pathophysiological processes of vascular smooth muscle cells (VSMCs). Pyrogallol (PG) induces the superoxide anion (O₂^{•-})-mediated cell death of numerous cell types. The present study aimed to investigate the effects of exogenous hydrogen peroxide (H₂O₂) and PG treatment on the cell growth and death of human pulmonary artery smooth muscle cells (HPASMCs), with regards to intracellular ROS and glutathione (GSH) levels, as determined by MTT and cell number assays. H₂O₂ led to reduced growth of HPASMCs, with a half maximal inhibitory concentration of 250-500 μM at 24 h, and induced apoptosis, as determined by Annexin V-staining and benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone treatment. However, PG did not strongly induce growth inhibition and death of HPASMCs. In addition, H₂O₂ led to increased ROS levels, including mitochondrial O₂^{•-}, and induced GSH depletion in HPASMCs. Treatment with N-acetyl cysteine (NAC) attenuated apoptotic cell death and ROS levels in H₂O₂-treated HPASMCs, and also prevented GSH depletion. Notably, PG treatment did not increase ROS levels, including mitochondrial O₂^{•-}. Furthermore, NAC induced a significant increase in mitochondrial O₂^{•-} levels in PG-treated HPASMCs, and cell death and GSH depletion were significantly increased. L-buthionine sulfoximine intensified cell death and GSH

depletion in PG-treated HPASMCs. In conclusion, exogenous H₂O₂ induced growth inhibition and cell death of HPASMCs via GSH depletion.

Introduction

Reactive oxygen species (ROS) are a class of oxygen-derived molecules, which include hydrogen peroxide (H₂O₂), superoxide anion (O₂^{•-}) and hydroxyl radical (•OH). These elemental molecules are considered to be deleterious or harmful to cells and tissues; however, it has been reported that ROS may regulate several cellular events, including gene expression, differentiation and cell proliferation (1,2). In addition, ROS may act as secondary messengers to manipulate distinct signal transduction pathways in the cardiovascular and pulmonary systems (3,4). ROS are usually generated as by-products of mitochondrial respiration; however, they may also be specifically produced by various oxidases (5). The major metabolic pathways contain superoxide dismutases, which metabolize O₂^{•-} to H₂O₂ (6). Further metabolism by catalase or glutathione (GSH) peroxidase, produces O₂ and H₂O (7). Cells possess diverse antioxidant systems to control their redox state, which is important for the balance between cell survival and death. Oxidative stress may be the result of overproduction of ROS or downregulation of antioxidants, which induces irreversible alterations to proteins, lipids and DNA, resulting in cell death and tissue damage (8-10).

Vascular smooth muscle cells (VSMCs) in the medial layer of blood vessels are a dynamic component of the vascular system. When these cells are cultured in normal media, they exhibit a contractile phenotype for the regulation of blood pressure. In response to pathological stimuli, VSMCs may undergo hypertrophy or proliferation, which leads to various vascular diseases, including hypertension, restenosis and atherosclerosis (3,4). VSMCs contain several sources of ROS, including NADPH oxidase and mitochondrial respiration. In VSMCs, ROS mediate several pathophysiological processes, including growth, migration, apoptosis and secretion of inflammatory cytokines, and physiological processes at numerous signaling levels (3,4). Particularly relevant to the pulmonary vascular system is modulation of ROS levels by tissue oxygen concentration (4). ROS induce an increase in intracellular calcium

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Abbreviations: HPASM, human pulmonary artery smooth muscle; PG, pyrogallol; ROS, reactive oxygen species; FITC, fluorescein isothiocyanate; CMFDA, 5-chloromethylfluorescein diacetate; Z-VAD-FMK, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; H₂DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; NAC, N-acetyl cysteine; BSO, L-buthionine sulfoximine; GSH, glutathione

Key words: smooth muscle cell, H₂O₂, pyrogallol, cell death, ROS

concentration and contraction in human pulmonary artery smooth muscle cells (HPASMCs), consequently contributing to the cellular response induced by various vasoconstrictor stimuli, including hypoxia (4). ROS are involved in the development of pulmonary hypertension, ultimately inducing right ventricular failure, which may result in fatality (4). Therefore, it is critical to understand the various functions of ROS in the physiology and pathophysiology of VSMCs. In particular, an improved understanding of how ROS regulate proliferation and apoptosis of VSMCs may allow for the development of novel strategies to treat or prevent vascular diseases.

Pyrogallol (PG; benzene-1,2,3-triol) is derived from hardwood plants. Due to its capability to generate free radicals, PG is frequently used to investigate the function of O₂^{•-} in several biological systems (11-13). For example, PG induces O₂^{•-}-mediated cell death in various types of cancer, including lung, gastric and cervical cancer (13-16). However, to the best of our knowledge, the effects of PG on normal VSMCs have not yet been elucidated. PG-induced cytotoxicity in VSMCs *in vitro* may be of interest for toxicological research, considering the toxic potential of PG on VSMCs. In the present study, the effects of exogenous H₂O₂ and PG on the cell growth and death of HPASMCs were investigated, with regards to changes in intracellular ROS and GSH levels. In addition, the effects of N-acetyl cysteine (NAC; an established antioxidant) and L-buthionine sulfoximine (BSO; an inhibitor of GSH synthesis) were examined on H₂O₂ or PG-induced HPASMC death.

Materials and methods

Cell culture. The primary HPASMCs were obtained from PromoCell GmbH (Heidelberg, Germany) and were maintained in a humidified incubator containing 5% CO₂ at 37°C. HPASMCs were cultured in Complete Smooth Muscle Cell Growth Medium 2 (PromoCell GmbH). The cells were grown in 100-mm plastic tissue culture dishes (Nunc; Sigma-Aldrich, St. Louis, MO, USA), and were washed and detached with 30 mM HEPES buffered saline solution, trypsin-EDTA and trypsin neutralization solution (PromoCell GmbH). HPASMCs between passages four and six were used for subsequent experiments.

Reagents. H₂O₂ and PG were purchased from Sigma-Aldrich. PG was dissolved in water. The Pan-caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-FMK) was obtained from R&D Systems, Inc. (Minneapolis, MN, USA) and was dissolved in dimethyl sulfoxide (Sigma-Aldrich). NAC and BSO were obtained from Sigma-Aldrich. NAC was dissolved in buffer [20 mM HEPES (pH 7.0)] and BSO was dissolved in water. Based on previous studies (14,17), cells were pretreated with or without 15 μM Z-VAD-FMK, 2 mM NAC or 10 μM BSO for 1 h at 37°C prior to treatment with H₂O₂ or PG.

Cell growth and cell number assays. The growth rate of HPASMCs treated with H₂O₂ or PG was indirectly determined according to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) dye absorbance, as previously described (18). Changes in viable

and dead cell counts were determined by trypan blue cell counting. Briefly, 5.0x10³ cells/well were seeded in 96-well microtiter plates (Nunc; Sigma-Aldrich) for the MTT assays, and 2x10⁵ cells/well were seeded in 24-well plates (Nunc; Sigma-Aldrich) for cell counting. Following exposure to the indicated concentrations of H₂O₂ or PG (0, 100, 250, 500, 750 and 1,000 μM) for 24 h at 37°C, cells in the 96-well plates were used for MTT assays, and cells in the 24-well plates were collected with trypsin for trypan blue cell counting.

Annexin V-fluorescein isothiocyanate (FITC) staining for cell death detection. Apoptosis was determined by staining cells with Annexin V-FITC (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA; excitation/emission=488/519 nm) as described previously (19). Cells were incubated with the indicated concentrations of H₂O₂ or PG (0, 100, 250, 500, 750 and 1,000 μM) for 24 h at 37°C in the presence or absence of Z-VAD-FMK, NAC or BSO. Annexin V-FITC staining was analyzed using a FACStar flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Detection of intracellular ROS levels. Intracellular ROS levels were detected using the oxidation-sensitive fluorescent probe, 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA; excitation/emission=495/529 nm; Invitrogen; Thermo Fisher Scientific, Inc.) as described previously (19). Briefly, 1.0x10⁶ cells/ml were aliquoted in a flow cytometer tube (BD Biosciences) and were treated with 500 μM H₂O₂ or PG in the presence of 20 μM H₂DCFDA at 37°C. The level of dichlorofluorescein (DCF) fluorescence was evaluated using a FACStar flow cytometer at 0, 10, 30, 60, 120 and 180 min. DCF (ROS) levels were expressed as mean fluorescence intensity (MFI). The levels of mitochondrial O₂^{•-} were specifically detected using MitoSOX Red mitochondrial O₂^{•-} indicator (excitation/emission=510/580 nm; Invitrogen; Thermo Fisher Scientific, Inc.) as previously described (20). Briefly, 1.0x10⁶ cells in a 60-mm culture dish were incubated with the indicated concentrations of H₂O₂ or PG (0, 100, 250, 500, 750 and 1,000 μM) at 37°C for 24 h in the presence or absence of Z-VAD-FMK or NAC. Cells were incubated with 5 μM MitoSOX Red at 37°C for 30 min. MitoSOX Red fluorescence was assessed using a FACStar flow cytometer and the levels were expressed as MFI.

Detection of intracellular GSH levels. GSH levels were analyzed using a 5-chloromethylfluorescein diacetate dye (CMFDA; excitation/emission=522 nm/595 nm; Invitrogen; Thermo Fisher Scientific, Inc.) as previously described (18,19). Briefly, 1.0x10⁶ cells/ml were aliquoted in a flow cytometer tube (BD Biosciences) and were treated at 37°C with 500 μM H₂O₂ or PG in the presence of 5 μM CMFDA. The level of 5-chloromethyl-fluorescein (CMF) fluorescence was evaluated using a FACStar flow cytometer at the indicated times (0, 10, 30, 60, 120 and 180 min). CMF (GSH) levels were expressed as MFI. In addition, 1.0x10⁶ cells in a 60-mm culture dish were incubated with the indicated amounts of H₂O₂ or PG (0, 100, 250, 500, 750 and 1,000 μM) for 24 h at 37°C in the presence or absence of Z-VAD-FMK, NAC or BSO. Following the treatment, cells were incubated with 5 μM CMFDA at 37°C for 30 min. CMF fluorescence was assessed using a FACStar flow cytometer. Negative

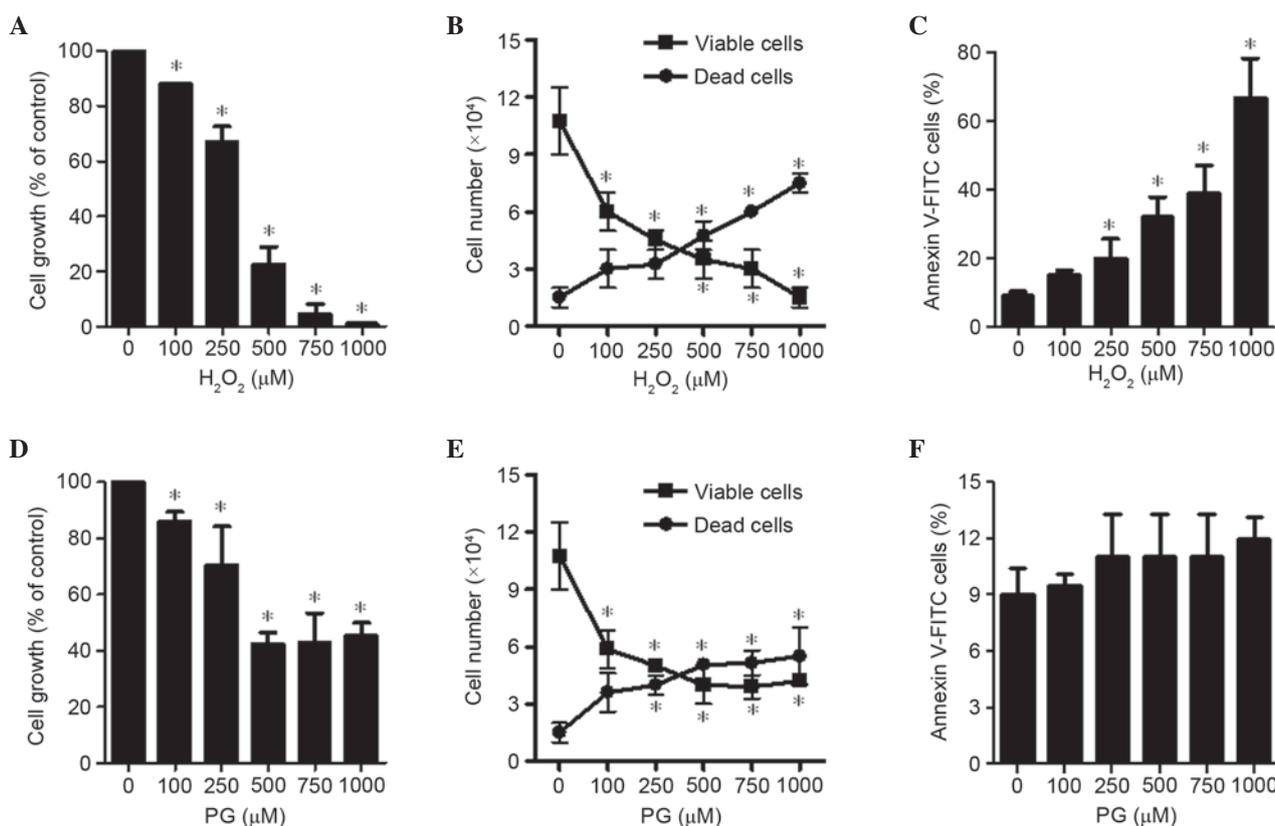


Figure 1. Effects of (A-C) H₂O₂ and (D-F) PG on cell growth and death in human pulmonary artery smooth muscle cells. (A and D) Cellular growth changes, as assessed by MTT assays. (B and E) Number of viable and dead cells, as assessed by trypan blue exclusion cell counting. (C and F) Percentages of Annexin V-FITC positive cells, as measured using a FACStar flow cytometer. Data are presented as the mean ± standard deviation. *P<0.05 vs. the control group. H₂O₂, hydrogen peroxide; PG, pyrogallol; FITC, fluorescein isothiocyanate.

CMF staining (GSH depletion) of cells is expressed as the percentage of (-) CMF cells.

Statistical analysis. Data are presented as the mean ± standard deviation of three independent experiments. Data were analyzed using InStat software, version 5 (GraphPad Software, Inc., La Jolla, CA, USA). Student's t-test, or one-way analysis of variance with Tukey's honest significant difference test as post-hoc analysis, was used to determine if there was a significant difference between the means of various treatment groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of H₂O₂ and PG on cell growth and death of HPASMCs.

The effects of H₂O₂ and PG were examined on HPASMCs 24 h after treatment. Treatment with H₂O₂ led to a dose-dependent inhibition of HPASMCs with a half maximal inhibitory concentration (IC₅₀) of 250-500 μM (P=0.006; Fig. 1A). In addition, as the concentration of H₂O₂ increased from 100 to 1,000 μM the population of viable (trypan blue-negative) HPASMCs was significantly reduced, whereas the number of dead (trypan blue-positive) cells increased in a dose-dependent manner (P<0.001; Fig. 1B). The ratio of dead cells to viable cells was increased by H₂O₂ treatment. Furthermore, the number of Annexin V-stained cells was increased in a dose-dependent manner (Fig. 1C). When HPASMCs were exposed to 500 μM

PG, their growth was decreased by ~50% (P<0.001; Fig. 1D). However, this effect was not dose-dependent, since 750 and 1,000 μM PG did not inhibit cell proliferation to the same extent as 500 μM PG (Fig. 1D). In addition, PG increased the ratio of dead to viable cells; however, higher doses of PG did not additionally increase the ratio (Fig. 1E). The doses of PG used did not significantly increase the proportion of Annexin V-stained cells (Fig. 1F).

Effects of H₂O₂ and PG on ROS levels in HPASMCs. To assess intracellular ROS levels in H₂O₂ and PG-treated HPASMCs, H₂DCFDA and MitoSOX Red dyes were used. Treatment with 500 μM H₂O₂ increased ROS (DCF) levels gradually from 10 until 60 min, followed by a reduction (Fig. 2A). In addition, H₂O₂ treatment led to significantly increased levels of mitochondrial O₂^{•-}, as detected by MitoSOX Red dye, in a dose-dependent manner (P=0.031; Fig. 2B). Conversely, 500 μM PG did not increase ROS (DCF) levels in HPASMCs at the 10, 30 or 60 min time points, and ROS levels were significantly decreased at 120 and 180 min (P=0.047; Fig. 2C). In addition, the levels of mitochondrial O₂^{•-} were significantly decreased following treatment with PG (P=0.013; Fig. 2D).

Effects of Z-VAD-FMK, NAC or BSO on cell death and ROS levels in H₂O₂ or PG-treated HPASMCs. For this experiment, 500 μM H₂O₂ and PG was selected as a suitable dose to differentiate the levels of cell death in the presence or absence of Z-VAD-FMK, NAC or BSO. Application of Z-VAD-FMK

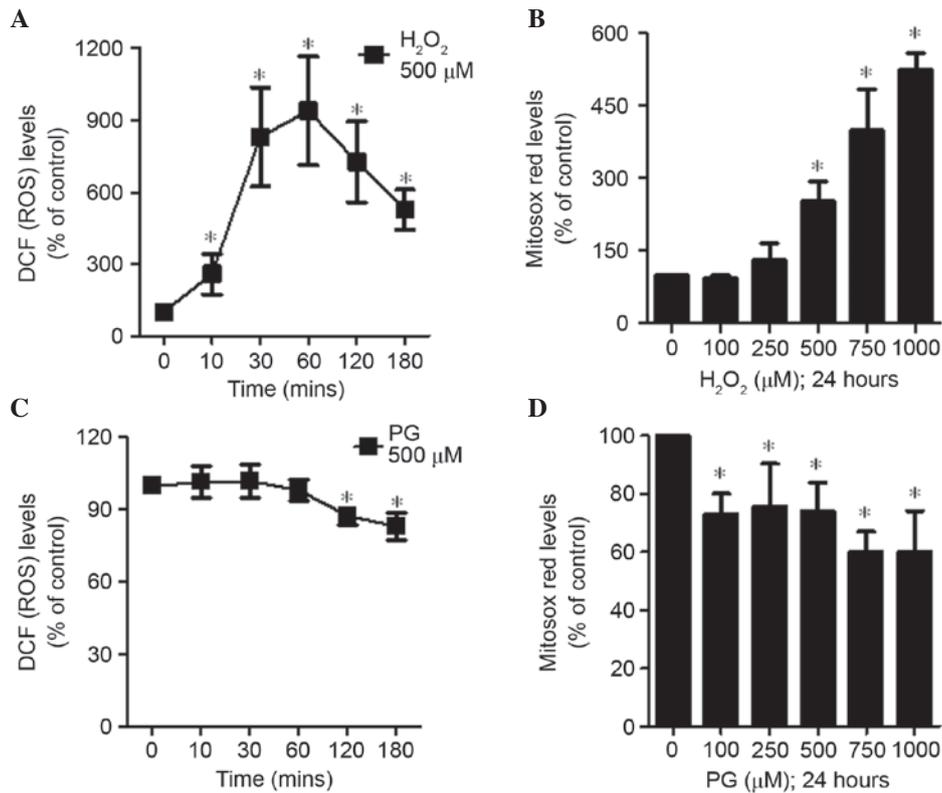


Figure 2. Effects of (A and B) H₂O₂ and (C and D) PG on ROS levels in HPASMCs; ROS levels were measured using a FACStar flow cytometer. (A and C) DCF (ROS) levels (% of control) at the indicated time points. (B and D) Levels of mitochondrial O₂⁻ (% of control), as detected by MitoSOX Red dye at 24 h. Data are presented as the mean ± standard deviation. *P<0.05 vs. the control group. H₂O₂, hydrogen peroxide; PG, pyrogallol, ROS, reactive oxygen species; HPASMCs, human pulmonary artery smooth muscle cells; DCF, dichlorofluorescein; O₂⁻, superoxide anion.

and NAC led to a decrease in apoptotic cell death in H₂O₂-treated HPASMCs (Fig. 3A). In addition, these agents attenuated ROS (DCF) levels in H₂O₂-treated HPASMCs from 10 min, with NAC having a strong effect (Fig. 3B). NAC also significantly attenuated the levels of mitochondrial O₂⁻ in H₂O₂-treated HPASMCs (P=0.049; Fig. 3C). In PG-treated HPASMCs, Z-VAD-FMK did not alter the proportion of Annexin V-stained cells, whereas NAC and BSO significantly increased the proportion (P=0.010; Fig. 3D). Z-VAD-FMK and NAC decreased ROS (DCF) levels in PG-treated HPASMCs at the earlier time points of 10, 30 and 60 min (Fig. 3E). However, NAC increased the ROS (DCF) levels at 120 and 180 min (Fig. 3E). In addition, Z-VAD-FMK did not affect the levels of mitochondrial O₂⁻ in PG-treated HPASMCs, whereas NAC significantly increased the O₂⁻ levels in these cells (P=0.004; Fig. 3F).

Effects of H₂O₂ and PG on GSH levels in HPASMCs. CMFDA dye was used to evaluate intracellular GSH levels in H₂O₂ and PG-treated HPASMCs. Treatment with 500 μM H₂O₂ decreased GSH (CMF) levels at 10 min compared with 0 min (P=0.022); however, the level was gradually recovered for the remainder of the treatment time (Fig. 4A). H₂O₂ dose-dependently increased the number of GSH-depleted cells in HPASMCs (Fig. 4B). Conversely, Z-VAD-FMK and NAC reduced GSH depletion in H₂O₂-treated HPASMCs (Fig. 4C). When cells were exposed to PG, GSH (CMF) levels were transiently decreased in HPASMCs at 10 min (P=0.005; Fig. 4D). The decreased level was gradually and partially recovered

from 30 min onwards; however, the GSH level was significantly reduced compared with the control group (P=0.013; Fig. 4D). PG (500 μM) also significantly increased the number of GSH-depleted cells (P=0.014; Fig. 4E). However, relatively high doses of PG (750 and 1,000 μM) did not strongly increase the number of GSH-depleted cells (Fig. 4E). Z-VAD-FMK did not affect GSH depletion in PG-treated HPASMCs; however, NAC and BSO significantly intensified GSH depletion in these cells (P=0.042; Fig. 4F).

Discussion

ROS are involved in various physiological and pathophysiological processes of VSM systems via the manipulation of cell proliferation, cell hypertrophy, migration, inflammation, contraction, and death of VSMCs (3,4). The present study aimed to elucidate the cytotoxic effects of exogenous H₂O₂ and PG on HPASMCs with respect to changes in intracellular ROS and GSH levels. Previous studies have reported that exogenous ROS generators lead to VSMC death (21,22). However, they may also be associated with proliferation of these cells (23,24), and intracellular ROS are essential for the survival of VSMCs (25). The present study demonstrated that H₂O₂ decreased the proliferation of HPASMCs, with an IC₅₀ of 250-500 μM at 24 h. In addition, these doses were determined to partially induce apoptosis, as exhibited by the Annexin V-staining of cells and Z-VAD-FMK treatment. However, the precise exposure times and concentrations of exogenous oxidants have not been precisely defined in order to

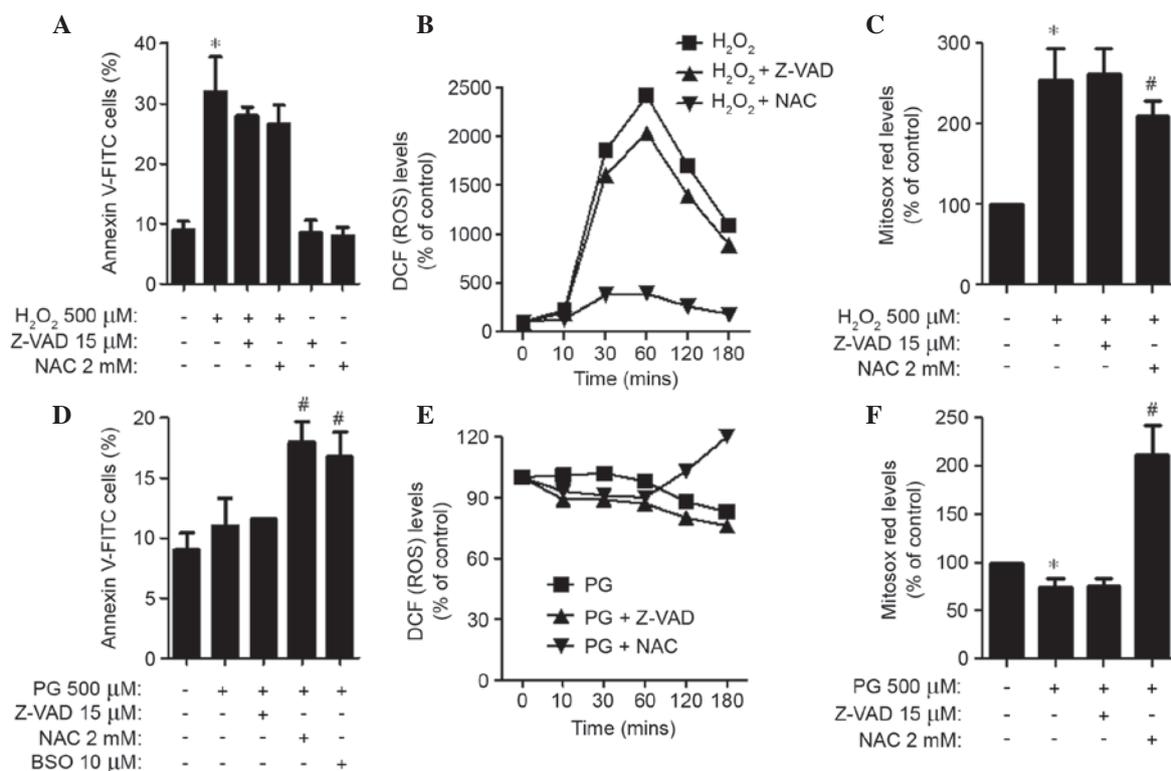


Figure 3. Effects of Z-VAD-FMK, NAC or BSO on cell death and ROS levels in (A-C) H₂O₂ or (D-F) PG-treated human pulmonary artery smooth muscle cells. (A and D) Percentages of Annexin V-FITC positive cells, as measured using a FACStar flow cytometer. (B and E) DCF (ROS) levels (% of control) at the indicated time points. (C and F) Levels of mitochondrial O₂⁻ (% of control), as detected by MitoSOX Red dye at 24 h. Data are presented as the mean ± standard deviation. *P<0.05 vs. the control group (three independent experiments). #P<0.05 vs. 500 μM H₂O₂ or PG groups. H₂O₂, hydrogen peroxide; PG, pyrogallol; NAC, N-acetyl cysteine; BSO, L-buthionine sulfoximine, Z-VAD-FMK, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; FITC, fluorescein isothiocyanate; ROS, reactive oxygen species; DCF, dichlorofluorescein; O₂⁻, superoxide anion.

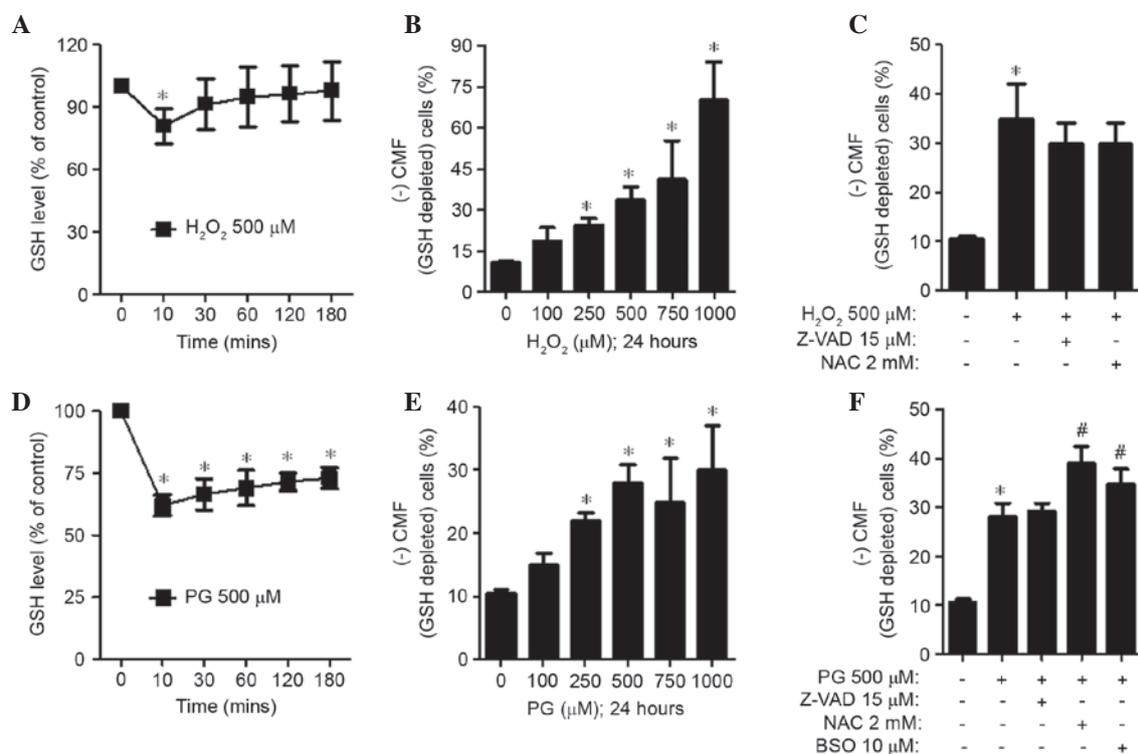


Figure 4. Effects of Z-VAD-FMK, NAC or BSO on GSH levels in (A-C) H₂O₂ or (D-F) PG-treated HPASMCs. GSH levels in HPASMCs were measured using a FACStar flow cytometer. (A and D) GSH (CMF) levels (% of control) at the indicated time points. (B, C, E and F) (-)CMF (GSH-depleted) cells (%) at 24 h. Data are presented as the mean ± standard deviation. *P<0.05 vs. the control group (three independent experiments). #P<0.05 vs. 500 μM PG groups. H₂O₂, hydrogen peroxide; PG, pyrogallol; HPASMCs, human pulmonary artery smooth muscle cells; NAC, N-acetyl cysteine; BSO, L-buthionine sulfoximine, Z-VAD-FMK, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; GSH, glutathione; CMF, 5-chloromethyl-fluorescein.

determine their effects on cell growth, cell survival and death of VSMCs. Notably, the relatively higher doses of PG (750 and 1,000 μ M) did not induce apoptosis or growth inhibition of HPASMCs. Previous studies have stated that the IC₅₀ values of PG range between 20 and 50 mM in lung, gastric and cervical cancer cells (13-16); however, the susceptibility of HPASMCs to PG is low compared with that of cancer cells. In addition, the susceptibility of HPASMCs is lower compared with normal endothelial cells (26). Therefore, HPASMCs appeared to be cytotoxically resistant to PG compared with other cell types, including cancer cells.

H₂O₂ and O₂^{•-} are among the primary ROS involved in various cell signaling pathways. The toxicity of ROS is usually mediated by [•]OH (4). In the present study, ROS levels (as determined by DCF) were increased in HPASMCs treated with 500 μ M H₂O₂ for 10 min. In addition, H₂O₂ increased the levels of mitochondrial O₂^{•-}. These results indicated that exogenous H₂O₂ may damage the mitochondria and induce cell death via the generation of O₂^{•-}. Exogenous H₂O₂ may lead to a greater production of ROS by a self-amplifying mechanism (ROS-induced ROS generation), which may be converted into the toxic ROS [•]OH via the Fenton reaction, leading to death of HPASMCs. As expected, NAC attenuated cell death in H₂O₂-treated HPASMCs and markedly decreased ROS (DCF) levels at the earlier time points; the levels of mitochondrial O₂^{•-} were also decreased. In addition, Z-VAD-FMK decreased apoptotic cell death and ROS (DCF) levels in H₂O₂-treated HPASMCs. Notably, although PG has been established to generate O₂^{•-} in biological systems (11-13), it did not increase ROS (DCF) levels in HPASMCs at the earlier time points in the present study. In addition, PG significantly decreased the levels of mitochondrial O₂^{•-}. It is possible that a failure in the generation of O₂^{•-} in PG-treated HPASMCs led to a cytotoxic resistance to PG compared with other cell types. Notably, NAC decreased ROS (DCF) levels in PG-treated HPASMCs at 10, 30 and 60 min; however, it increased the levels at 120 and 180 min. NAC significantly increased the levels of mitochondrial O₂^{•-} and cell death of PG-treated HPASMCs. In the present study, NAC acted as a pro-oxidant in PG-treated cells, which led to an increase in O₂^{•-} levels, as opposed to an antioxidant in PG-treated HPASMCs, consequently intensifying cell death. In addition, a previous study reported that NAC enhances growth inhibition and death in gallic acid-treated lung cancer, which was accompanied by an increase in O₂^{•-} levels (17). Therefore, NAC may act as an antioxidant or a pro-oxidant depending on co-incubated agents and should be used with caution since it may induce vascular toxicity via the increased generation of O₂^{•-}.

The GSH content of cells is inversely proportionate to the induction of apoptosis (27). In the present study, H₂O₂ treatment led to a dose-dependent increase in the number of GSH-depleted cells. Conversely, Z-VAD-FMK and NAC prevented H₂O₂-induced GSH depletion. In addition, PG increased the number of GSH-depleted cells. Treatment with NAC led to the PG-induced death of HPASMCs and significantly intensified GSH depletion. Furthermore, BSO increased GSH depletion in PG-treated HPASMCs and enhanced cell death. These results supported the conclusions of previous studies, which stated that the intracellular GSH content has an important effect on cell death (20,28-30). H₂O₂ decreased

GSH levels at 10 min; however, the levels were recovered from 30 min onwards. Conversely, the transient decrease of GSH levels in PG-treated HPASMCs was significant and the levels did not fully recover. Since PG did not strongly affect ROS (DCF) levels at the earlier time points, it may reduce GSH levels as opposed to generating ROS in HPASMCs.

In conclusion, H₂O₂ and PG induced growth inhibition and death of HPASMCs via GSH depletion. However, when exposed to PG, HPASMCs were not significantly affected compared with other cell types investigated in previous studies. NAC attenuated cell death and GSH depletion in H₂O₂-treated HPASMCs, whereas it intensified cell death and GSH depletion in PG-treated HPASMCs. The results of the present study indicated that exogenous oxidants may disturb the various physiological properties of VSMCs through altering the balance between cell survival and death. Therefore, it is imperative that future research efforts aim to define precise signaling pathways and mechanisms involved in vascular toxicity triggered by endogenous and exogenous ROS. Future research efforts may allow for the elucidation of more effective prevention and therapeutic strategies for vascular diseases in response to oxidative stress.

Acknowledgements

The present study was supported by a grant from the National Research Foundation of Korea (NRF) funded by the Korean government (MSIP; grant no. 2008-0062279).

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