The effects of exogenous $\text{H}_2\text{O}_2$ on cell death, reactive oxygen species and glutathione levels in calf pulmonary artery and human umbilical vein endothelial cells

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Abstract. Enhanced oxidative stress contributes to endothelial dysfunction via the apoptotic induction of endothelial cells (ECs). However, the precise molecular mechanisms underlying its important effect remain unclear. Here, we evaluated the effects of exogenous hydrogen peroxide ($\text{H}_2\text{O}_2$) on cell growth and death in ECs such as calf pulmonary artery endothelial cells (CPAECs) and human umbilical vein endothelial cells (HUVECs) and investigated its mechanism of action in CPAECs. $\text{H}_2\text{O}_2$ inhibited the growth of CPAECs and HUVECs at 24 h with IC$_{50}$ of approximately 20 and 300 µM, respectively. $\text{H}_2\text{O}_2$ induced cell death in both ECs, which was accompanied by the loss of mitochondrial membrane potential ($\Delta\Psi_m$). $\text{H}_2\text{O}_2$-induced CPAEC death occurred via apoptosis, demonstrated by Annexin V-staining cells and Z-VAD (a pan-caspase inhibitor) treatment. $\text{H}_2\text{O}_2$ increased superoxide anion levels in HUVECs but not in CPAECs. Treatment with 30 µM $\text{H}_2\text{O}_2$ significantly decreased the activities of superoxide dismutases and catalase in CPAECs. $\text{H}_2\text{O}_2$ induced glutathione (GSH) depletion in both ECs. Z-VAD and N-acetyl cysteine (NAC; a well-known antioxidant) attenuated apoptotic cell death and GSH depletion in $\text{H}_2\text{O}_2$-treated CPAECs. In conclusion, $\text{H}_2\text{O}_2$ induced growth inhibition and death in ECs via GSH depletion. HUVECs were relatively resistant to $\text{H}_2\text{O}_2$ compared with CPAECs. $\text{H}_2\text{O}_2$-induced CPAEC apoptosis required the activation of various caspases.

Introduction

Several cells that consist of the vasculature produce reactive oxygen species (ROS), which are a class of oxygen-derived molecules including hydrogen peroxide ($\text{H}_2\text{O}_2$), superoxide anion ($\text{O}_2^{•−}$) and hydroxyl radical (‘OH). These elemental molecules have been regarded as deleterious to the vasculature, leading to pathological processes such as atherosclerosis, restenosis, hypertension, diabetic vascular complications and heart failure (1,2). However, it has become evident that ROS in vascular cells play both a physiological and pathophysiological role in vascular homeostasis via the regulation of numerous cellular events including cell death, differentiation, contraction and cell proliferation (1,3,4). They can also act as second messengers, influencing distinct signal transduction pathways in the cardiovascular and pulmonary systems (1,5). In particular, vascular endothelial cells (ECs) are involved in various regulatory responsibilities such as vascular permeability for gases and metabolites, vascular smooth muscle tone, blood pressure, blood coagulation, inflammation and angiogenesis (6). Endothelial dysfunction has been implicated in the initiation and propagation of various vascular diseases (7). Thus, enhanced oxidative stress may contribute to endothelial dysfunction in vascular diseases via the apoptotic induction of ECs (1).

ROS are mostly generated as by-products of mitochondrial respiration or are specifically produced by oxidases such as nicotine adenine diphosphate (NADPH) oxidase and xanthine oxidase (8). The major metabolic pathways embrace superoxide dismutases (SODs), which metabolize $\text{O}_2^{•−}$ to $\text{H}_2\text{O}_2$ (9). Further metabolism by catalase or glutathione (GSH) peroxidase yields $\text{O}_2$ and $\text{H}_2\text{O}$ (10). Among ROS, $\text{H}_2\text{O}_2$ can diffuse freely through cellular membranes to a distance of numerous cell diameters before reacting with specific molecular targets due to its solubility in both lipid and aqueous environments.
and its comparatively low reactivity. Tissue concentrations of H$_2$O$_2$ for the duration of inflammation are likely to reach near millimolar levels, whereas minute amounts of H$_2$O$_2$ generated by NADPH oxidase are believed to act only in microenvironments of the plasma membrane such as lipid rafts (11,12). Nonetheless, in both cases, H$_2$O$_2$ may modulate essential cellular functions of cell growth, proliferation and differentiation or it can trigger cell death by apoptosis or necrosis.

H$_2$O$_2$ modulates endothelial cell function via elaborate mechanisms. Ambient production of O$_2^\cdot$ and subsequently H$_2$O$_2$ at low levels is crucial for endothelial cell growth and proliferation (2). On the other hand, the mode of action of H$_2$O$_2$ in provoking endothelial dysfunction and death has also been extensively investigated. However, the precise molecular mechanisms underlying these important effects remain largely unclear. Therefore, it is critical to understand the different roles ROS play in the physiology and pathophysiology of ECs. A fuller understanding of how H$_2$O$_2$ affects apoptosis in ECs may aid in the development of novel strategies to treat or prevent vascular diseases. In the present study, we evaluated the effects of exogenous H$_2$O$_2$ on cell growth and death in ECs such as calf pulmonary artery endothelial cells (CPAECs) and human umbilical vein endothelial cells (HUVECs) in relation to changes in intracellular ROS and GSH levels, and investigated its mechanism in CPAECs.

Materials and methods

Cell culture. CPAECs obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea) were maintained in a humidified incubator containing 5% CO$_2$ at 37°C. CPAECs were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich Chemical Co., St. Louis, MO, USA) and 1% penicillin-streptomycin (Gibco-BRL, Grand Island, NY, USA). The primary HUVECs from PromoCell GmbH (Heidelberg, Germany) were maintained in a humidified incubator containing 5% CO$_2$ at 37°C. HUVECs were cultured in complete endothelial cell growth medium containing 2% FBS, which was purchased from PromoCell GmbH. CPAECs and HUVECs were grown in 100-mm plastic tissue culture dishes (Nunc, Roskilde, Denmark) containing 10 ml media and washed twice with PBS and incubated with the indicated amounts of H$_2$O$_2$ with or without 15 µM each caspase inhibitor or 2 mM NAC 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. Five microliters of Annexin V-FITC was then added to these cells, which were analyzed with a FACStar flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA).

Measurement of mitochondrial membrane potential (MMP; ΔΨ$_m$). MMP (ΔΨ$_m$) levels were measured by a rhodamine 123 fluorescent dye (Ex/Em=485/535 nm; Invitrogen Corporation, Camarillo, CA, USA). In brief, 1x10$^6$ cells in 60-mm culture dishes were incubated with the indicated amounts of H$_2$O$_2$ for 24 h. Cells were washed twice with cold PBS and then incubated with rhodamine 123 (0.1 µg/ml) at 37°C for 30 min. Rhodamine 123 staining intensity was determined by a FACStar flow cytometer (Becton-Dickinson). Rhodamine 123 negative cells indicated the loss of MMP (ΔΨ$_m$) in cells.

Detection of intracellular ROS levels. Intracellular ROS level including O$_2^\cdot$ was detected by means of an oxidation-sensitive fluorescent probe dye, dihydroethidium (DHE; Ex/Em=518/535 nm; Invitrogen/Molecular Probes). In brief, 1x10$^6$ cells in 60-mm culture dishes were incubated with the indicated amounts of H$_2$O$_2$ for 24 h. Cells were then washed in PBS and incubated with 20 µM DHE at 37°C for 30 min. DHE fluorescence intensities were detected using a FACStar flow cytometer (Becton-Dickinson). ROS (DHE) level was expressed as mean fluorescence intensity (MFI), which was calculated by CellQuest software (Becton-Dickinson).

Detection of the intracellular GSH. Cellular GSH levels were analyzed using a 5-chloromethylfluorescein diacetate
as previously described (17). In brief, 1x10^6 cells in 60-mm culture dishes (Nunc) were incubated with the indicated amounts of H_2O_2 with or without 15 µM each caspase inhibitor or 2 mM NAC for 24 h. Cells were then washed with PBS and incubated with 5 µM CMFDA at 37˚C for 30 min. CMF fluorescence was assessed using a FACStar flow cytometer (Becton-Dickinson). Negative CMF staining (GSH-depleted) of cells is expressed as the percentage of (-) CMF cells.

Measurement of cellular SOD and catalase activities. SOD enzyme activity was measured using the SOD assay kit-WST (Fluka Chemical Corp., Milwaukee, WI, USA) and catalase enzyme activity was measured using the catalase assay kit from Sigma-Aldrich Chemical Co., as previously described (18). In brief, 1x10^6 cells were incubated with 30 µM H_2O_2 for 24 h. The cells were then washed in PBS and suspended in five volumes of lysis buffer [20 mM HEPES (pH 7.9), 20% Glycerol, 200 mM KCl, 0.5 mM EDTA, 0.5% NP-40, 0.5 mM DTT, 1% protease inhibitor cocktail (from Sigma-Aldrich Chemical Co.)]. Supernatant protein concentration was determined by the Bradford method. Supernatant samples containing 100 µg of total protein were used for determination of SOD and catalase enzyme activities. These were added to each well in 96-well microtiter plates (Nunc) with the appropriate working solutions according to the manufacturer's instructions at 25˚C for 30 min. The color changes were measured at 450 or 520 nm using a microplate reader (SpectraMax 340; Molecular Devices Co., Sunnyvale, CA, USA). The value for the experimental group was converted to the percentage of the control group.

Statistical analysis. The results represent the means of at least three independent experiments (means ± SD). The data were analyzed using Instat software (GraphPad Prism 4; GraphPad Software, 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. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of H_2O_2 on the growth, death and MMP (ΔΨ_m) of CPAECs and HUVECs. We examined the effect of H_2O_2 on the growth and death of CPAECs and HUVECs at 24 h. When the growth of ECs after treatment with H_2O_2 was assessed by MTT assays, the reduction of cell growth was observed in both ECs in a dose-dependent manner, and the IC_{50} (the half maximal inhibitory concentration) of H_2O_2 in CPAECs and HUVECs was ~20 and 300 µM, respectively (Fig. 1A and D). When ECs were stained with Annexin V-FITC to evaluate the induction of apoptosis, the number of Annexin V-staining cells was increased in H_2O_2-treated ECs (Fig. 1B and E). At a 50 µM dose of H_2O_2, the number of Annexin V-staining cells in CPAECs increased ~30% compared with control CPAECs and the number in HUVECs increased ~5% compared with control HUVECs (Fig. 1A and D). When ECs were stained with Annexin V-FITC to evaluate the induction of apoptosis, the number of Annexin V-staining cells was increased in H_2O_2-treated ECs (Fig. 1B and E). At a 50 µM dose of H_2O_2, the number of Annexin V-staining cells in CPAECs increased ~30% compared with control CPAECs and the number in HUVECs increased ~5% compared with control HUVECs (Fig. 1B and E). When ECs were stained with Annexin V-FITC to evaluate the induction of apoptosis, the number of Annexin V-staining cells was increased in H_2O_2-treated ECs (Fig. 1B and E). At a 50 µM dose of H_2O_2, the number of Annexin V-staining cells in CPAECs increased ~30% compared with control CPAECs and the number in HUVECs increased ~5% compared with control HUVECs (Fig. 1B and E). Since apoptosis is closely related to the collapse of MMP (ΔΨ_m) (19), we assessed the effect of H_2O_2 on MMP (ΔΨ_m) using rhodamine 123. Although 5 or 10 µM H_2O_2 did not induce the loss of MMP (ΔΨ_m) in CPAECs, 30 or 50 µM H_2O_2 strongly increased the MMP (ΔΨ_m) loss (Fig. 1C).
In HUVECs, 50 or 100 µM H$_2$O$_2$ did not induce the loss of MMP ($\Delta$Ψ$_m$), but 200 or 300 µM H$_2$O$_2$ did (Fig. 1F).

**Effects of H$_2$O$_2$ on intracellular ROS and GSH levels in CPAECs and HUVECs.** To assess levels of intracellular ROS including O$_2^\cdot$ in H$_2$O$_2$-treated ECs at 24 h, we used a DHE fluorescence dye, which specifically reflects O$_2^\cdot$ accumulation in cells. As shown in Fig. 2A, all the tested doses of H$_2$O$_2$ decreased DHE (O$_2^\cdot$) levels in CPAECs. However, 100-300 µM H$_2$O$_2$ significantly increased the DHE (O$_2^\cdot$) levels in HUVECs (Fig. 2C). Next, we analyzed the changes of GSH levels in ECs using a CMF fluorescence dye. All the tested doses of H$_2$O$_2$ significantly increased the number of GSH-depleted cells in CPAECs (Fig. 2B). The relatively higher doses of 200 or 300 µM H$_2$O$_2$ also increased the number of GSH-depleted cells in HUVECs (Fig. 2D). Furthermore, we measured the activities of SOD and catalase in H$_2$O$_2$-treated CPAECs. As shown in Fig. 3, 30 µM H$_2$O$_2$ significantly decreased the activities of SOD and catalase.

**Effects of caspase inhibitors on cell death, MMP ($\Delta$Ψ$_m$) and GSH depletion in H$_2$O$_2$-treated CPAECs.** To determine which caspases were involved in apoptotic cell death in H$_2$O$_2$-treated CPAECs, cells were pretreated with pan-caspase inhibitor (Z-VAD), caspase-3 inhibitor (Z-DEVD), caspase-8 inhibitor (Z-IETD) or caspase-9 inhibitor (Z-LEHD) prior to treatment with H$_2$O$_2$. For this experiment, 30 µM H$_2$O$_2$ was selected as a suitable dose to differentiate the levels of cell death, MMP ($\Delta$Ψ$_m$) and GSH depletion in the presence or absence of each caspase inhibitor. While only Z-VAD significantly prevented apoptotic cell death in H$_2$O$_2$-treated CPAECs, other caspase inhibitors did not affect the apoptotic cell death (Fig. 4A). In addition, Z-VAD significantly attenuated the loss of MMP ($\Delta$Ψ$_m$) by H$_2$O$_2$ whereas other caspase inhibitors did not alter the loss (Fig. 4B). In relation to GSH depletion, only Z-VAD, no other caspase inhibitor, significantly decreased GSH depletion in H$_2$O$_2$-treated CPAECs (Fig. 4C).

**Effects of NAC on cell death, MMP ($\Delta$Ψ$_m$) and GSH depletion in H$_2$O$_2$-treated CPAECs.** Next, we investigated the effects
of NAC (a well-known antioxidant or GSH precursor) on cell death, MMP ($\Delta \psi_m$) and GSH depletion in H$_2$O$_2$-treated CPAECs at 24 h. NAC significantly reduced the number of Annexin V-FITC positive cells in H$_2$O$_2$-treated CPAECs (Fig. 5A). NAC also significantly attenuated the loss of MMP ($\Delta \psi_m$) in these cells (Fig. 5B). Moreover, NAC decreased GSH depletion in H$_2$O$_2$-treated CPAECs (Fig. 5C).

**Discussion**

ROS are involved in several physiological and pathophysiological processes in vascular endothelium by influencing cell proliferation, hypertrophy, migration, inflammation, contraction and death (1,2,5). In the present study, we elucidated the cytotoxic effect of exogenous H$_2$O$_2$ on ECs such as CPAECs and HUVECs in relation to cell death, ROS and GSH. Other studies have reported that ROS not only lead to cell death in ECs (20-22) but they are also involved in the survival of ECs (20). Our current results demonstrate that H$_2$O$_2$ inhibited the growth of CPAECs and HUVECs with an IC$_{50}$ of approximately 20 and 300 μM, respectively. H$_2$O$_2$ also provoked cell death in both ECs, as evidenced by Annexin V-staining cells and trypan blue cell counting (data not shown) and triggered the loss of MMP ($\Delta \psi_m$). In addition, H$_2$O$_2$ induced apoptosis in CPAECs in a caspase-dependent manner. However, the susceptibility of H$_2$O$_2$ between these ECs was different. HUVECs were more resistant to H$_2$O$_2$ than CPAECs. The difference in susceptibility may be due to the dissimilar basal antioxidant enzymes each cell has. Thus, the cytotoxic effects of H$_2$O$_2$ may differ depending on various endothelial cell types, such as artery vs. vein, large vessels vs. small vessels, human vs. other species, coronary vs. pulmonary. It is imperative that such effects of ROS, especially H$_2$O$_2$, be defined and characterized in the future.

When determining which caspase was involved in apoptosis in H$_2$O$_2$-treated CPAECs, only pan-caspase inhibitor Z-VAD significantly prevented apoptotic cell death in H$_2$O$_2$-treated CPAECs. Other caspase inhibitors did not affect the apoptotic cell death. In addition, Z-VAD attenuated the loss of MMP ($\Delta \psi_m$) whereas other caspase inhibitors did not alter the loss of MMP ($\Delta \psi_m$). These results suggest that H$_2$O$_2$-induced CPAEC apoptosis requires the activation of various caspases containing both caspase-8, necessary for the death receptor pathway, and caspase-9, related to the mitochondrial pathway. We observed that 10 μM H$_2$O$_2$ significantly increased the number of Annexin V-staining cells in CPAECs but this dose did not induce the MMP ($\Delta \psi_m$) loss. In addition, 50 and 100 μM H$_2$O$_2$ significantly increased...
the number of Annexin V-staining cells in HUVECs but those concentrations did not induce the MMP (ΔΨm) loss. By contrast, 30 or 50 µM H2O2 strongly increased the proportion of MMP (ΔΨm) loss in CPAECs compared with that of Annexin V-staining cells. Therefore, the effect of MMP (ΔΨm) loss in H2O2-induced EC apoptosis is likely concentration specific. It appears that relatively higher concentrations in each EC induce cell death via steadily inducing MMP (ΔΨm) loss.

The main ROS involved in cell signaling pathways are H2O2 and O2·-. ROS toxicity is usually mediated by ‘OH (5). According to our present results, H2O2 increased DHE (O2·-) levels in HUVECs. H2O2 appeared to induce the potential leakage of electron from mitochondrial respiratory transport chain and/or activated oxidases such as NADPH oxidase and xanthine oxidase in HUVECs. By contrast, although H2O2 reduced the activity of SOD in CPAECs, it did not increase DHE (O2·-) levels in these cells. Thus, H2O2 did not affect both mitochondrial respiratory transport chain and various oxidases to generate O2·- in CPAECs. Instead, H2O2 decreased DHE (O2·-) levels in CPAECs via an unidentified mechanism. The different effects may be due to different basal mitochondrial activity and antioxidant enzymes between two ECs. As H2O2 significantly induced apoptosis and decreased the activity of catalase in CPAECs, it is possible that exogenous H2O2 can be efficiently converted into the toxic ROS of ‘OH via the Fenton reaction to kill CPAECs. The intracellular GSH content has a decisive effect on anticancer drug-induced apoptosis, indicating that apoptotic effects are inversely proportional to GSH content (23,24). Similarly, H2O2 increased the number of GSH-depleted cells in both ECs. At 50 µM H2O2-treated ECs, the GSH-depleted cell number in CPAECs was higher than that in HUVECs. These results seem to be correlated with Annexin V-FITC results from ECs treated with H2O2. In addition, Z-VAD reduced GSH-depleted cell numbers in H2O2-treated CPAECs. NAC showing an anti-apoptotic effect on H2O2-treated CPAECs significantly decreased GSH depletion.

In conclusion, H2O2-induced growth inhibition and death in ECs via GSH depletion. HUVECs were relatively resistant to H2O2 compared with CPAECs. H2O2-induced CPAEC death occurs via apoptosis, which requires the activation of various caspases.

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References