Propofol Reduces Hydrogen Peroxide-Induced Apoptosis through Down-Regulating Bim Expression in Alveolar Epithelial Cells

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Abstract
Propofol is an intravenous anesthetic agent widely used to introduce and maintain anesthesia during surgical procedures, and it has often been used in recent years as a sedative in patients with acute lung injury (ALI). A common cause of ALI is sepsis, and apoptosis in vascular endothelium and alveolar epithelial cells (AECs) during sepsis plays an important role in the pathologic formation of ALI. Propofol has drawn greater attention due to its anti-inflammatory effect and anti-apoptosis capability. It was already found that propofol inhibited MCP-1 mRNA expression and secretion, as well as the phosphorylation of p38 MAPK, SAPK/JNK, ATF-2, and c-Jun induced by lipopolysaccharide (LPS) in AECs.

The present study investigated the effect and mechanism of propofol on apoptosis induced by hydrogen peroxide (H2O2) in AECs. AECs were treated with 0.1 or 1nM H2O2 for 1, 12, or 24h following pretreatment with 25μM propofol for 1h. The cell proliferation assay was performed using the TetraColor ONE. The percentage of apoptotic cells was measured by flow cytometry analysis. The phosphorylation of c-Jun and expression of Bim, Bcl-2, and Bcl-xL were measured by a Western blot analysis. Propofol down-regulated c-Jun phosphorylation and Bim (as a known c-Jun-mediated induction of pro-apoptotic and anti-apoptotic target genes) expression and up-regulated the reduction of Bcl-2 and Bcl-xL expression induced by H2O2 in AECs. These results suggest that the application of propofol has potential to reduce apoptosis through the JNK pathway and Bim down-regulation.

Introduction
Propofol is an intravenous anesthetic agent widely used to introduce and maintain anesthesia during surgical procedures. Several studies have reported that propofol show anti-inflammatory effects through inhibiting mitogen-activated protein kinase (MAPK) signaling. Propofol inhibits the activation of p38 MAPK up-regulating the expression of Annexin A1 in human monocytic THP-1 cells (1) and attenuates the granulocyte-macrophage colony-stimulating factor (GM-CSF) production by suppressing MAPK/extracellular signal-regulated kinase (ERK) activity and NF-κB translocation in hepatocytes (2). In the previous report, we demonstrated that propofol inhibited monocyte chemoattractant protein-1 (MCP-1) mRNA expression and secretion, as well as the phosphorylation of p38 MAPK, Stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK), ATF-2, and c-Jun induced by lipopolysaccharide (LPS) in alveolar epithelial cells (AECs) (3).

Propofol has often been used in recent years as a sedative in patients with acute lung injury (ALI), which is a common and critical pulmonary complication. ALI is an inflammatory disorder characterized by an excessive infiltration of neutrophils, the release of inflammatory mediators, and destruction of the alveolar–capillary membrane with severe consequences for pulmonary gas exchange (4–6). A common cause of ALI is sepsis, and apoptosis in vascular endothelium and AECs during sepsis plays an important role in the pathologic formation of ALI. In mouse septic lungs, the surface expression of death receptors is up-regulated and
the adaptor molecule Fas-associated death domain is highly expressed, and also the apoptotic effectors such as caspase-8 and caspase-3 are expressed and activated. Thus, the death receptor apoptotic pathway is critical for the development of ALI during sepsis (7). In addition, alveolar epithelial injury in humans with ALI is in part associated with local up-regulation of the Fas/FasL system and activation of the apoptotic cascade in the epithelial cells that line the alveolar air spaces (8).

Several studies have reported that propofol has antioxidant potential to attenuate hydrogen peroxide-induced PC12 cell death through p38 MAPK and protects against oxidative stress in human umbilical vein endothelial cells and human hepatic cells (9–12). However, there is only limited information about the effect of propofol on apoptosis in AECs.

The present study investigated the effect and mechanism of propofol on apoptosis induced by hydrogen peroxide (H₂O₂) in AECs.

Materials and Methods

Cell culture
AECs, L2 cells (JCRB9053, Japanese Collection of Research Bioresources, Japan), were derived from an adult female rat lung. AECs were incubated in an atmosphere of 5% CO₂-95% air at 37°C in F-12K Nutrient Mixture Kaighn’s Modification Medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml). AECs were grown in a 75 cm² culture flask to >95% confluence and routinely passaged with 0.25% trypsin and 1 mM EDTA-4Na in the Hanks solution. All the chemicals and reagents for the tissue culture were purchased from Invitrogen Corporation (Carlsbad, CA, USA).

Cell proliferation assay
AECs were treated with 0.1 μM H₂O₂ (Wako Pure Chemical Industries, Ltd., Japan) for 24h following pretreatment with 25 μM propofol (2, 6-disopropylphenol, Tokyo Chemical Industry Co., Ltd., Japan) for 1h in a medium with 0.5% FBS. The cell proliferation assay was performed using the Cell Proliferation Assay System, TetraColor ONE (Seikagaku Biobusiness Corporation, Japan) and Microplate Reader MTP-450 (Corona Electric Co., Ltd., Japan).

Flow cytometry analysis
AECs were treated with 1 μM H₂O₂ for 12h following pretreatment with 25 μM propofol for 1h in a medium with 0.5% FBS. The number of apoptotic cells and the cell cycle phase distribution in AECs were quantified using the CycleTEST™ PLUS DNA reagent kit (BD; Becton, Dickinson and Company, USA) and the FACS Calibur (BD). The percentage of cells in the Sub-G₁ phase out of 20,000 cells was determined by CellQuest software (BD) and ModFit LT™ (Verity Software House, USA).

Western blot analysis
AECs were treated with 1 μM H₂O₂ for 1h following pretreatment with 25 μM propofol for 1h in a medium with 0.5% FBS. After that, AECs were lysed in a 200 μl Tris-SDS-β-Me sample buffer (Daichi Pure Chemicals Co., Ltd, Tokyo, Japan) with 0.1% pepstatin, aprotinin and leupeptin as a protease inhibitor (Sigma-Aldrich Co., MO, USA). The protein concentration was determined by the Bradford methods using the Bio-Rad Protein Assay. Equal amounts of proteins were separated electrophoretically on Mini-PROTEAN Precast Gels and transferred to supported nitrocellulose membrane. All chemicals and reagents used to measure volumes of protein and electrophoresis were purchased from Bio-Rad Laboratories, Inc. (CA, USA). Membranes were probed with the respective primary antibodies to phospho-SAPK/JNK (Thr183/Thr185), phospho-c-Jun (Ser73), Bim, Bcl-2, Bcl-xL, and β-actin, and anti-rabbit IgG HRP-linked secondary antibody (Cell Signaling Technology Inc, MA, USA). Immunocomplexes were visualized by ECL Plus Western Blotting Detection Reagents (GE Healthcare UK Ltd., Buckinghamshire, UK).

Statistical analysis
Data were expressed as the mean ± SD. Differences among multiple groups were expressed by one-way ANOVA, followed by the Bonferroni post hoc test. P values of < 0.05 were considered as statistically significant.

Results
Effect of propofol on cell proliferation in H₂O₂-treated AECs
The effect of propofol on cell proliferation in H₂O₂-treated AECs is shown in Fig.1. H₂O₂ at 0.1 μM significantly decreased cell proliferation in AECs (P < 0.001). Propofol at 25 μM significantly enhanced cell proliferation at 24h in H₂O₂-treated AECs (P < 0.001).
Effect of propofol on cell cycle phase distribution in H$_2$O$_2$-treated AECs

The cell cycle phase distribution of AECs at 12h was analyzed by flow cytometry. The histogram shows a representative cell cycle phase distribution in AECs, including the cells in the Sub-G$_1$ phase (apoptotic cells; left low peak), the cells in G$_0$/G$_1$ phases (sharp high peak), the cells in G$_2$/M phases (right low peak), and the cells in the S phase (between the high and right low peaks) (Fig.2a). The percentages of Sub-G$_1$ phase cells are summarized in Fig.2b. H$_2$O$_2$ at 1μM significantly increased the percentage of Sub-G$_1$ phase cells compared to the control (P < 0.001). Propofol at 25μM significantly reduced the percentage of Sub-G$_1$ phase cells at 12h in H$_2$O$_2$-treated AECs (P < 0.001).

Effect of propofol on the expression of apoptosis and cell cycle regulators in H$_2$O$_2$-treated AECs

The phosphorylation of c-Jun and expression of Bcl-2 family proteins (Bim, Bcl-2, and Bcl-xL) in AECs treated with 25μM propofol and/or 1μM H$_2$O$_2$ were evaluated by Western blot analysis. A representative image of these protein levels are shown in Fig.3. H$_2$O$_2$ at 1μM increased SAPK/JNK and c-Jun phosphorylation and Bim expression compared to the control, while propofol down-regulated H$_2$O$_2$-induced SAPK/JNK and c-Jun phosphorylation and Bim expression. In contrast, H$_2$O$_2$ at 1μM decreased Bcl-2 and Bcl-xL expressions compared to the control, while propofol up-regulated the reduction of Bcl-2 and Bcl-xL expression induced by H$_2$O$_2$. 

Fig.1 The effect of propofol on cell proliferation in L2 cell. L2 cells were treated with 0.1μM H$_2$O$_2$ for 24h following pretreatment with 25μM propofol for 1h. Optical density at 450nm was measured using TetraColor ONE and Microplate Reader MTP-450. Results are mean ± S.D. of four trials. PPF: propofol. ***P < 0.001, compared to H$_2$O$_2$. ###P < 0.001, compared to control.

Fig.2 The effect of propofol on cell cycle phase distribution in L2 cell. L2 cells were treated with 1μM H$_2$O$_2$ for 12h following pretreatment with 25μM propofol for 1h. H$_2$O$_2$-induced apoptosis (Sub-G$_1$) and distribution of cell cycle phases in L2 cell were evaluated by flow cytometry. A: A representative data. B: Effect of propofol on H$_2$O$_2$-induced apoptosis (Sub-G$_1$) in L2 cell. The percentage of cells in the Sub-G$_1$ phase was determined by CellQuest software and ModFit LTTM. Results are mean ± S.D. of four trials. PPF: propofol. ***P < 0.001, compared to H$_2$O$_2$. ###P < 0.001, compared to the control.

Fig.3 The effect of propofol on the expression of apoptosis and cell cycle regulators in L2 cell. L2 cells were treated with 1μM H$_2$O$_2$ for 1h following pretreatment with 25μM propofol for 1h. The phosphorylation of SAPK/JNK and c-Jun, and the expression of Bim, Bcl-2, and Bcl-xL were measured by Western blot analysis. These figures are the representative images of the visualized immunocomplexes of three trials. PPF: propofol.
**Discussion**

Propofol, a widely used anesthetic and sedative, has recently drawn greater attention due to its anti-apoptosis capability. Several studies have reported that propofol limits myocardial ischemia/reperfusion injury with an associated reduction in apoptotic cell deaths in rats (13), and it attenuates intestinal ischemia/reperfusion induced intestinal epithelial apoptosis which might be attributable to its antioxidant property modulating the ceramide pathway (14). Moreover, propofol protects hepatic L02 cells from H$_2$O$_2$-induced apoptosis partly through activating the MEK-ERK pathway and further suppressing Bad and Bax expression (12), and it exerts protective effects against H$_2$O$_2$-enhanced TNF-$\alpha$ cell toxicity by reducing oxidative injury (15).

In the present study, we investigated the effect and mechanism of propofol on apoptosis induced by H$_2$O$_2$ in AECs. Preliminary experiments were tested with H$_2$O$_2$ at 0.1, 1, and 10 $\mu$M in AECs. H$_2$O$_2$ at 0.1 $\mu$M for cell proliferation assay, and 1 $\mu$M for flow cytometry and Westernblot analysis were the appropriate concentrations in this study (data not shown). H$_2$O$_2$ decreased cell proliferation, while propofol inhibited it decreased by H$_2$O$_2$ at 24 h in AECs. In addition, H$_2$O$_2$ increased the percentage of Sub-G1 phase cells, while propofol inhibited it increased by H$_2$O$_2$ at 12 h in AECs. It has been reported that propofol shows the protective effect to the glucose oxidase-induced rat embryonic ventricular myocardial cell death on cell viability (13). Also, propofol suppressed H$_2$O$_2$-induced apoptosis in human hepatic L02 cells (12), and the tumor necrosis factor (TNF) -$\alpha$ and H$_2$O$_2$-induced apoptosis in human vascular endothelial cells measured by TUNEL assay (15). These results suggest that H$_2$O$_2$ induced apoptosis and cell deaths as well as propofol reduced those by H$_2$O$_2$ in AECs, same as human hepatic L02 cells and vascular endothelial cells.

As is widely known, H$_2$O$_2$ induces apoptosis in AECs. It has been demonstrated that H$_2$O$_2$ was able to sensitize A549 cells to TRAIL-mediated apoptosis and caspase-3 activation (16), and it was involved in the elevation of p53 levels, the Bax/Bcl-xL ratio, and TRAIL receptor levels (17). The induction of apoptosis is controlled by the balance of the pro- and anti-apoptotic family of Bcl-2 proteins, which includes the pro-apoptotic members Bim, Bax, Bak, Bad and Bcl-xS, and the anti-apoptotic members Bcl-2, Bcl-w, Mcl-1 and Bcl-xL at the mitochondria (18-20). Bcl-2 and its closest relatives, Bcl-xL and Bcl-w, exert a survival function in response to a wide range of apoptotic stimuli through the inhibition of mitochondrial cytochrome c release (21). On the other hand, Bim induces apoptosis by binding to and antagonizing anti-apoptotic members of the Bcl-2 family by interaction with Bcl-2, Bcl-xL, Mcl-1, Bcl-w, Bfl-1 and BHRF-1 (22, 23). It activates the mitochondrial pathway, releases cytochrome c, and activates caspases leading to apoptosis (19, 24).

Furthermore, the JNK pathway is a critical mediator of cell apoptosis in response to H$_2$O$_2$ in AECs (25). Bim provides a molecular link between the JNK signal transduction pathway and the Bax/Bak-dependent mitochondrial apoptotic machinery (26). JNKs regulate the pro-apoptotic activity of Bim during Trophic factor deprivation, both transcriptionally and posttranslationally (27). The role of c-Jun in apoptosis and survival might be more of a balancing act between the c-Jun-mediated induction of pro-apoptotic and anti-apoptotic target genes, including FasL, Bcl3, and Bim (28).

In the present study, H$_2$O$_2$ increased SAPK/JNK and c-Jun phosphorylation and Bim expression compared to the control, while propofol down-regulated H$_2$O$_2$-induced SAPK/JNK and c-Jun phosphorylation and Bim expression. In contrast, H$_2$O$_2$ decreased Bcl-2 and Bcl-xL expressions compared to the control, while propofol up-regulated the reduction of Bcl-2 and Bcl-xL expression induced by H$_2$O$_2$. In our previous study, it was demonstrated that propofol inhibited the phosphorylation of SAPK/JNK and c-Jun induced by LPS in AECs (3). These results suggest that propofol shows an anti-apoptotic effect on Bcl-2 family members through the JNK pathway.

It has been reported that propofol protects erythrocytes against oxidative stress by the aid of ascorbate-driven recycling, as well as against physical stress by increasing membrane fluidity, indicating that propofol has a high potential as an efficient antioxidant (29). Propofol at 200 $\mu$M had significant cytotoxic effects, however 10 $\mu$M propofol up-regulated the expression of Bcl-2, and activated pERK and pAkt in SH-SY5Y cells. These results suggest that propofol at a low concentration protects SH-SY5Y cells against stresses and improves cell proliferation by elevating the expression of Bcl-2, pAkt, and pERK (30). Therefore, the concentration at 25$\mu$M (4.46 $\mu$g/ml) is the range of clinically relevant concentrations during anesthesia using propofol. These results suggest that propofol may have the
effect of anti-apoptosis at clinically achievable concentrations.

In conclusion, it was demonstrated that propofol down-regulated c-Jun phosphorylation and Bim (as a known cJun-mediated induction of pro-apoptotic and anti-apoptotic target genes) expression, and up-regulated the reduction of Bcl-2 and Bcl-xL expression induced by H2O2 in AECs. These results suggest that the application of propofol has potential to reduce apoptosis through the JNK pathway and Bim down-regulation.

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