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Propofol attenuation of hydrogen peroxide-induced injury in human umbilical vein endothelial cells involves aldose reductase

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Propofol is a widely used intravenous anesthetic agent with antioxidant/antiapoptotic properties. Aldose reductase (AR) has been implicated in oxidative stress and apoptosis in endothelial cells. AR inhibition may protect cells from cardiovascular injury. Although the cytoprotective effect of propofol against hydrogen peroxide (H_2O_2)-induced injury has been widely studied, there is no information about the effects of propofol on AR. We therefore investigated the effect of propofol on H_2O_2-mediated injury and on aldose reductase expression. We found that propofol protected HUVECs against H_2O_2-induced damage and apoptosis and ameliorated AR expression induced by H_2O_2. Propofol also inhibited H_2O_2-induced p38 MAPK, JNK and Akt phosphorylation. Epalrestat (an AR inhibitor) or ablation of AR siRNA had a similar effect to propofol. The results suggest that propofol may be a preemptive anesthetic in patients with cardiovascular disease and inhibition of AR might be a new cytoprotective pathway for propofol.

1. Introduction

Vascular endothelial cells have important physiologic functions in maintaining cardiovascular stability. Many risk factors may cause endothelial cell dysfunction and result in apoptosis. Oxidative stress and reactive oxygen species (ROS) are regarded as critical pathogenic factors in endothelial cell damage and the development of cardiovascular diseases (Cai 2005). As one of the major ROS, hydrogen peroxide (H_2O_2) plays a central role in vascular pathophysiology. Many studies have revealed that H_2O_2 can induce cell damage and apoptosis in endothelial cells, and the underlying mechanisms are related to Mitogen activated protein kinases (MAPKs) and Akt (Chen et al. 2010; Wang et al. 2007). Among the MAPK members, JNK and p38 MAPK are preferentially activated in response to various stresses and pro-apoptotic signals in numerous cell types. The Akt pathway is generally associated with survival pathways against multiple pro-apoptotic stimuli that induce its activation (Ryter et al. 2007). Therefore, blockade of these pro-apoptotic/pro-oxidative pathways in endothelial cells is considered to be an attractive therapeutic strategy to prevent or ameliorate the progression of H_2O_2-induced cardiovascular diseases.

Propofol (2,6-diisopropylphenol) is a widely used intravenous agent for anesthesia and sedation. Its structure is similar to the endogenous antioxidant α-tocopherol (vitamin E) (Murphy et al. 1992). Propofol has antioxidant and antiapoptotic effects, but its effect varies among different cell types and involves multiple mechanisms (Chen et al. 2010; Ansley et al. 1999; Chen et al. 2011; Wang et al. 2009). Propofol differentially activates certain MAPKs, ERK, p38-MAPK, and JNK, depending on the cell type and experimental conditions (Chen et al. 2010; Gu et al. 2013; Liang et al. 2011). Although the cytoprotective effect of propofol against H_2O_2-induced injury has been widely studied, the underlying mechanisms remain unclear.

Aldose reductase (AR), a rate-limiting enzyme in the polyol pathway of glucose metabolism, has been studied for its involvement in the pathogenesis of secondary diabetic complications. AR activation may cause oxidative stress and mediates cardiovascular dysfunction and complications *via* oxidative stress (Ramasamy and Goldberg 2010). Conversely, oxidative stress has been shown to activate AR (Kaiserova et al. 2006). AR inhibition may therefore be a useful therapy against oxidative stress. On the other hand, AR, as a member of the NADPH-dependent aldo-keto reductase family, exhibits broad substrate specificity for reactive aldehydes that are down-stream products of lipid peroxidation, suggesting that this enzyme may be involved in protection against oxidative injury (Ramana et al. 2000). Moreover, it has been reported that AR can attenuate cell apoptosis by inhibiting oxidative stress and AR is a critical regulator of apoptotic signaling in endothelial cells (Ramana et al. 2004). Despite the above contradictory reports, AR may play an important role in ROS-mediated cytotoxicity.

However, no evidence has been reported concerning the direct effect of propofol on AR. We hypothesized that propofol attenuates oxidative stress and cell apoptosis *via* AR and thereby confers its protection. The results suggested that the protective effect of propofol on H_2O_2-induced HUVEC dysfunction was involved in AR. Further study demonstrated the involvement of p38 MAPK, JNK and Akt pathways in this process.

2. Investigations and results

2.1. Effects of propofol or epalrestat on H_2O_2-induced cytotoxicity in HUVECs

After incubation with 100, 200, 300, 400 and 500 μM H_2O_2 for 4 h, the viability of HUVECs was reduced significantly in a

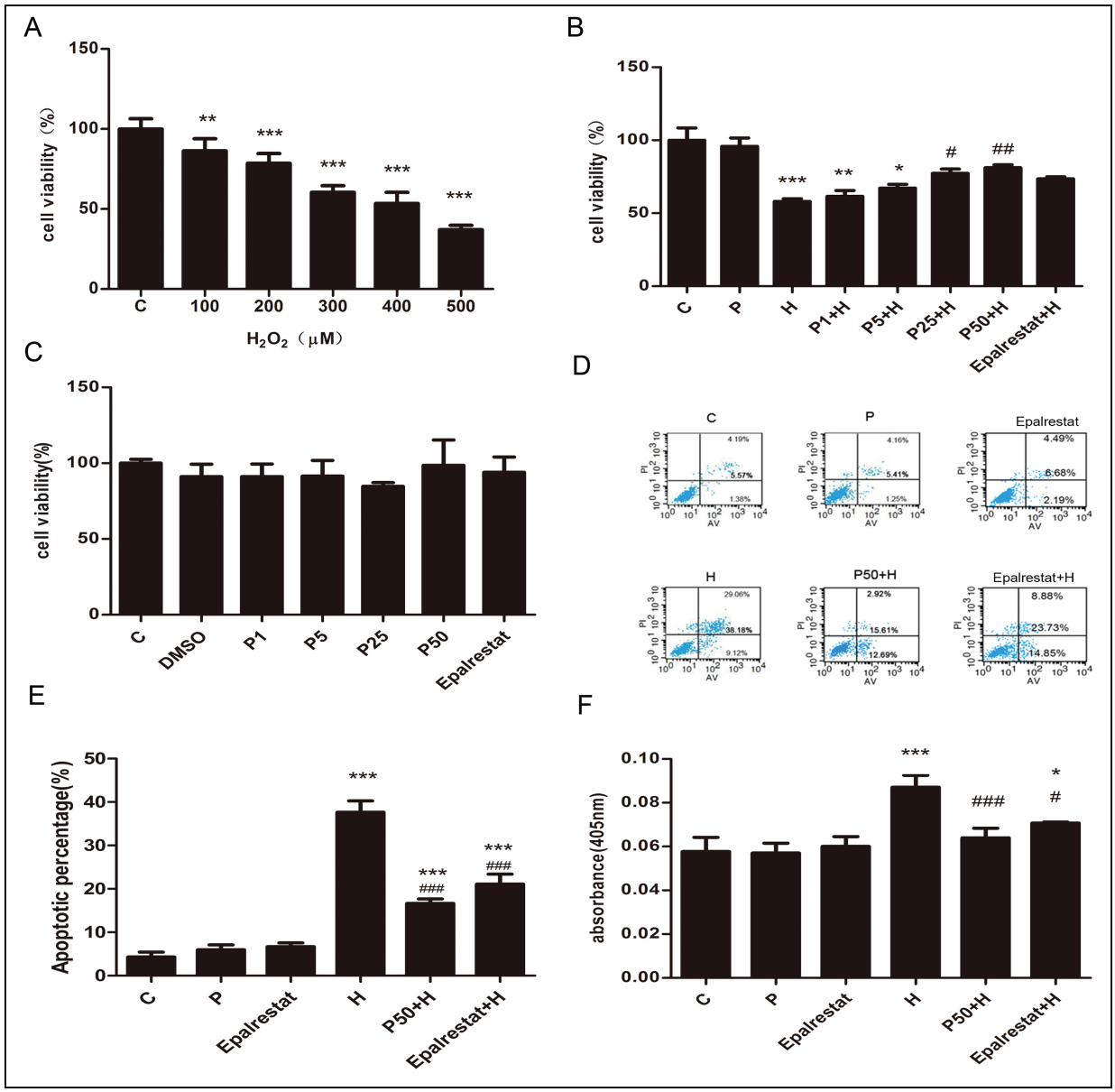

Fig. 1: Effects of H_2O_2 and/or propofol/epalrestat on HUVEC viability and apoptosis. (A-C) Cell viability was measured using a CCK-8 assay. HUVECs were incubated with or without H_2O_2 for 4 h. C, DMSO, P, and epalrestat are plain control, vehichle control, propofol control, and epalrestat control, respectively. (A) H_2O_2 decreased the HUVEC viability in a concentration-dependent manner. (B) HUVECs were pretreated for 30 min with propofol or epalrestat in the presence or absence of H_2O_2 (400 μM) for 4 h. (C) Cell viability was not significantly affected by propofol (1, 5, 25, 50 μM), epalrestat (25 μM) or vehicle (DMSO). (D, F) Cell apoptosis was evaluated using Annexin V/PI staining. (E) Caspase-3 activity was measured using Fluorometric CaspACE^{TM}. The results were expressed as mean ± SD (n = 5). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs C group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs H group.

dose-dependent manner (Fig. 1A). When the cells were treated with 400 μM H_2O_2 for 4 h, the cell viability was approximately 50%. Thus, 400 μM H_2O_2 was chosen for subsequent experiments based on these results.

Because the initial concentration of propofol for sedation is approximately 1 μM and steady-plasma propofol concentrations in the clinical setting are 10 to 50 μM (McMurray et al. 2004; McDermott et al. 2007), we initially examined the effect of propofol from sedation to anesthesia at concentrations of 1–50 μM. Propofol pretreatment inhibited the H_2O_2-induced cytotoxicity in a concentration-dependent manner. Pretreatment with 25 μM epalrestat (an AR inhibitor) also suppressed HUVEC viability, but there was no significant difference (Fig. 1B).

Propofol or epalrestat alone did not affect cell viability. Propofol was diluted in dimethyl sulfoxide (DMSO), whose concentration in the medium was 0.1%. DMSO had no effect on H_2O_2-induced cell viability (Fig. 1C). Moreover, we eventually chose the level of 50 μM propofol for subsequent experiments based on the following AR expression and on similar methods described in the scientific literature (Wang et al. 2007). Because similar results were obtained using AR-antisense and inhibitors, an AR inhibitor was used for most of the subsequent experiments.

2.2. Effects of propofol or epalrestat on H_2O_2-induced apoptosis in HUVECs

Annexin V/PI staining was used to examine the protective role of propofol or epalrestat in H_2O_2-induced apoptosis. The apoptosis rate was the sum of both early and late apoptosis events. As shown in Fig. 1D and 1E, after 4 h treatment with 400 μM H_2O_2, the proportion of apoptotic/necrotic cells was more than three times higher than that of the untreated control. Pretreatment with propofol or epalrestat significantly decreased the percentage of apoptotic cells following H_2O_2 treatment.

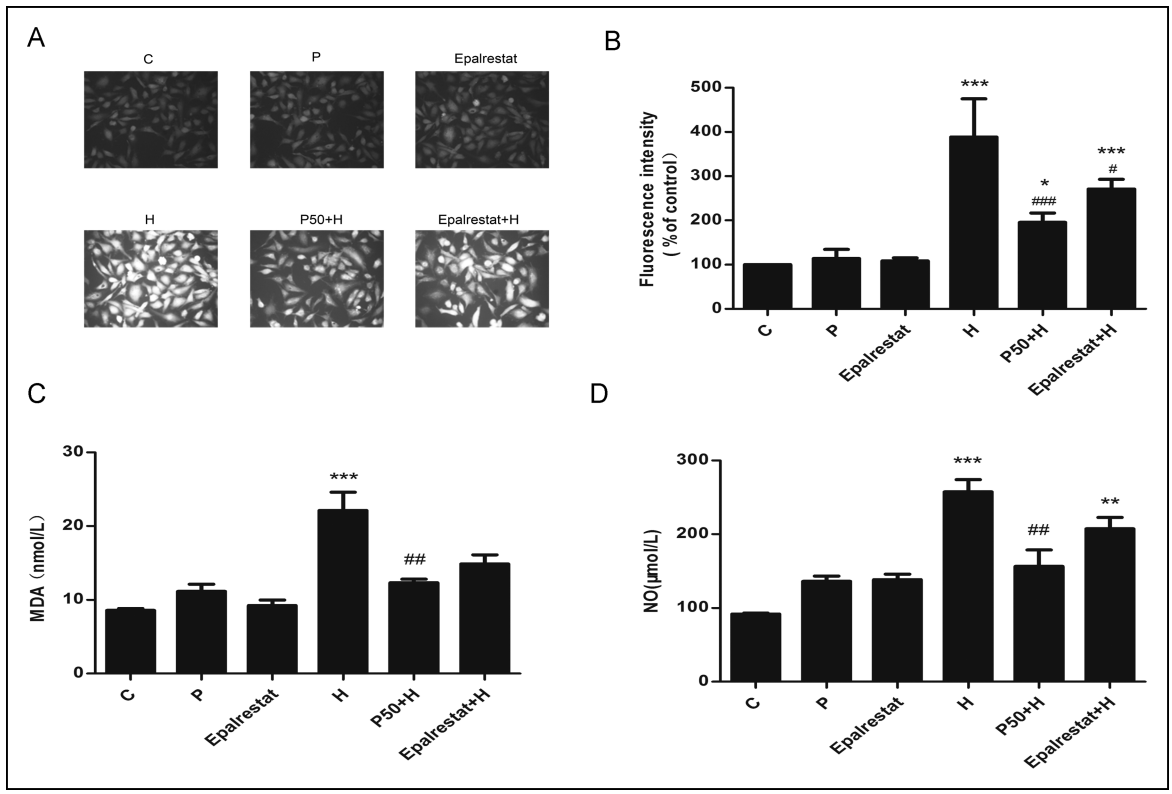

Fig. 2: Effects of Propofol or epalrestat on H_2O_2-induced production of ROS in HUVECs, MDA and NO in cell culture medium. HUVECs were pretreated with propofol or epalrestat for 30 min followed by 400 μM H_2O_2 treatment for 4 h. C, P, and epalrestat are plain control, propofol control, and epalrestat control, respectively. (A) Morphological observation using a fluorescent microscope (magnification 200 ×). (B) Quantification of ROS production. (C, D) The levels of MDA and NO. The results were expressed as mean ± SD (n = 5). $^{**}P < 0.01$, $^{***}P < 0.001$ vs C group; $^{\#\#}P < 0.01$ vs H group.

We also examined the effect of propofol or epalrestat on H_2O_2-induced caspase-3 activity. As shown in Fig. 1F, H_2O_2 treatment significantly increased caspase-3 activity compared with the control. Propofol or epalrestat pretreatment significantly decreased this H_2O_2-induced caspase-3 activation.

2.2.1. Effects of propofol or epalrestat on H_2O_2-induced ROS generation

To investigate whether ROS are involved in H_2O_2-induced HUVECs injury, we examined ROS levels in the presence or absence of propofol or epalrestat. H_2O_2 induced a significant increase in intracellular ROS levels, which was attenuated in cells pretreated with propofol or epalrestat (Fig. 2A and 2B). Propofol or epalrestat alone caused no significant changes in the ROS level in HUVECs.

2.2.2. Effects of propofol or epalrestat on H_2O_2-induced the production of MDA and NO

It has been reported that AR is an important component of antioxidant enzymes involved in the detoxification of reactive aldehydes generated by lipid peroxidation (Wen et al. 2013), and MDA is widely used as a marker of lipid peroxidation. AR and nitric oxide synthase share NADPH as an obligate cofactor. Therefore, we examined the production of MDA and NO in the presence or absence of propofol or epalrestat. H_2O_2 significantly increased the levels of MDA and NO, which were attenuated in cells pretreated with propofol or epalrestat (Fig. 2C and 2D). These results indicate that propofol or inhibition of AR sequesters oxidative stress to protect against H_2O_2-induced cell damage. Propofol or epalrestat alone have no evident effects on MDA and NO.

2.3. Induction of AR expression in H_2O_2-treated HUVECs

When HUVECs were incubated with 400 μM H_2O_2, AR expression increased significantly, reaching a maximum of 5.7-fold after 12 h (Fig. 3A and3B). Considering the majority of operation time and anesthesia duration, we chose HUVECs incubation with H_2O_2 for 4 h for all experiments.

2.4. Effects of propofol on H_2O_2-induced AR expression in HUVECs

H_2O_2 significantly increased AR expression in HUVECs, which was attenuated by propofol in a dose-dependent manner. Propofol pretreatment (50 μM) significantly decreased AR protein expression induced by H_2O_2 (Fig. 3C and 3D).

To further examine the role of AR in H_2O_2-induced cellular damage, the effects of gene silencing were examined (data not shown). A pool of siRNA against human AR, but not of control siRNA, reduced AR protein expression in HUVECs.

2.5. Effects of propofol, epalrestat or AR siRNA on H_2O_2-induced p38 MAPK/JNK /Akt phosphorylation in HUVECs

To determine the mechanisms of propofol's action, p38 MAPK, p-p38 MAPK, JNK p-JNK, Akt, and p-Akt (serine 473) were detected using western blotting. The results showed that H_2O_2 significantly activated p38 MAPK, JNK and Akt signaling pathways. Pretreatment of cells with propofol or epalrestat attenuated the effect of H_2O_2 (Fig. 4).

To rule out nonspecific effects of epalrestat, we silenced AR in HUVECs using AR siRNA to investigate whether the phenotypic absence of AR will have similar effects in HUVECs as do

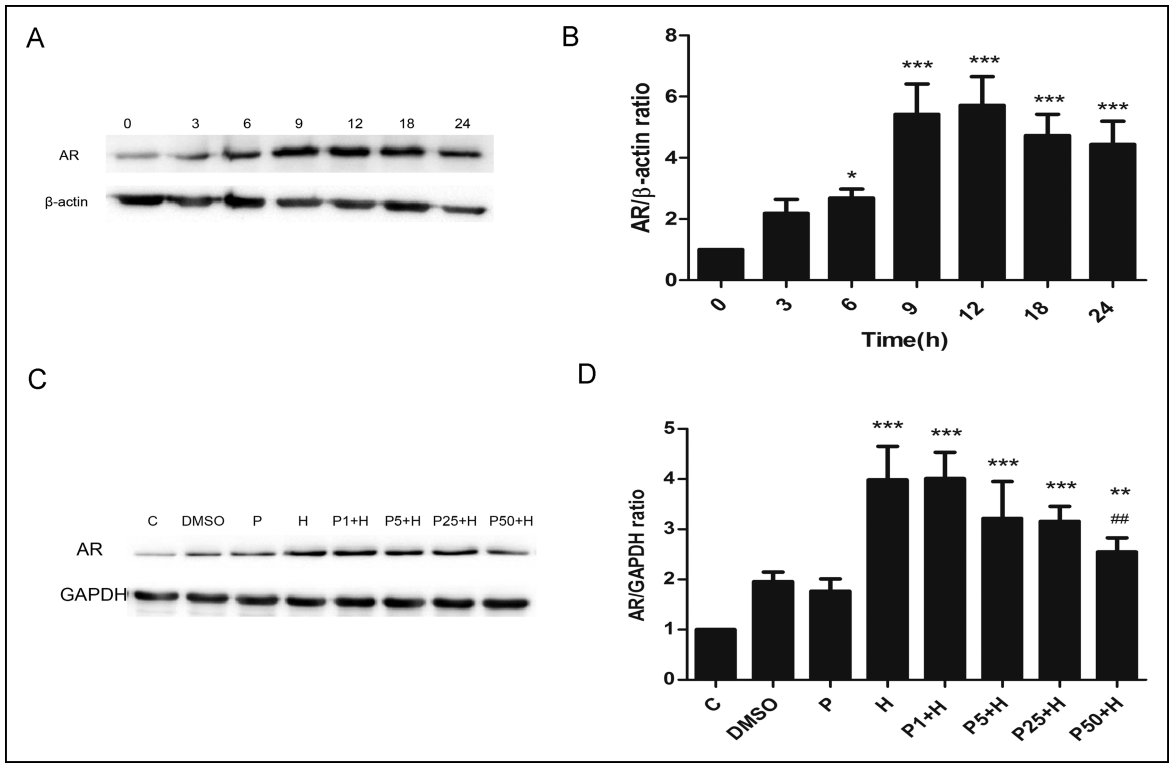

Fig. 3: Induction of AR expression in HUVECs by H_2O_2 and effects of propofol on H_2O_2-induced AR protein expression. C, DMSO, and P are plain control, vehicle control, and propofol control, respectively. (A, B) Cells was incubated with 400 μM H_2O_2 for the indicated times. (C, D) HUVECs were pretreated with propofol for 30 min followed by H_2O_2 (400 μM) for 4 h. Western blotting was performed to examine AR protein expression. GAPDH expression or β-actin was also examined for protein loading control. The results were expressed as mean ± SD (n = 5). $^{*}P < 0.05$, $^{***}P < 0.001$ vs C group; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ vs H group.

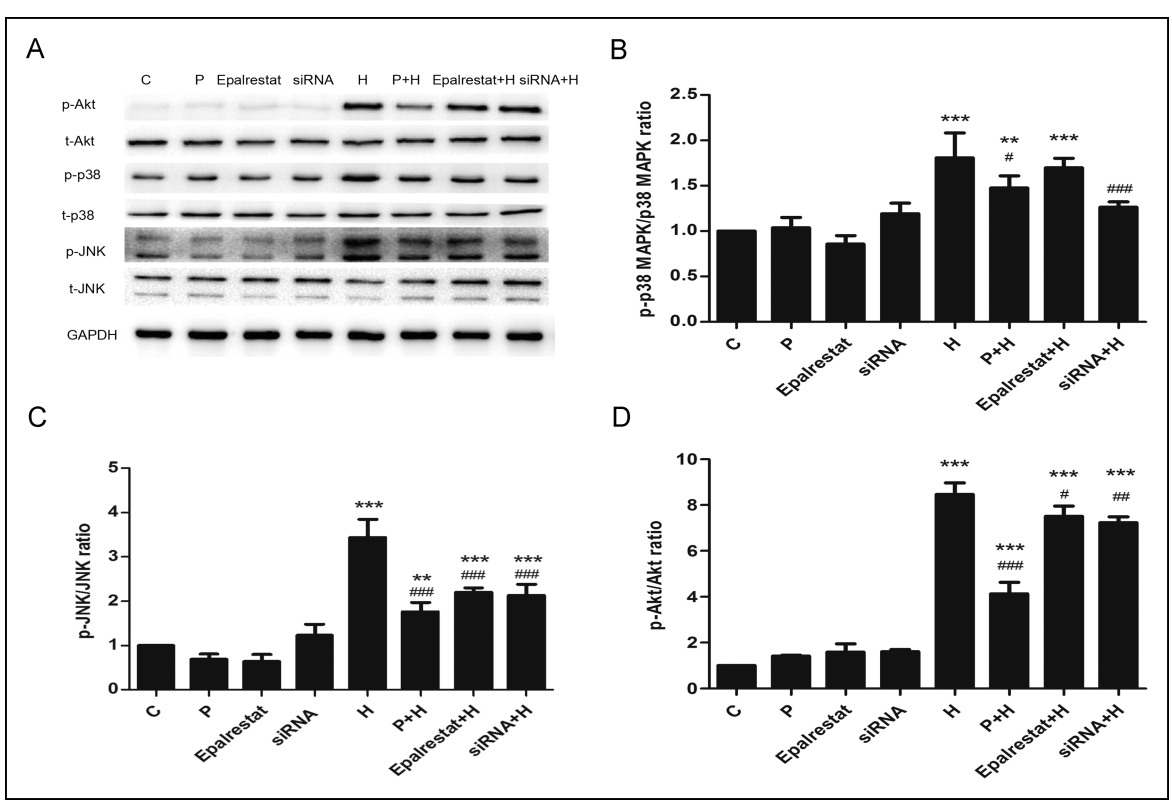

Fig. 4: Effects of propofol/epalrestat/AR siRNA on the H_2O_2-induced phosphorylation of p38 MAPK/JNK/Akt. HUVECs were pretreated with propofol or epalrestat or AR siRNA for 30 min followed by 400 μM H_2O_2 treatment for 4 h. C, P, epalrestat and AR siRNA are plain control, propofol control, epalrestat control and AR siRNA control, respectively. (A) Representative western blot of phosphorylated p38 MAPK/JNK/Akt. (B,C,D) Densitometric analysis of p-p38 MAPK/p-p JNK/p-p Akt, and the bands are normalized against GAPDH. The results were expressed as mean ± SD (n = 5). $^{**}P < 0.01$, $^{***}P < 0.001$ vs C group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ $^{\#\#\#}P < 0.001$ vs H group.

AR inhibitors. As shown in Fig. 4, AR ablation using a siRNA significantly inhibited p38 MAPK, JNK and Akt signaling pathways induced by H_2O_2 in HUVECs, which was similar to AR inhibition.

3. Discussion

Cultured HUVECs are particularly prone to oxidative damage and H_2O_2 has been extensively used as an apoptotic inducer in *in vitro* models. Propofol has been shown to have anti-oxidative and antiapoptotic effects (Marik 2005). In this study, after exposure to a high H_2O_2 concentration, HUVECs showed remarkable cytotoxicity, which is characterized by loss of cell viability, and an increase in caspase-3 activity and the apoptosis rate. This cytotoxic effect could be attenuated by propofol pretreatment, which was consistent with previous reports (Wang et al. 2007, 2009; Gu et al. 2013). In addition, incubation of HUVECs with H_2O_2 strikingly increased the production of ROS, MDA and NO, and this increase was significantly suppressed by pretreatment with propofol. Chen et al. (2010) also reported that propofol had the ability to scavenge ROS that were induced by H_2O_2 in endothelial cells. These results indicate that propofol can protect HUVECs against H_2O_2-induced injury. In addition, we found that propofol ameliorated cell injury accompanied by a down-regulation of AR expression.

It has been reported that AR overexpression, even without hyperglycemia, is sufficient to perturb the redox balance and induce cell death. Oxidative stress has been shown to increase glucose flux through the polyol pathway (Ramasamy and Goldberg 2010), and to activate AR in ischemic conditions (Kaiserova et al. 2006). In turn, AR activation may cause oxidative stress (Chung et al. 2003). In the present study, AR expression was activated by H_2O_2 in HUVECs, which was inhibited by propofol in a concentration-dependent manner. To further determine the role of AR, investigation of AR inhibition's role in preventing or exacerbating H_2O_2 injury in exposed HUVECs is required. Epalrestat, an AR inhibitor, exhibited similar effects to propofol on apoptosis and oxidative stress; epalrestat attenuated HUVEC injury similar to propofol. The observation that AR inhibition prevents endothelial injury suggests that AR inhibitor could be useful in preventing endothelial injury. In agreement with a previous study, they reported that AR inhibition suppressed lipopolysaccharide (LPS)-induced NO synthesis in macrophages (Ramana et al. 2007). Ramana et al. (2004) found that AR inhibition protects vascular endothelial cells against TNF-α-mediated apoptosis. Other studies also show that AR inhibition or ablation prevents LPS-induced apoptosis in macrophages and human lens epithelial cells (Ramana et al. 2007; Pladzyk et al. 2006). The mechanisms by which AR inhibition promotes cell survival remain unclear, but they may relate to AR-induced changes in the cellular redox state. Inhibition of AR catalysis that consumes NADPH has been thought to improve the intracellular reducing environment.

However, the roles of AR in oxidative damage are controversial. Our findings showed that AR inhibition decreased MDA production while some studies reported that AR inhibition increased the lipid peroxidation products and led to increased cell apoptosis (Wen et al. 2013; Kang et al. 2011). AR overexpression suppressed the increase in intracellular ROS and attenuated apoptotic cell death induced by ultraviolet B radiation (Kang et al. 2011). Wen et al. (2013) found that AR inhibition resulted in redox imbalance and further exacerbated the oxidative stress-induced damage and apoptosis in the ischemic intestine. AR was also shown to be an innate protective factor in an intestinal ischemia/reperfusion model. These researchers suggested that AR up-regulation may represent an adaptive response that removes toxic aldehydes and contributes to an endogenous and complex self-defense mechanism, ensuring an increased cellular resistance against toxic injury. The disparities between our findings and these reports may be related to the inconsistent and uncertain effects of AR inhibitors on different organs or tissues. These may include differences in organs or tissues and animal models, and differences in the specificity and selectivity of AR inhibitors in different tissues or organs. Most likely, AR is a significant regulator of the cellular redox state and apoptotic signaling, and its pro-oxidant/pro-apoptotic or antioxidant/antiapoptotic effects depend on the metabolic context that requires AR catalysis.

The regulatory mechanisms of AR expression under oxidative stress are poorly understood in endothelial cells. Among cell signaling pathways, MAPKs and Akt are essential for the death or survival of endothelial cells, respectively (Chavakis and Dimmeler 2002). A previous study has shown that H_2O_2 activates p38 proteins in HUVECs, and the mediation of cell death by ROS may be closely related to MAPK signaling (Chen et al. 2010). MAPKs and Akt pathways are involved in AR expression (Kang et al. 2011; Nishinaka and Yabe-Nishimura 2001; Ramana et al. 2004; Pladzyk et al. 2006; Yadav et al. 2013). Our results showed that p38 MAPK, JNK and Akt were significantly activated by oxidative stress in cultured HUVEC, which were inhibited by epalrestat or AR siRNA.

Propofol inhibition of p38 MAPK, JNK and Akt is similar to epalrestat and AR siRNA. Moreover, the effect of propofol on JNK in H_2O_2-treated HUVECs has never been investigated. We observed that activation of p38 MAPK and JNK phosphorylation was induced by H_2O_2 and suppressed by pretreatment with propofol. We also found that treatment with H_2O_2 for 4 h significantly increased Akt phosphorylation and pretreatment with propofol dramatically inhibited H_2O_2-induced Akt phosphorylation in HUVECs. This effect was not expected and it seems to contradict the results of other studies (Wang et al. 2007, 2009; Jun et al. 2011). Cell types, extracellular conditions, the changed cellular redox status and/or timing of Akt measurement may explain the discrepancy between these results.

Based on the above findings, propofol inhibits up-regulation of H_2O_2-induced AR expression. Propofol seems to play a similar role to that of epalrestat or AR siRNA in cytoprection of HUVECs. These cytoprotective effects might be in part mediated by AR inhibition or induction. Therefore, AR inhibition or induction using pharmacological inhibitors or inducers may represent a novel target for therapeutic intervention. Propofol, a frequently used anesthetic, which has antioxidant and antiapoptotic effects, may act as the target for AR therapeutic intervention.

There are several limitations to this study. First, propofol in the presence or absence of an AR inhibitor was required to enhance or reverse the protective effect. Second, further studies involving the use of specific signal pathway inhibitors, the measurement of AR activity, and AR at mRNA levels that are involved in various signaling pathways for varying periods are required to clarify the effects of propofol on endothelial cell injury.

In conclusion, propofol protected HUVECs from H_2O_2-induced injury. Propofol's protective effects on H_2O_2-induced injury may involve AR. The mechanism may be mediated *via* the p38 MAPK, JNK and Akt signaling pathways. These findings suggest that propofol may be a preemptive anesthetic in patients with cardiovascular disease, and highlight AR as a potential and promising target for therapeutic interventions.

4. Experimental

4.1. Chemicals

Propofol (purity > 97%; Lot number: D126608) was purchased from Sigma Aldrich (St. Louis, MO, USA). Epalrestat was obtained from Key Organ-

ics (Camelford, UK). The Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Laboratories (Kumamoto, Japan), and the bicinchoninic acid (BCA) protein assay kit was purchased from Beyotime Institute of Biotechnology (Haimen, China). Rabbit polyclonal antibodies against Akt, p-Akt, p38MAPK, p-p38MAPK, JNK, p-JNK, β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Cell Signaling Technology (Beverly, MA, USA). AR siRNA was purchased from Ribo Bio (Guangzhou, China). Dimethyl sulfoxide (DMSO) was purchased from Biosharp (Hefei, China). All other chemicals and reagents used were commercially available and of standard biochemical quality.

4.2. Cell culture and treatment

HUVECs were obtained from human umbilical cord veins by digestion with 0.1% collagenase type II (Sigma). The cells were cultured in Dulbecco's modified eagle medium (DMEM, Hyclone, UT, USA) supplemented with heat-inactivated 15% fetal bovine serum, endothelial cell growth supplement, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were then incubated at 37 °C in 5% CO_2 and 95% air. HUVECs at passage 2–5 were used for the experiments. Blood vessels were labeled using the anti-von Willebrand factor antibody. After 1 week of culture, HUVECs were identified using immunofluorescence staining with anti-von Willebrand factor antibody (Al-Mohanna et al. 1997).

HUVECs were cultured and divided into several groups depending on the treatment: Group C without any treatment was used as the control; cells in group DMSO, P, epalrestat and H were incubated with 0.1% DMSO, 50 μM propofol, 25 μM epalrestat or 400 μM H_2O_2, respectively, for 4 h; cells in group AR siRNA were incubated with 50 nM AR siRNA for 48 h. In Group P1 + H, P5 + H, P25 + H and P50 + H, cells were treated with 1, 5, 25, 50 μM propofol, respectively, for 30 min, and then incubated with 400 μM H_2O_2 for 4 h. In group AR siRNA+H, cells were treated with 50 nM AR siRNA and then incubated with 400 μM H_2O_2 for 4 h. Propofol was prepared in 0.1% DMSO and diluted with a culture medium immediately before the experiment.

4.3. Cell viability assay

Cell viability was tested using CCK-8, according to the manufacturer's instructions. Briefly, 2×10^3 cells/well were seeded in a 96-well flat-bottomed plate and treated with different conditions at 37 °C. After 24 h, 10 μL WST-8 dye was added into each well and the cells were incubated at 37 °C for 1 h, followed by measurement of optical density at 450 nm using a microplate absorbance reader (Bio-Tek, Elx800, USA). Cells stained positively with the CCK-8 solution were considered viable and are presented as a percentage compared with the control cells.

4.4. Annexin V-fluorescein isothiocyanate/propidium iodide (AV-FITC/PI) double- staining assay

HUVECs treated with propofol and/or H_2O_2 were collected *via* trypsin digestion, washed with cold phosphate-buffered solution (PBS), and resuspended in Annexin V binding buffer. AV-FITC (Becton Dickinson (BD) Biosciences, USA) at a final concentration of 1 μg/mL and 250 ng of PI was added to a mixture containing 100 μL each of cell resuspension and binding buffer (BD Biosciences). The mixture was incubated in the dark for 15 min at room temperature. Cells were washed once with binding buffer and resuspended in 400 μL of binding buffer prior to flow cytometric analysis using a BD FACScan Flow Cytometer (BD, Mountain View, CA, USA). AV-FITC-negative/PI-negative cells were considered to be healthy cells. Annexin V-FITC-positive/PI-negative cells were mainly early apoptotic cells. Annexin V-FITC-positive/PI-positive population represented late apoptotic or necrotic cells.

4.5. Caspase-3 activity assay

HUVECs were lysed and caspase-3 activity was measured in the cell lysate using the colorimetric CaspACE^{TM} Assay System. The colorimetric, caspase-3 specific substrate N-acetyl-Asp-Glu-Val-Asp-p-nitroaniline that was provided within the assay system is labeled with the chromophore p-nitroaniline (pNA). Free pNA produces a yellow color that is monitored using a spectrophotometer at 405 nm. The amount of yellow color produced upon cleavage is proportional to the amount of DEVDase (caspase-3) activity present in the sample.

4.6. ROS measurement

Intracellular ROS were measured fluorometrically with the DCFH-DA probe. After the indicated treatments, the cells were washed with PBS and incubated with 10 μmol/L DCFH-DA at 37 °C for 30 min. and excess DCFH-DA was removed. Subsequently, the cells were washed three times using PBS and fluorescence was measured using a fluorescence plate reader at excitation (485 nm) and emission (538 nm) wavelengths. Relative ROS production is expressed as a change in fluorescence.

4.7. Measurement of MDA and NO

The levels of malondialdehyde (MDA) and nitric oxide (NO) in the supernatants of cultured HUVECs were measured by spectrophotometry using kits from Jiancheng Bioengineering Institute (Nanjing, China) according to the manufacturer's recommendations.

4.8. Gene silencing with small interfering RNA

Small interfering RNAs (siRNAs) were designed to target the human aldose reductase coding sequence. The nucleotide RNAs were designed and then chemically synthesized, purified, and annealed (5′-AAC GCA TTG CTG AGA ACT TTA-3′) by Ribo Bio, China. Control, nonsilencing siRNA was also obtained from Ribo Bio. HUVECs were grown in DMEM that contained 15% FBS and 1% penicillin and streptomycin at 37 °C and 5% CO_2 and seeded on 6-well plates or 96-well plates. When the cells reached 70–80% confluence, the medium was replaced with fresh DMEM without serum, and the cells were transfected with 50 nmol/L control siRNA or siRNA designed against nucleotides of the human AR mRNA sequence, and the RNAiFect transfection reagent (lipo 2000) as per the supplier's instructions. After incubation for 15 min at 25 °C, the medium was aspirated and replaced with fresh DMEM that contained 10% serum added drop-wise to the cells. The cells were cultured for 48 h at 37 °C and 5% CO_2, and changes in aldose reductase were determined by measuring aldose reductase protein on western blots using anti-aldose reductase antibodies.

4.9. Western blotting

Cells in a 6-well plate were washed with PBS and lysed using lysis buffer containing phenylmethanesulfonyl fluoride (PMSF) at 4 °C for 5 min. Then the lysates were collected and centrifuged at 14,000 rpm and 4 °C for 15 min, and the supernatant was collected for protein analysis using an enhanced BCA protein assay kit. The whole protein samples were boiled for 15 min in 5 × loading buffer and separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples (50 μg/lane) were loaded on 10% SDS-PAGE, and proteins were transferred to polyvinylidene fluoride (PVDF) membranes at a current of 300 mA for 90 min. The membranes were incubated in Tris-buffered saline (TBS) containing 0.1% Tween-20 and 5% dry milk, and then incubated overnight with anti-GAPDH, AR, p38MAPK, p-p38MAPK, JNK, p-JNK, Akt or p-Akt antibody. After 2 h of incubation with anti-rabbit horse-radish peroxidase (HRP)-conjugated secondary antibodies, the protein bands were detected using an enhanced chemiluminescence (ECL) kit. The immune complexes were visualized via fluorography using an enhanced ECL system (Millipore, USA).

4.10. Statistical analysis

Results were analyzed using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). The data are expressed as the mean ± standard deviation (SD), and statistical analyses were performed using a one-way analysis of variance (ANOVA). $P < 0.05$ was considered significant.

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