

# Propofol attenuates angiotensin II-induced apoptosis in human coronary artery endothelial cells

J. Chen, W. Chen, M. Zhu, Y. Zhu, H. Yin and Z. Tan\*

Department of Anaesthesiology and Oncology, Fudan University Shanghai, China

\* Corresponding author. E-mail: anesthesiology2009@hotmail.com

## Editor's key points

- Activation of the renin–angiotensin system results in oxidative stress and apoptosis.
- The ability of propofol to reduce apoptosis induced by angiotensin II (Ang II) was assessed in human coronary artery endothelial cells.
- Propofol protected endothelial cells from Ang II-induced apoptosis by reducing oxidative stress and redox-sensitive apoptotic pathways.

**Background.** Angiotensin II (Ang II) induces oxidative stress and apoptosis in vascular endothelial cells. We hypothesized that propofol may attenuate Ang II-induced apoptosis in human coronary artery endothelial cells (HCAECs) and aimed to identify the underlying mechanisms.

**Methods.** Endothelial cells were pre-treated with propofol and then stimulated with Ang II. Apoptosis was examined by TUNEL, DNA laddering, and caspase-3 activity assays. The effect of propofol on Ang II-modulated NADPH oxidase expression and activity, nitric oxide synthase III (NOSIII) expression and phosphorylation and activity, lipid peroxidation, superoxide anion generation, nitric oxide production, caspase activity, and protein expression of cytochrome c, Bcl-2, and C-IAP-1 were measured.

**Results.** Ang II induced apoptosis, which was attenuated by 50  $\mu$ M propofol ( $P < 0.05$ ). Propofol ameliorated Ang II-induced NADPH oxidase expression and activation ( $P < 0.01$ ), lipid peroxidation ( $P < 0.05$ ), and superoxide anion generation ( $P < 0.05$ ), whereas restoring NOSIII phosphorylation and activity ( $P < 0.01$ ) were down-regulated by Ang II. Propofol attenuated Ang II-modulated cytochrome c release, and the expression of Bcl-2 and C-IAP-1. In addition, propofol inhibited Ang II-induced caspase-9 ( $P < 0.01$ ) and caspase-3 activity ( $P < 0.01$ ).

**Conclusions.** Propofol protected HCAECs from Ang II-induced apoptosis by interfering with the generation of oxidative stress and redox-sensitive apoptotic pathways.

**Keywords:** anaesthetics i.v.; angiotensin II; apoptosis; endothelial cell; propofol; reactive oxygen species

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Although the renin–angiotensin system is an important adaptive system for maintaining the cardiovascular system in a haemodynamically stable state, over-activation with the synthesis and release of angiotensin II (Ang II) followed by target cell activation is a key feature of hypertension, heart failure, and coronary artery disease, which may cause high mortality and morbidity.<sup>1–3</sup> At a molecular level, Ang II has been shown to induce cardiomyocyte hypertrophy,<sup>4</sup> cardiac fibroblast proliferation,<sup>5</sup> and vascular endothelial cell apoptosis,<sup>6</sup> resulting in the dysfunction of the cardiovascular system.

Endothelial apoptosis is recognized as an independent risk factor for cardiovascular disease. Dimmeler and colleagues<sup>7</sup> reported that Ang II-induced endothelial apoptosis was mediated through oxidative stress. The major source of oxidative stress in the vessel wall is the membrane-associated enzyme NADPH oxidase, which consists of multiple subunits (gp91phox, p22phox, p47phox, and p67phox). Ang II was shown to activate NADPH oxidase and induce oxidative stress in endothelial cells, and these effects were mediated by increased expression of gp91phox and p22phox.<sup>8, 9</sup>

Under physiological conditions, nitric oxide may inactivate superoxide anion and limit reactive oxygen species (ROS) formation, thus functioning as an endogenous antioxidant.<sup>10</sup> However, it is noted that when cells are exposed to excessive amount of nitric oxide and oxidative stress, peroxynitrite is formed which is very toxic and damaging. Also nitric oxide could stimulate endothelial cell growth and survival, acting as an antiapoptotic factor.<sup>7, 11</sup> Nitric oxide is predominantly produced by endothelial nitric oxide synthase III, NOSIII.<sup>12</sup> Li and colleagues<sup>6</sup> showed that Ang II enhanced apoptosis of human coronary artery endothelial cells (HCAECs) via modulation of protein expression of NOSIII. Another study showed that Ang II enhanced the phosphorylation of NOSIII, resulting in reduced nitric oxide availability in endothelial cells.<sup>13</sup> Moreover, a series of studies focused on the intracellular mechanisms responsible for Ang II-induced endothelial apoptosis found the involvement of the antiapoptotic factor Bcl-2 and proapoptotic protease caspase-3.<sup>6, 7</sup>

Propofol is an i.v. sedative and hypnotic agent and is indicated for the induction and maintenance of general anaesthesia.<sup>14</sup> It has a phenolic structure similar to that of

$\alpha$ -tocopherol and has antioxidant properties both *in vitro* and *in vivo*.<sup>15–17</sup> Propofol has been demonstrated to prevent oxidative stress-mediated endothelial cell activation and dysfunction induced by hydrogen peroxide,<sup>17</sup> tumour necrosis factor- $\alpha$  (TNF- $\alpha$ )<sup>18</sup> and ischaemia–reperfusion injury.<sup>19</sup> Considering the fact that patients during the perioperative period are likely to be exposed to activation of the renin–angiotensin system and oxidative stress, we wondered whether propofol was likely to affect oxidative stress. The aims of this study were to investigate the antioxidative and antiapoptotic effects of propofol in Ang II-treated endothelial cells and to identify the mechanisms responsible for any effects.

## Methods

### Cell culture

HCAECs were obtained from Clonetics (Cambrex, MD, USA) in a cryopreserved vial, seeded in culture dishes containing endothelial basal medium with 20% fetal bovine serum and growth factors, and cultured in a humidified 37°C/5% CO<sub>2</sub> incubator. After reaching 80% confluence, cells were passaged and cultured. Cells at passage 5 were used in this study.

### Apoptosis by TUNEL assay

TUNEL assay was performed as described previously.<sup>6</sup> In brief, after treatment, cells were harvested, plated on slides, and fixed with formaldehyde. After quenching endogenous peroxidase, cells were incubated with fluorescein-12-dUTP and immersed in propidium iodide. The slides were viewed under a fluorescence microscope. At least 200 cells from randomly selected fields were analysed to determine the percentage of apoptotic cells.

### DNA fragmentation gel electrophoresis (DNA laddering) assay

DNA laddering was performed as described previously.<sup>6</sup> In brief, cells were harvested and lysed. The fragmented DNA was separated from intact chromatin by centrifugation at 20 000g for 10 min at 4°C, and the supernatants were collected and treated with RNase A at 56°C for 2 h, followed by proteinase K at 37°C for 2 h. DNA was precipitated with ethanol and dissolved in gel loading buffer. DNA fragments were separated by electrophoresis in 1.5% agarose gel.

### NADPH oxidase activity

NADPH oxidase activity was measured by lucigenin-enhanced chemiluminescence as described previously.<sup>20</sup> Briefly, cells were incubated in lysis buffer, followed by centrifugation, then NADPH (100  $\mu$ mol litre<sup>-1</sup>) was added to the buffer containing cell homogenate suspension and lucigenin, and the photon emission was measured. NADPH oxidase activity was calculated by subtracting the basal values from those in the presence of NADPH. Data were expressed as nmol O<sub>2</sub><sup>-</sup> min<sup>-1</sup> mg protein<sup>-1</sup>.

### Lipid peroxidation

Malondialdehyde (MDA) was used as an index of lipid peroxidation.<sup>17</sup> In brief, cells were collected and suspended in phosphate buffer. After adding *N*-methyl-2-phenylindole and HCl, the reaction mixture was vortexed, followed by centrifugation. The absorbance of the supernatant was measured by spectrophotometry at 586 nm and compared with a calibration curve to determine MDA concentrations.

### Superoxide anion generation

Intracellular superoxide anion generation was measured with the superoxide anion-sensitive chemiluminescent probe coelenterazine.<sup>17</sup> In brief, cells were collected, washed with phosphate-buffered saline, and washed in the Krebs–Ringer buffer containing 10  $\mu$ M coelenterazine. The chemiluminescence was detected on a scintillation counter. Data were expressed as milliunits of light min<sup>-1</sup> mg protein<sup>-1</sup>.

### NOSIII activity

NOSIII activity was determined by measuring the conversion of <sup>3</sup>H-arginine to <sup>3</sup>H-citrulline with an NOSIII detection assay kit (Calbiochem, NJ, USA) according to the manufacturer's instructions. Briefly, cells were incubated with 10  $\mu$ M <sup>3</sup>H-arginine, harvested, and lysed. Protein concentration was determined, and the converted <sup>3</sup>H-citrulline was eluted and measured for radioactivity with liquid scintillation counting. Enzyme activity was expressed as <sup>3</sup>H-citrulline production in pmol min<sup>-1</sup> mg protein<sup>-1</sup>.

### Protein preparation and analysis by western blot

After treatment, cells were pelleted and lysed. Protein concentration was measured with the Bio-Rad protein assay system (Bio-Rad, CA, USA). For western blot analysis, proteins (~60  $\mu$ g) were mixed with electrophoresis buffer, separated by 6–10% SDS–PAGE, and transferred to polyvinylidene difluoride membranes. After incubation in 5% skimmed milk, the membranes were incubated with primary antibody overnight at 4°C. Primary antibodies used were monoclonal antibody against NOSIII, phosphorylated NOSIII Thr<sup>495</sup>, phosphorylated NOSIII Ser<sup>1177</sup>, NADPH oxidase p22phox, NADPH oxidase gp91phox, NADPH oxidase p47phox, Bcl-2, C-IAP-1, cytochrome c, and GAPDH. The membranes were then incubated with a corresponding secondary antibody. Protein bands were detected using enhanced chemiluminescence.

### Measurement of intracellular nitric oxide production

Intracellular nitric oxide levels were measured with a quantitative spectrofluorometer, as described earlier.<sup>17</sup> In brief, cells were cultured in 24-well plates, washed with Hanks' balanced salt solution, and incubated with 10  $\mu$ M 4,5-diaminofluorescein diacetate at 37°C for 40 min. The fluorescence was determined with a spectrofluorometer at excitation/emission wavelengths of 485/530 nm. Data were expressed as fluorescence intensity and reported in arbitrary units.

### Measurement of caspase activity

The activity of caspase-3, -8, and -9 was measured with a Caspase activity assay kit (Calbiochem). In brief, after treatment, cells were harvested and lysed. The supernatants were transferred to a tube to which specific substrate conjugates for caspase-3, -8, or -9 were added. Samples for caspase-3 and -9 activity were read by spectrophotometry at 405 nm. Samples for caspase-8 activity were read in a fluorescent plate reader at 400/505 nm for excitation/emission. The absorbance was referred to a calibration curve to determine units of caspase activity.

### Statistical analysis

Statistical comparisons were made by the Kruskal–Wallis test with SPSS V11.0 software. A value of  $P < 0.05$  was considered significant.

## Results

### Effects of propofol on Ang II-induced apoptosis

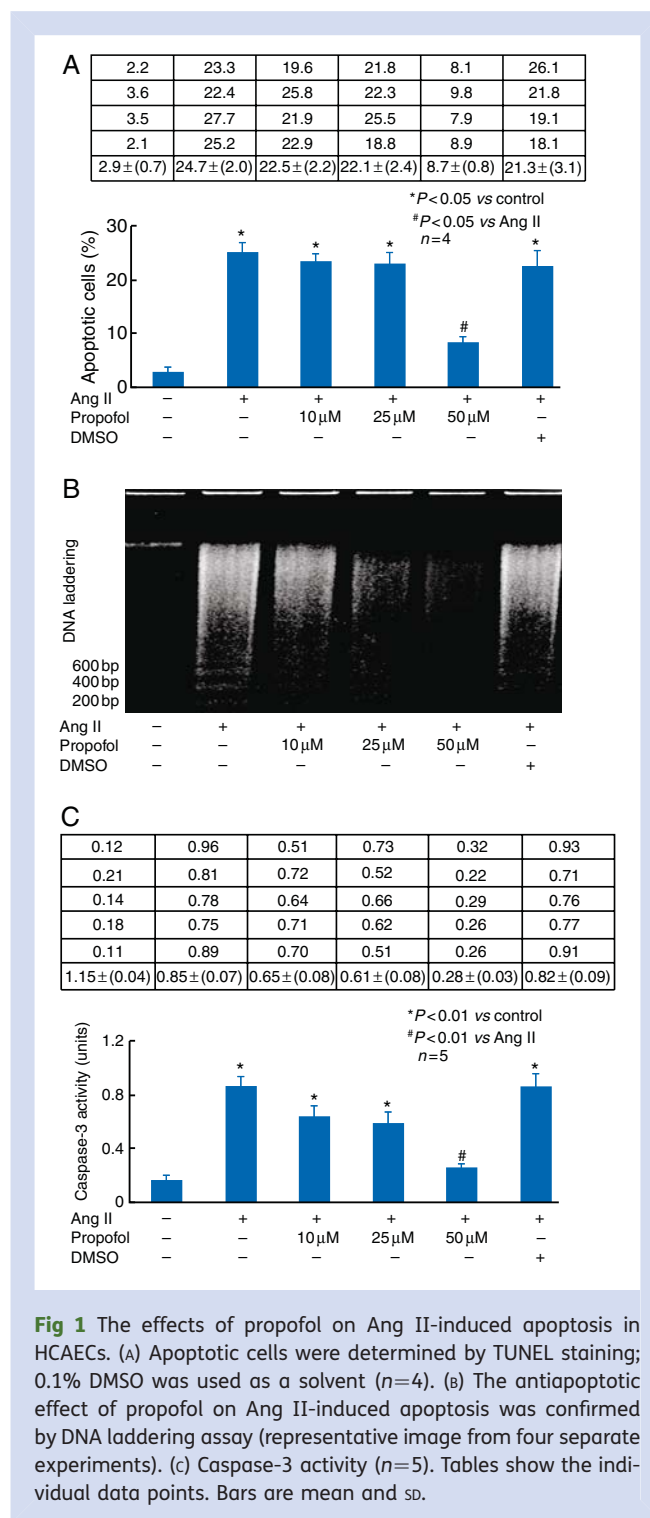
Ang II (1  $\mu\text{M}$ , 24 h) significantly induced the percentage of apoptotic cells by about nine-fold ( $P < 0.05$  vs control), which concurs with a previous study.<sup>5</sup> Pre-treatment of cells with 10 or 25  $\mu\text{M}$  propofol had no effect on Ang II-induced apoptosis. However, 50  $\mu\text{M}$  propofol markedly inhibited Ang II-induced apoptosis ( $P < 0.05$ ) (Fig. 1A). The effect of Ang II and propofol on apoptosis was confirmed by the DNA laddering assay (Fig. 1B). As shown in Figure 1C, Ang II greatly induced caspase-3 activity ( $P < 0.01$ ). While 10 or 25  $\mu\text{M}$  propofol pre-treatment had no effect on Ang II-induced caspase-3 activation, but 50  $\mu\text{M}$  propofol significantly reduced caspase-3 activity ( $P < 0.01$ ) (Fig. 1C). Propofol was diluted in dimethyl sulphoxide (DMSO), whose concentration in the medium was  $< 0.1\%$ . Note that 0.1% DMSO had no effect on Ang II-induced apoptosis (Fig. 1).

### Effects of propofol on Ang II-induced NADPH oxidase expression and activation and oxidative stress

As shown in Figure 2A, Ang II increased NADPH oxidase activity, which was ameliorated by 50  $\mu\text{M}$  propofol ( $P < 0.01$ ). We also examined the expression of NADPH oxidase p22phox, p47phox, and gp91phox subunits, which have previously been shown to be up-regulated by Ang II. We found that Ang II-induced up-regulation of p22phox and gp91phox was attenuated by 50  $\mu\text{M}$  propofol, whereas Ang II-induced up-regulation of p47phox was unaffected (Fig. 2B). Ang II also significantly induced superoxide anion generation and lipid peroxidation ( $P < 0.01$ ), which were inhibited by 50  $\mu\text{M}$  propofol pre-treatment ( $P < 0.05$ ; Fig. 2C).

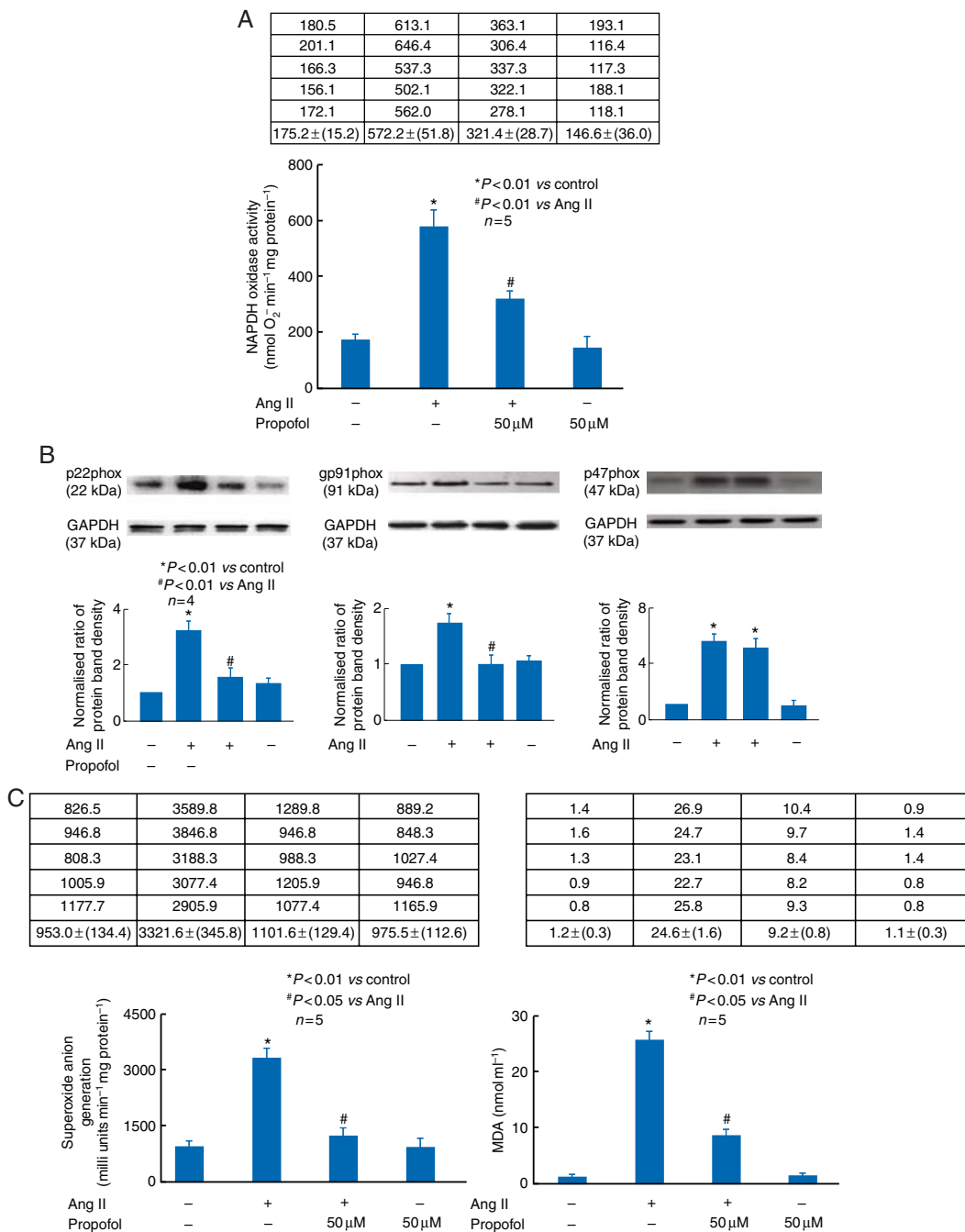
### Effects of propofol on Ang II-mediated NOSIII and nitric oxide

NOSIII has numerous potential phosphorylation sites, and phosphorylation of a serine residue (NOSIII Ser<sup>1177</sup>) and a threonine residue (NOSIII Thr<sup>495</sup>) is widely studied. Ser<sup>1177</sup> phosphorylation activates NOSIII, whereas Thr<sup>495</sup>



**Fig 1** The effects of propofol on Ang II-induced apoptosis in HCAECs. (A) Apoptotic cells were determined by TUNEL staining; 0.1% DMSO was used as a solvent ( $n = 4$ ). (B) The antiapoptotic effect of propofol on Ang II-induced apoptosis was confirmed by DNA laddering assay (representative image from four separate experiments). (C) Caspase-3 activity ( $n = 5$ ). Tables show the individual data points. Bars are mean and SD.

phosphorylation decreases NOSIII activity. As shown in Figure 3A, Ang II significantly induced NOSIII expression ( $P < 0.05$ ), which was not affected by propofol pre-treatment. However, Ang II markedly induced NOSIII phosphorylation at Thr<sup>495</sup>, whereas decreased NOSIII phosphorylation at Ser<sup>1177</sup> ( $P < 0.01$ ). The Ang II-mediated modulations of NOSIII phosphorylation were markedly reduced by 50  $\mu\text{M}$  propofol pre-

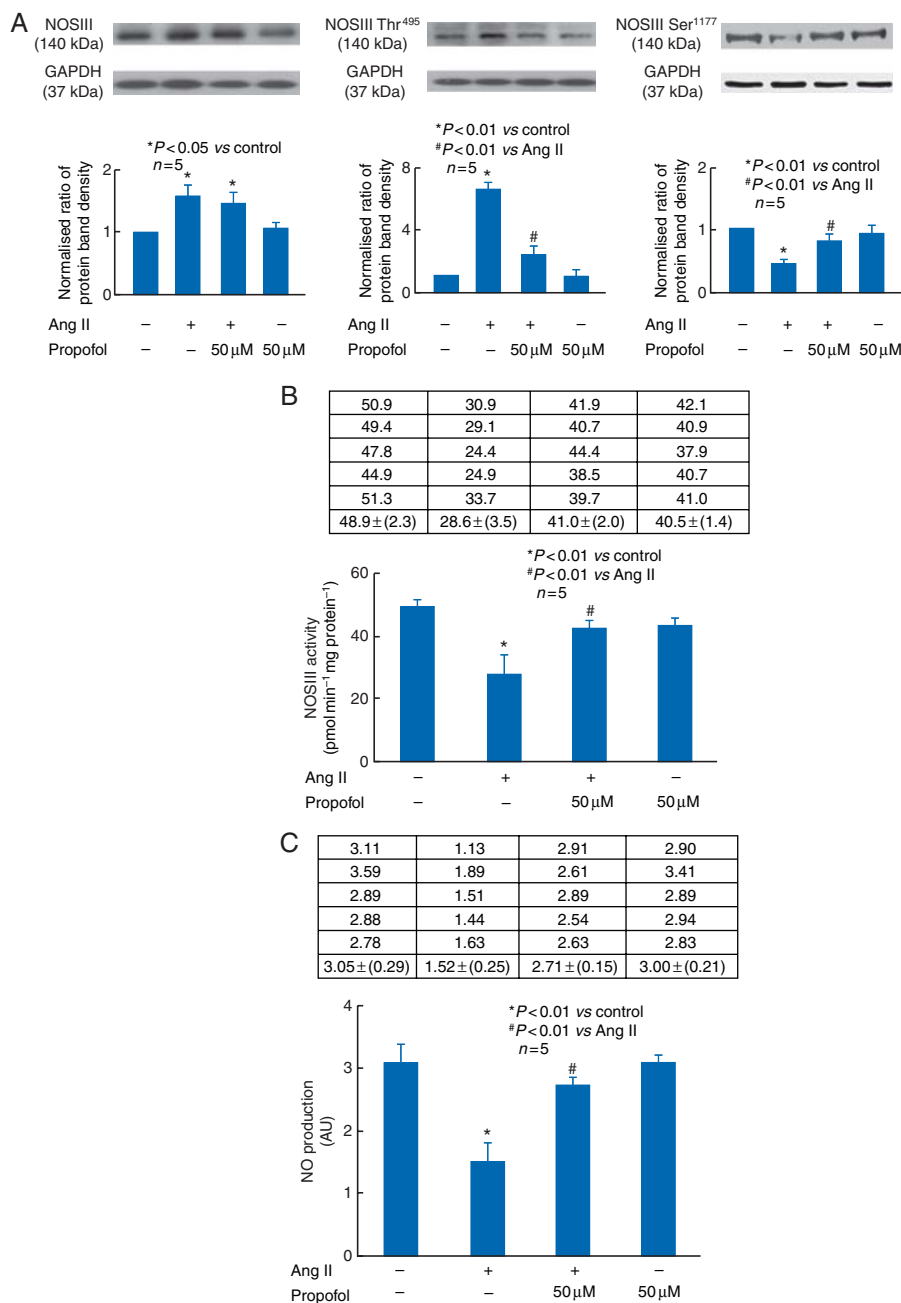


**Fig 2** The effects of propofol on Ang II-induced NADPH oxidase expression and activity and oxidative stress in HCAECs. (A) Propofol attenuated Ang II-induced NADPH oxidase activity ( $n=5$ ). (B) The effect of propofol on Ang II-induced protein expression of NADPH oxidase p22phox subunit (left), gp91phox subunit (middle), and p47phox subunit (right). GAPDH served as a loading control. The upper panel shows a representative experiment. The lower panel is the summary of densitometric data from four separate experiments. (C) Inhibitor effect of propofol on Ang II-induced oxidative stress as demonstrated by superoxide anion generation (left) and MDA (right) ( $n=5$ ). Tables show the individual data points. Bars are mean and SD.

treatment ( $P<0.01$ ). Ang II treatment inhibited NOSIII activity, which was attenuated by propofol pre-treatment (Fig. 3B). In addition, we found that Ang II decreased NO production, which was increased by 50  $\mu$ M propofol (Fig. 3C).

### Effects of propofol on Ang II-induced caspase activation and apoptosis regulator proteins

We found that Ang II greatly activated caspase-9 (by about five-fold) and caspase-3 (by about nine-fold), while having

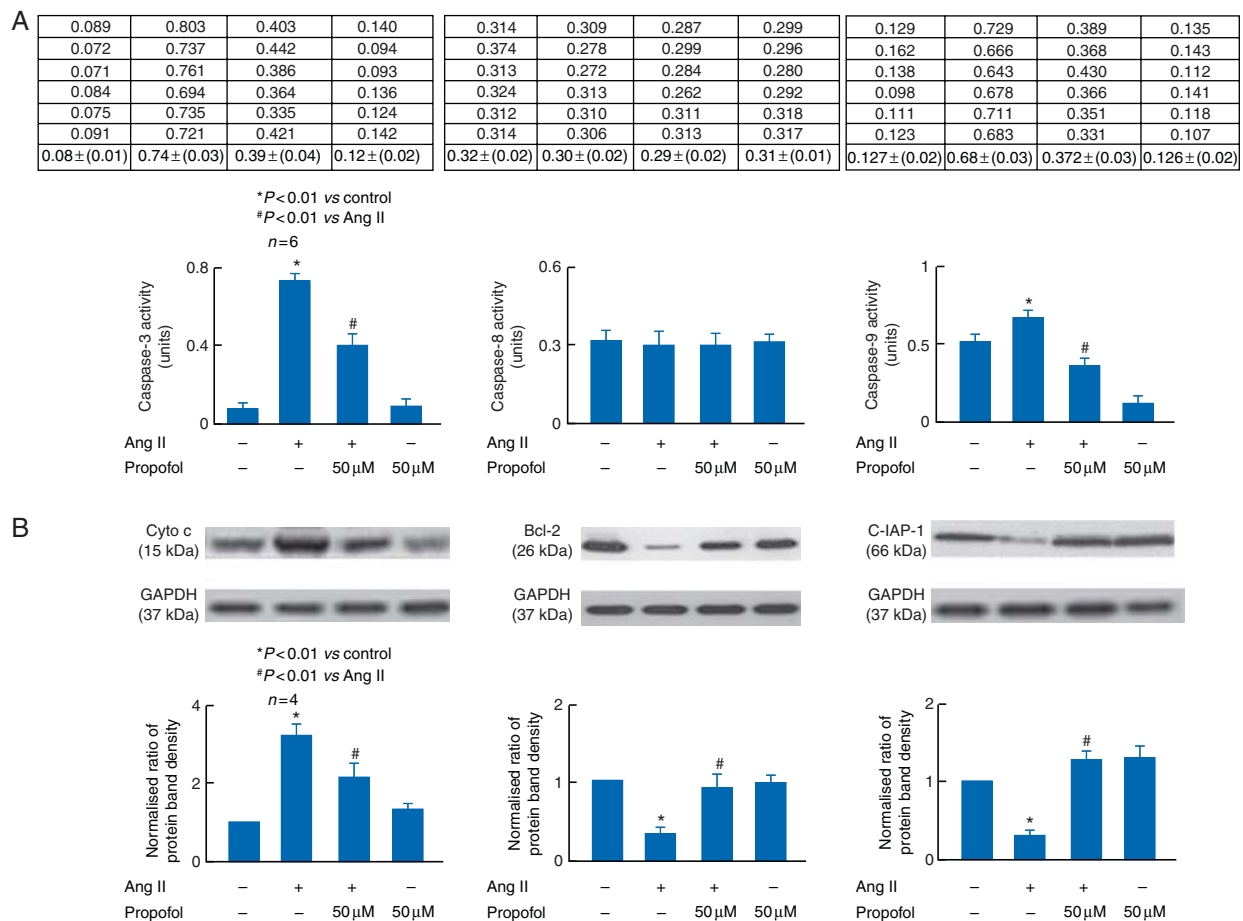


**Fig 3** The effects of propofol on Ang II-mediated NOSIII expression, phosphorylation, and activity and NO production in HCAECs. (A) Effect of propofol on Ang II-regulated NOSIII expression (left), and phosphorylation at Thr<sup>495</sup> (middle) and at Ser<sup>1177</sup> (right). GAPDH served as a loading control. The upper panel is a representative experiment. The lower panel is the summary of densitometric data from five separate experiments. (B) Effect of propofol on Ang II-modulated NOSIII activity (n=5). (C) Effect of propofol and Ang II on NO production (n=5). Tables show the individual data points. Bars are mean and SD.

no effect on caspase-8. Also 50 μM propofol pre-treatment inhibited Ang II-induced caspase-9 and -3 activation (Fig. 4A). The release of Ang II also induced the release of cytochrome c and reduced the expression of antiapoptotic protein Bcl-2 and C-IAP-1. Furthermore, the effects of Ang II on the modulation of these apoptosis regulator proteins were inhibited by 50 μM propofol (Fig. 4B).

### Discussion

Ang II is a major pathological factor and is closely associated with cardiovascular complications, especially during peri-operative period when Ang II is released due to acute or chronic stress. It has been reported that Ang II induced oxidative stress in endothelial cells.<sup>21</sup> Although the main source of ROS is aerobic respiration within mitochondria, they are



**Fig 4** The effect of propofol on Ang II-activated mitochondria-dependent caspase-9 pathway. (a) Effect of propofol on Ang II-modulated caspase-3 (left), caspase-8 (middle), and caspase-9 (right) activity (*n*=6). (b) Effect of propofol and Ang II on the release of cytochrome c (Cyto c) from mitochondria (left), and the protein expression of Bcl-2 (middle) and C-IAP-1 (right). GAPDH served as a loading control. The upper panel is a representative experiment. The lower panel shows densitometric data (*n*=4). Tables show the individual data points. Bars are mean and sd.

also produced by NADPH oxidase. Increasing experimental evidence has pointed to the critical role of NADPH oxidase in Ang II-induced oxidative stress in vascular endothelial cells. We demonstrated here that pre-treatment of HCAECs with propofol (50  $\mu$ M) significantly attenuated Ang II-induced NADPH oxidase expression and activity, and also inhibited Ang II-induced ROS formation (Fig. 2). In the present *in vitro* study, the beneficial concentration of propofol was found to be 50  $\mu$ M, which is  $\sim$ 9  $\mu$ g ml<sup>-1</sup>. In clinical practice, a serum concentration of propofol kept at 2–5  $\mu$ g ml<sup>-1</sup> has been shown to provide adequate plasma levels to achieve general anaesthesia.<sup>22 23</sup> The concentration used in this *in vitro* study is slightly higher than those observed clinically, but it is difficult to extrapolate the *in vitro* dose to the *in vivo* situation.

Many molecular mediators are responsible for the functional properties of the normal endothelium. Among them, nitric oxide is of central importance in that it contributes to principal endothelial functions, and is generally thought to

be protective against noxious stimuli, such as oxidative stress. Nitric oxide is synthesized by the NOS family of oxidoreductases. The endothelial isoform, NOSIII, is expressed constitutively in the normal endothelial cells. Accordingly, NOSIII is considered to be protective against oxidative stress. We demonstrated that Ang II decreased NOSIII phosphorylation at Ser<sup>1177</sup>, while increasing NOSIII phosphorylation at Thr<sup>495</sup>, leading to decreased NOSIII activity and nitric oxide generation. More importantly, we reported that these Ang II-mediated modifications were attenuated by propofol (Fig. 3). Combined with the findings that propofol reduced Ang II-stimulated NADPH oxidase expression and activity as well ROS generation (Fig. 2), we concluded that propofol may reduce Ang II-induced oxidative stress in endothelial cells.

In addition to phosphorylation, NOSIII activity is modulated by a variety of cofactors, including heat shock protein 90 (Hsp90) and 5,6,7,8-tetrahydrobiopterin. Hsp90 functions as a chaperone that regulates NOSIII activity<sup>24–26</sup> and

tetrahydrobiopterin is also critical for NOSIII enzymatic activity. In the absence of adequate levels, NOSIII becomes 'uncoupled' from L-arginine oxidation and molecular oxygen is reduced to form superoxide rather than nitric oxide.<sup>27–29</sup> To our knowledge, no existing study investigated the effects of Ang II and propofol on Hsp90 and tetrahydrobiopterin expression and on NOSIII 'coupling'.

In this study, we detected a seemingly conflicting phenomenon in that Ang II increased NOSIII expression, but decreased its activity. It is possible that modulation of protein expression may not always result in functional change. In this case, we assumed that the induced NOSIII expression may be a compensatory change in response to decreased activity. Nevertheless, this requires further investigation.

Apoptosis is an important process of many pathological conditions. A series of studies have provided convincing evidence, suggesting that Ang II induced apoptosis of vascular endothelial cells.<sup>7,30</sup> There are two major pathways for apoptosis: mitochondria-dependent caspase-9 pathway and the mitochondria-independent caspase-8 pathway. The mitochondria-dependent pathway is activated by agents such as ROS which trigger the release of cytochrome c from mitochondria.<sup>31</sup> The released cytochrome c complexes with apoptotic protease activating factor-1 (APAF-1) and stimulates the activation of caspase-9. The mitochondria-independent pathway is activated by extracellular apoptosis-inducing ligands, such as TNF- $\alpha$ .<sup>31</sup> The binding of ligands with cytoplasmic death domain of cell surface receptors activates caspase-8. Caspase-9 and -8 activate caspase-3, which cleaves vital cellular proteins or other caspases, leading to apoptosis. In our study, we detected the activation of caspase-3 by Ang II (Fig. 2). Furthermore, we identified that Ang II induced apoptosis of endothelial cells by a mitochondria-dependent pathway rather than a mitochondria-independent pathway (Fig. 4). The involvement of the mitochondria-dependent pathway was further confirmed by the findings that Ang II induced the release of cytochrome c, whereas reduced the expression of antiapoptotic protein Bcl-2 and C-IAP-1 (Fig. 4). Since Bcl-2 and C-IAP-1 function as blockers for the interaction between cytochrome c and APAF-1, reduced expression of Bcl-2 and C-IAP-1 may promote caspase-9 activation and apoptosis. Combined with our findings that Ang II induced ROS in endothelial cells, we believe that Ang II may induce apoptosis in HCAECs via oxidative stress which caused the release of cytochrome c and induced the expression of Bcl-2 and C-IAP-1, leading to the activation of caspase-9 and -3, thus initiating apoptosis.

Although the antiapoptotic effect of propofol in vascular endothelial cells has been widely studied, the underlying mechanism is far from clear. We have previously shown the antioxidant effects of propofol in endothelial cells.<sup>17</sup> Wang and colleagues<sup>32</sup> revealed that propofol significantly increased NOSIII activation and significantly protected endothelial cells from hydrogen peroxide-induced caspase-3 activation and apoptosis. Luo and colleagues reported that propofol, at concentrations higher than 12  $\mu$ M, significantly

attenuated TNF- $\alpha$ -induced increase in apoptosis and decrease in nitric oxide production and altering the Bcl-2 (antiapoptotic)/Bax (proapoptotic) ratio.<sup>18</sup> Considering the fact that hydrogen peroxide and TNF- $\alpha$  both induce oxidative stress in endothelial cells<sup>17</sup> and that NOSIII, nitric oxide, and Bcl-2/Bax ratio are both involved in oxidative stress-related signalling pathways, we think that propofol exerts its antiapoptotic effects via antioxidant activity. In this study, we demonstrated that propofol alleviated Ang II-induced apoptosis in HCAECs. Furthermore, we identified that propofol attenuated Ang II-induced oxidative stress and oxidative stress-related apoptotic pathway. Accordingly, our findings suggest that the antioxidant effect may be at least one potential mechanism responsible for the antiapoptotic effect of propofol in endothelial cells.

There are several limitations of this study. First, it was performed in cultured endothelial cells and *in vivo* studies, such as animal studies, are required to verify any beneficial effect. Secondly, in this study, endothelial cells were treated with propofol before exposure to Ang II. In the clinical situation, propofol is usually given to patients who already have activation of the renin-angiotensin system, and thus, the effect of propofol after exposure to Ang II needs to be evaluated. Another limitation of this study is that we observed the antioxidative effect and antiapoptotic effect of propofol in endothelial cells and implied that the antioxidant effect is responsible for the antiapoptotic effect of propofol. However, there is still lack of direct evidence suggesting the causal relationship. To verify this, the effect of NOSIII inhibitors, nitric oxide donors and ROS scavengers could be examined in future studies.

### Summary

We found that propofol could prevent Ang II-induced apoptosis by decreasing NADPH oxidase expression and activity and increasing NOSIII phosphorylation and activity but not expression, resulting in decreased oxidative stress. Propofol could inhibit redox-sensitive caspase-9- and -3-involved apoptotic pathway, which is activated by Ang II. Our findings have potential clinical relevance.

### Conflict of interest

None declared.

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