Original Article Protective effects of propofol on rat renal tubule epithelial cell line NRK-52E with hypoxia-reoxygenation

Shengdong Guo¹, Wenjin Chen¹, Mei Zheng^{1,2}, Shenggang Wang¹

¹Department of Anesthesiology, Linyi People's Hospital, Linyi 276002, Shandong, P. R. China; ²Department of Reproductive Medicine Center, Linyi People's Hospital, Linyi 276002, Shandong, P. R. China

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Abstract: Proximal tubule epithelial cells are the most vulnerable renal cells to ischemia-reperfusion injury, during which hypoxia and the subsequent reoxygenation are prone to induce cell death and inflammatory responses. As an extensively usedanesthetic, propofol has been demonstrated to play protective roles in myocardial cells with hypoxia-reoxygenation (HR), while little is known about its roles in renal cells. This study aimed to uncover the effect of propofol on rat renal tubule epithelial cells NRK-52E with HR. NRK-52E cells were treated with 0.5 or 5.0 µg/mL propofol and cultured under HR conditions. The viability and apoptosis of these cell groups were detected using MTT assay and flow cytometry. Expression changes of tumor necrosis factor (*Tnf*), anti-apoptosis factors B-cell lymphoma 2 (BCL2) and protein kinase B (AKT), and pro-apoptosis factor BCL2-associated X protein (BAX), were examined. Results showed that HR inhibited viability and promoted apoptosis of NRK-52E cells, while propofol markedly mitigated these injuries, with cell viability promoted and apoptosis suppressed (P < 0.01). *Tnf* mRNA level was up-regulated byHR, and was reduced to normal levels by propofol. Consistent to cell apoptosis changes, BAX was inhibited, and BCL-2 and phospho-AKT were up-regulated by propofol. Besides, 0.5 µg/mL propofol was sufficient to make these obvious changes, since no more significant effect was found when using 5.0 µg/mL propofol. These results indicated propofol was capable of inhibiting apoptosis and regulating apoptosis-related factors in NRK-52E cells with HR, providing basic evidence for the protective roles of propofol against renal ischemia-reperfusion injuries.

Keywords: Hypoxia-reoxygenation, propofol, cell apoptosis, renaltubule epithelial cell, ischemia-reperfusion

Introduction

Renal ischemia-reperfusion is the primary cause of acute renal failure. It is inevitable after kidney transplantation, resulting in the high morbidity and mortality. The most important feature of renal ischemia-reperfusion is the accelerated cell death with consequent tissue injury and dysfunction. When threatened by ischemic and other stimulus, renal tubule epithelial cells are prone to enter the death processes. Necrosis is the main form of cell death during acute renal failure, and is likely to cause inflammation [1]. Differing from necrosis, cell apoptosis also contributes to the aggravated cell death during renal ischemia-reperfusion, but without the induction of inflammation. The molecular mechanism regulating cell death and inflammation in renal ischemia-reperfusion is a hotspot of kidney nephropathy studies, and various problems are still to be elucidated.

Earlier studies have observed a number of aberrantly expressed genes in the murine renal ischemia-reperfusion injury model [2], which associate with the anabatic cell death and inflammation. Under ischemia and hypoxia conditions, renal epithelial cells highly express bunches of pro-apoptotic mediators, such as Toll-like receptors, to induce cell apoptosis [3, 4]. They also produce reactive oxygen species to trigger apoptosis [5]. These mediators modulate further downstream Caspase family via intrinsic or extrinsic apoptosis pathways to accelerate cell apoptosis [6]. From another aspect, renal tubule epithelial cells can participate in inflammatory responses by producing pro-inflammatory cytokines such as interleukins and tumor necrosis factor (TNF), exacerbating the resultant renal injury [7]. Despite these studies on depicting molecular mechanisms, great efforts are still necessary to develop effective strategies for treating and preventing renal ischemia-reperfusion injuries.

Propofol is a powerful general anesthetic widely used in various clinical departments for sedation and anesthesia. Recently, it is found to have anti-inflammatory and anti-oxidative effects in lipopolysaccharide-activated macrophages through regulating the biosynthesis of TNF, interleukins and nitric oxide [8]. Besides, it plays protective roles in brain, liver, lung and heart of ischemia-reperfusion rat models [9-12]. However, the function of propofol in kidney protection remains unclear. In this study, we aimed to investigate the role of propofol in renal cells with ischemia-reperfusion injuries. We performed hypoxia-reoxygenation (HR) on rat renal proximal tubule epithelial cell line NRK-52E to imitate in vitro the ischemia-reperfusion conditions. Different doses of propofol were added to the culture medium for cell viability and apoptosis assays. Expression levels of the pro-inflammatory mediator TNF, and apoptosis regulators B-cell lymphoma 2 (BCL2), BCL2-associated X protein (BAX) and protein kinase B (AKT) were examined. These results could provide fundamental information for the protective role of propofol in renal tubule epithelial cells and offer a potential therapeutic strategy for preventing renal ischemia-reperfusion injuries.

Materials and methods

Cell and treatment

Rat renal proximal tubule epithelial cell line NRK-52E purchased from ATCC (Manassas, VA) was cultured in Dulbecco's modified eagle medium (DMEM)/F12 (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gibco) in atmosphere with 5% CO_2 at 37°C. Cells were grouped into Control (cultured in the original condition throughout the experiment), HR (undergone HR), P1 (treated with 0.5 µg/mL propofol and HR) and P2 (treated with 5.0 µg/ mL propofol and HR). For P1 and P2 groups, propofol (Sigma-Aldrich, Shanghai, China) of a certain concentration was added at 2 h before hypoxia. Then HR, P1 and P2 groups were all cultured in 95% N₂ with 5% CO₂ for 1 h, after which they were transferred into atmospherewith 5% CO₂ for 2 h. At the end of the experiment, cells of all the four groups were collected for further analysis.

Cell viability assay

Cells of the four groups were adjusted to 5 \times 10⁴/mL and cultured in 96-well plates, with

200 µL suspension in each well. Four parallel samples were performed for each group. Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method conducted with MTT Cell Proliferation and Cytotoxicity Assay Kit (Beyotime, Shanghai, China). Briefly, fresh medium with 0.5% MTT was added to each sample for 4 h incubation. Then the medium was removed and 150 µL dimethylsulfoxide was added to each well. The plates were shaken for 10 min to fully dissolve the crystals, and then the optical density (OD) at 490 nm was measured using iMarkreader (Bio-Rad, Hercules, CA). Results of HR, P1 and P2 groups were compared to Control group.

Cell apoptosis assay

Cell apoptosis was analyzed using Annexin V: FITC Apoptosis Detection Kit (BD Biosciences, San Jose, CA) according to the manuals. Briefly, the cells were digested by trypsin (Gibco) and collected after centrifugation. Then cells were washed with cold phosphate buffer saline and resuspended in binding buffer. Annexin V-FITC was added to the cells for 15 min incubation in dark and then prodium iodide (PI) was added for 5 min incubation, after which binding buffer was added and the cells were detected on BD FACSCanto II (BD Biosciences). Total apoptotic cells was the sum of cells in early apoptotic stages (annexin V positive and PI negative) and cells in late stages (annexin V positive and PI positive).

Real-time quantitative PCR (qPCR)

Cells of the four groups were lysed in TRIzol (Invitrogen, Carlsbad, CA) for total RNA extraction. The extracted RNAs were digested by DNase I (Invitrogen) to remove DNA contamination, and detected by agarose gel electrophoresis and NanoDrop 2000 (Thermo Scientific, Carlsbad, CA) for quantification. First strand complementary DNAs (cDNAs) were synthesized from 1 µg RNAs using PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). gPCR was performed on LightCycler 480 (Roche, Basel, Switzerland) with 20 ng cDNAs and the specific primers for tumor necrosis factor (Tnf, Fw: 5'-ATG GAT CTC AAA GAC AAC CA-3' and Rv: 5'-TCC TGG TAT GAA ATG GCA AA-3'). Data were processed with the 2-DACt method compared to Control group, using Gapdh (Fw: 5'-CGC ATT GCC AGA CAT ATC AGC-



Figure 1. Propofol promotes cell viability and inhibits cell apoptosis in NRK-52E after HR. HR, hypoxia-reoxygenation. P1, cells treated by 0.5 μ g/mL propofol and HR. P2, cells treated by 5.0 μ g/mL propofol and HR. ***P* < 0.01. A. Percent of viable cells was decreased by HR and increased by propofol. B. Percent of apoptotic cells was increased by HR and decreased by propofol.



Figure 2. Propofol down-regulates the HR-promoted *Tnf* mRNA level. Tnf, tumor necrosis factor. HR, hypoxia-reoxygenation. P1, cells treated by 0.5 μ g/mL propofol and HR. P2, cells treated by 5.0 μ g/mL propofol and HR. **P < 0.01.

3' and Rv: 5'-AGG TGA AGC AGG CTC AAT CAA-3') as the endogenous reference.

Western blot

Proteins were extracted from cell samples using M-PER Mammalian Protein Extraction Reagent (Thermo Scientific) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The protein bands were transferred to polyvinylidene fluoride membranes and blocked in 5% skim milk for 2 h at room temperature. Then the membranes were incubated in the specific primary antibodies, anti-Bcell lymphoma 2 (BCL2), anti-BCL2-associated X protein (BAX), anti-pan-protein kinase B (AKT) or anti-pan-AKT (phospho T308) (Abcam, Cambridge, UK), at 4°C overnight. The membranes were washed and incubated in the horse reddish peroxidase-conjugated secondary antibodies for 1 h at room temperature and the signals were developed using ECL Plus Western Blotting Substrate (Thermo Scientific). The density of bands was analyzed with ImageJ software 1.49 (National Institutes of Health, Bethesda, MD). GAPDH was used as the endogenous reference and densities were compared to Control group.

Statistical analysis

All the experiments were conducted in triplicate unless otherwise specified. Results were indicated as the mean \pm standard deviation. *t* test was performed using SPSS 20 (IBM, New York, USA) and *P* < 0.05 was considered statistically significant.

Results

Propofol promotes viability and inhibits apoptosis of NRK-52E cells

After reoxygenation for 2 h, cells of the four groups were collected for viability and apoptosis assay. MTT results showed a significant decrease of viable cells in HR group compared to Control group (P < 0.01), which was obviously mitigated by propofol treatment (P < 0.01, **Figure 1A**). Accordingly, the percent of apoptotic cells was promoted by HR (P < 0.01), and again decreased by propofol treatment (P < 0.01, **Figure 1B**). Besides, there was no signifi-



Figure 3. Propofol inhibits BAX, and promotes BCL2 and AKT-activation in NRK-52E after HR. HR, hypoxia-reoxygenation. P1, cells treated by 0.5 µg/mL propofol and HR. P2, cells treated by 5.0 µg/mL propofol and HR. BCL2, B-cell lymphoma 2. BAX, BCL2-associated X protein. AKT, protein kinase B. p-AKT, phospho-AKT at T308. **P < 0.01. ***P < 0.001. A. Western blot showing the protein level changes of BAX, BCL2 and p-AKT. GAPDH is used as the endogenous reference. B. Histogram showing the statistical significance of Western blot results.

cant change in cell viability or apoptosis between P1 and P2 groups (P > 0.05). Taken together, these results indicated that propofol could significantly mitigate the HR-induced viability suppression and apoptosis promotion in NRK-52E cells, implying its protective role in renal tubular epithelial cells with HR. In addition, 0.5 µg/mL propofol was sufficient for its effective functions.

Propofol suppresses TNF expression

Since HR is usually accompanied with anabatic inflammatory responses, which can be reflected by the up-regulated TNF level [13], so the *Tnf* mRNA levels of the four groups were examined in this study. Results showed that HR significantly induced the expression of *Tnf* mRNA (P < 0.01), while the *Tnf* levels in both P1 and P2

groups were down-regulated (P < 0.01, **Figure 2**), but no significance was observed between P1 and P2 groups (P > 0.05). The changes in *Tnf* indicated the aggravated inflammatory responses by HR in NRK-52E cells, and that 0.5 µg/mL propofol was sufficient to bring the *Tnf* levels down to the regular state. So propofol might control the expression of pro-inflammatory *Tnf* induced by HR.

Propofol regulates apoptosisrelated factors

To explain the potential regulatory mechanism of propofol in suppressing NRK-52E cell apoptosis, we performed western blot to analyze the expression of pro-apoptoticfactor BAX and anti-apoptotic factors BCL2 and p-AKT. In accordance with the changes in cell apoptosis (Figure 1B), the relative density of BAX signal was up-regulated by HR and down-regulated by propofol (Figure 3A), while BCL2 was inhibited by HR and recovered by propofol. The p-AKT level was inhibited in

HR group and up-regulated by propofol, while the total AKT level almost remained unchanged, suggesting the activation of AKT by propofol. In the histogram, significant changes could be observed between Control group and HR or propofol treated groups (P < 0.01 or P < 0.001, **Figure 3B**), while no statistical difference was detected between P1 and P2 groups (P < 0.05). Taken together, propofol could regulate apoptosis-related factors including BAX, BCL2 and p-AKT, which might be its potential regulatory mechanism in preventing NRK-52E cell apoptosis after HR.

Discussion

In spite of the wide usage as an effective general anesthetic, propofol has been proved to be protective against ischemia-reperfusion in brain, liver, lung and heart. In this study, we find evidence for the protective effects of propofol in renal tubule epithelial cells NRK-52E with HR, which may suggest its roles in preventing renal ischemia-reperfusion injuries. In NRK-52E cells, propofol promotes cell viability and inhibits cell apoptosis after HR. It also suppresses the HR-induced *Tnf* mRNA levels, inhibits BAX, and promotes BCL2 and the activation of AKT.

The protective roles of propofol in renal tubule epithelial cells against HR could be incarnated in two aspects, one of which was the promoted cell viability and suppressed cell apoptosis. As was shown in MTT and flow cytometry results, HR could severely affected NRK-52E cells, reducing the viable cell percent and increasing the apoptotic cell percent, both of which, however, could be abrogated by propofol treatment. As abovementioned that the accelerated cell death induced by ischemia-reperfusion could be reflected from cell apoptosis and cell necrosis, the MTT and flow cytometry results suggested the protective roles of propofol in preventing renal tubule epithelial cell apoptosis after HR. Another aspect embodying propofol roles was its suppression on Tnf levels. TNF is well-known as a pro-inflammatory mediator, contributing to the elevated inflammatory responses and the promoted cell death after cells are stimulated. During liver injury, TNF increases inducible nitric oxide synthases mRNA levels and induces liver cell apoptosis [14]. TNF is hardly detected in normal cells but is up-regulated by myocardial ischemia-reperfusion and induces vascular endothelial cell apoptosis, thus being a valuable potential target for heart diseases [15]. In addition, the cell apoptosis caused by renal ischemia-reperfusion can be regulated by inflammatory responses, where TNF plays vital roles [16]. Based on the existed information, the Tnf level detected in this study implied that HR could elevated the TNF-related inflammatory responses, while propofol showed significant effects in controlling TNF levels, suggesting its roles in controlling inflammatory responses and the subsequent cell apoptosis. Besides, the elevated inflammatory response might indicate the aggravated cell necrosis, which also implied the anti-cell death role of propofol against cell HR injuries and renal ischemia-reperfusion injuries.

In order to investigate the possible mechanism of propofol in preventing cell apoptosis, we per-

formed Western blot to analyze the expression change of apoptosis-related factors. Both BAX and BCL2 are members of the BCL-2 family, but with distinct functions in regulating cell apoptosis: BAX promotes apoptosis while BCL2 is antiapoptotic. Knock-down BAX protects liver from ischemia-reperfusion injuries [17], and overexpression of BCL2 helps hippocampal neuron survival after global ischemia [18]. BAX and BCL2 were differently regulated in renal ischemia-reperfusion [19], during which the overexpression of BCL2 is capable of inhibiting the ischemia-reperfusion-induced oxidative stress and cell apoptosis [20]. So the up-regulated BAX and down-regulated BCL2 by HR again proved the promoted renal tubule epithelial cell apoptosis. But propofol could reverse the influences of HR, suggesting its roles of suppressing cell apoptosis, which is possibly via regulating BAX and BCL2. Further analysis would be helpful to verify the necessity of BAX and BCL2 in the anti-apoptotic function of propofol.

We also detected the activation of AKT by western blot using the antibody against T308 phosphorylation, since AKT was activated by S473 and T308 phosphorylation [21, 22]. The T308phosphorylated p-AKT is down-regulated after myocardial ischemia-reperfusion, while AKT activation can suppress cardiomyocytes apoptosis and protect them against ischemia-reperfusion [23, 24]. Our experiments showed propofol could reverse the HR-induced downregulation of p-AKT, indicating propofol was able to promote AKT activation, which was a potential mechanism of its regulation on NRK-52E cell apoptosis. In fact, AKT is involved in the mitochondria apoptosis pathway, which mediates the release of cytochrome C and further regulates cell apoptosis [25]. Bcl-2 family members such as BAX and BCL2 are translocated to the outer mitochondrial wall to modulate the release of cytochrome C [26-28]. From this point of view, propofol was likely to affect the mitochondria apoptosis pathway, which was a possible mechanism of its role in preventing renal tubule epithelial cell apoptosis after HR.

In this study, we applied two different doses of propofol to NRK-52E cells, and found that 5.0 μ g/mL propofol did not achieve better effects than 0.5 μ g/mL propofol, indicating that 0.5 μ g/mL propofol was sufficient to prevent HR-induced apoptosis and regulate *Tnf*, BAX, BCL2 and p-AKT expression. Given the possible

side effects caused by improper usage of propofol [29, 30], it was of great significance to determine the proper dosage before the clinical application. So there would be a lot of work even after the function of propofol was thoroughly understood.

In summary, this study indicates the protective roles of a general anesthetic, propofol, in suppressing renal tubule epithelial cell apoptosis induced by HR, possibly via activating AKT and regulating BAX and BCL2. These results provide evidence for the potential usageof propofol in preventing renal ischemia-reperfusion injuries. More detailed mechanisms are still to be investigated.

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Disclosure of conflict of interest

None.

Address correspondence to: Shenggang Wang, Department of Anesthesiology, Linyi People's Hospital, No. 27, Jiefang Road, Linyi 276002, Shandong, P. R. China. Tel: +86-0539-8078062; E-mail: wangshenggang6372@126.com

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