Propofol Prevents Renal Ischemia-Reperfusion Injury via Inhibiting the Oxidative Stress Pathways

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Key Words
Anesthetic • Propofol • Renal ischemia/reperfusion injury • NRK-52E cell • Mitochondrial stress • Endoplasmic reticulum stress

Abstract
Background/Aims: Renal ischemia/reperfusion injury (IRI) is a risk for acute renal failure and delayed graft function in renal transplantation and cardiac surgery. The purpose of this study is to determine whether propofol could attenuate renal IRI and explore related mechanism. Methods: Male rat right kidney was removed, left kidney was subjected to IRI. Propofol was intravenously injected into rats before ischemia. The kidney morphology and renal function were analyzed. The expression of Bax, Bcl-2, caspase-3, cl-caspase-3, GRP78, CHOP and caspase-12 were detected by Western blot analysis. Results: IR rats with propofol pretreatment had better renal function and less tubular apoptosis than untreated IR rats. Propofol pretreated IR rats had lower Bax/Bcl-2 ratio and less cleaved caspase-3. The protein expression levels of GRP78, CHOP and caspase-12 decreased significantly in propofol pretreated IR rats. In vitro cell model showed that propofol significantly increased the viability of NRK-52E cells that were subjected to hypoxia/reoxygenation (H/R) in a dose-dependent manner. The effect of propofol on the expression regulation of Bax, Bcl-2, caspase-3, GRP78, CHOP was consistent in both in vitro and in vivo models. Conclusion: Experimental results suggest that propofol prevents renal IRI via inhibiting oxidative stress.

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Introduction

IRI is one of the major causes of organ failure and high mortality, which is encountered in various clinical conditions such as coronary artery bypass grafting, thrombolytic therapy, cardiopulmonary resuscitation and transplantation [1-6]. Renal IRI often occurs in partial nephrectomy, renal transplantation and various urological vascular surgeries that may transiently reduce renal blood flow [7]. Renal IRI is a risk for acute renal failure and delayed graft function clinically [8-10].

The detail mechanisms in which renal IRI occurs are still unclear. Multiple factors involved in the pathogenesis include oxidative stress [11, 12], inflammation [13-15], cellular necrosis [16], apoptosis [17, 18] and so on. Based on these possible mechanisms, a lot of candidate drugs have been reported having protective effects on renal IRI [19-22]. Erythropoietin pretreatment ameliorates renal IRI by activating PI3K/Akt signaling and inhibiting tubulointerstitial inflammation [23, 24]. By anti-inflammatory effects and by reducing oxidative stress, atorvastatin, baicalein and mangiferin reduce renal IRI [25-28]. Preconditioning with triiodothyronine and gabexate is also reported to ameliorate renal IRI in rats [29-31]. However, these compounds are not effective enough to prevent and treat renal IRI clinically. Therefore it is necessary to develop novel drugs to protect kidney from IRI [32].

Propofol (2,6 di-isopropyl phenol) is a type of rapid, short-acting intravenous anesthetic, widely used in clinical anesthesia as well as for sedation in the intensive care unit [33]. The rapid and complete recovery and lower incidence of postoperative nausea make propofol one of the most common anesthesias. In addition to its use for maintenance of sedative effects as an anesthetic, propofol shows effectiveness in anti-oxidative, antiemetic and anti-anxiety activities [34]. Some studies have found the protective effects of propofol on myocardial ischemia, lipopolysaccharide-induced acute lung injury, and brain slice injury associated with its antioxidant properties and reducing intracellular calcium [35-38]. Propofol also has renoprotective effects on renal injury via inhibiting gap junction composed of connexin 32 [39]. Therefore, we propose that propofol might prevent renal IRI.

To examine our conjecture, we use in vivo rat model under renal IR and in vitro cell model under H/R and endoplasmic reticulum (ER) stress to investigate whether propofol has effective protection on renal IRI. In our study, we found that propofol improved renal function, inhibited oxidative stress, decreased mitochondrial stress and ER stress and increased the viability of the cells with H/R. Our experimental results suggest that propofol could prevent renal IRI via reducing oxidative stress and may be used to prevent renal IRI clinically.

Materials and Methods

Materials

Propofol, its lipidic vehicle, Annexin V-fluorescein isothiocyanate (annexin V-FITC) and propidiumiodide (PI) were supplied by Peking University Health Science Center (Beijing, China). We purchased dulbecco’s modified eagle’s medium (DMEM) from Invitrogen (Carlsbad, CA, USA) and fetal bovine serum (FBS) from Hyclone (Logan, Utah, USA). The commercial kits for measuring SOD, MDA, urea nitrogen (BUN), myeloperoxidase (MPO), and creatinine were purchased from NJJC Biology (Nanjing, China). In Situ Cell Death Detection Kit and cocktail were purchased from Roche (Indianapolis, IN, USA). Cell Counting Kit-8 kit was purchased from DOJINDO (Tokyo, Japan). Tunicamycin and 4-phenylbutyric acid (4-PBA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The antibodies against Bax, Bcl-2, β-actin, GRP78, caspase-12, CHOP, Goat anti-rabbit IgG and goat anti-mouse IgG were purchased from Santa Cruz Biotechnology (CA, USA). The antibody against caspase-3 was purchased from Cell Signaling Technology (Danvers, MA, USA).
Ethic Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of China Association for Laboratory Animal Science (SCXK (Beijing) 2011-0012). All animal care and protocols were approved by the Animal Care Committee of Peking University Health Science Center. All sacrifice was performed under pentobarbital anesthesia, and every effort was made to minimize animal suffering (SYXK (Beijing) 2011-0039).

Animal models of renal IR injury

Male Wistar rats (8 weeks old), weighting 220±20 g were purchased from the Animal Center of Peking University Health Science Center. The male rats were fed in a 12/12 h light/dark cycle and free access to food and water. The rats were randomly divided into four groups: sham-operated group; IR group; IR with propofol-pretreated group; IR with vehicle solution-pretreated group. Each group has 8 rats. The rats were suffered the warm renal IRI after anesthetizing by intraperitoneal injections of sodium pentobarbital (80 mg/kg). Then, the renal pedicle was exposed by flank incision. We first removed the right kidney, and then used a non-traumatic vascular clip to clamp the left renal pedicle for 60 min. For reperfusion, the clamp was taken away and the kidney was monitored for color change to confirm blood reflow before suturing the incision. The same surgical procedure was undergone in the sham-operated animals, except for the occlusion of the renal arteries. In propofol-pretreated group and vehicle-pretreated group as the solvent contrast, 20 mg/kg•h of propofol and vehicle solution were respectively injected into caudal vein continuously by micro-pump 30 minutes before renal ischemia. Rats in sham-operated group and IR group received physiological saline. During the surgery all animals were placed on a heating blanket to maintain body temperature at 37 °C.

Measurement of creatinine, BUN, MPO, SOD and MDA

Renal function was evaluated by measuring serum creatinine and BUN. After reperfusion for 24 h, we collected the serum samples (8 samples each group) for determination of urea and creatinine. Commercial kits and quantitative colorimetric urea determination kit were used to measure the concentration of serum creatinine and BUN, according to the instructions. The kidney tissue MPO, SOD and MDA levels were measured using commercial kits, according to the instructions.

Hematoxylin-eosin staining and TUNEL assay

Kidneys after reperfusion for 24 h were obtained and fixed in 4% formaldehyde for paraffin embedding. Paraffin-embedded tissues were sectioned at 7 µm for hematoxylin and eosin staining. Histological changes were evaluated by analyzing the percentage of renal tubules that displayed cell lysis and brush border loss. Representative fields were displayed. TUNEL assay was also conducted using the In Situ Cell Death Detection Kit (Roche Applied Science) following the instruction. Then, we identified positive staining in cell nucleus with DNA breakage under fluorescence microscopy.

Cell culture, H/R model, ER stress model

Normal rat renal tubular epithelial cell line NRK-52E cells were purchased from the Cell Resource Center of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. NRK-52E cells were cultured in DMEM containing 10% FBS (Invitrogen), 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, in an atmosphere with 5% CO₂ and air at 37 °C. For H/R protocol, an incubator (Thermo Fisher Scientific) was used to make the concentration of O₂ in the culture environment down to 1%. Cells were cultured to 70% to 80% confluence and then serum deprived for 24 h, and then cells were cultured for 12 h in media without glucose, in a hypoxic atmosphere as the H/R group. In the propofol groups various concentrations of propofol including 4 μM, 10 μM and 25 μM were added 1h before exposure to H/R. After hypoxia, cells were maintained in 5% CO₂ and air for reoxygenation for 4 h. The control cells were incubated at 37 °C in an atmosphere of 5% CO₂ and air for the same duration as hypoxic cells. For endoplasmic reticulum stress protocol, we cultured NRK-52E cells in the media with 2 μg/ml tunicamycin for 24 h to simulate endoplasmic reticulum stress. Likewise, different concentrations of propofol (4 μM, 10 μM and 25 μM) were added 1h previously. 4-phenylbutyric acid was used as positive control.
Cell viability assay

NRK-52E cells were harvested using 0.25% trypsin. Cells were suspended (50,000 cells/ml, 0.1 ml/well) in DMEM containing 10% FBS, plated in 96-well culture plates, and incubated at 37 °C, 5% CO₂ for 24 h. The media were replaced with 0.1 ml of DMEM without FBS and incubated for 24 h with propofol at different concentrations (1.56, 3.12, 6.25, 12.5, 25, 50, 100 μM). Cell viability was assayed according to the manufacturer’s instructions from CCK-8 Kit (Dojindo, Japan).

Annexin V-FITC/PI assay

Cell apoptosis was analyzed by staining with annexin V-FITC and PI staining. Cells were collected, washed in PBS and binding buffer respectively, then resuspended in 200 μl of binding buffer containing 10 μl of annexin V-FITC and incubated for 30 min on the ice in the dark. 5 μl of PI and 200 μl of binding buffer were added to each sample before flow cytometric analysis. Cells were analyzed using a FACS calibur (Becton Dickinson, Franklin, NJ, USA). In each sample, a minimum of 10,000 cells were counted. Data analysis was collected by using Cell Quest software (Becton Dickinson).

Western blot analysis

Tissues or cells were homogenized in RIPA lysis buffer containing protease inhibitor cocktail. Total protein was measured by BCA (Pierce Biotechnology, Rockford, IL, USA) and size separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were blotted to polyvinylidene difluoride membranes (Amersham Biosciences, Piscataway, NJ, USA). Blots were incubated with antibodies against Bax, Bcl-2, caspase-3, and β-actin, which was used as an internal control for equal protein loading. GRP-78, CHOP, caspase-12. Goat anti-rabbit IgG and goat anti-mouse IgG were added and the blots were developed with ECL plus kit (Amersham Biosciences).

We measured proteins Bax, Bcl-2, caspase-3 to demonstrate the level of apoptosis. The proteins GRP-78, CHOP, caspase-12 were also measured to indicate the level of ER stress.

Statistics

Data are expressed as mean ± SEM. All experiments were repeated at least 3 times on each specimen and there were 3 specimens from each group. Data obtained from each group were tested by one-way ANOVA and, whenever needed, post-hoc LSD test to detect any between-group differences. A p value < 0.05 was considered statistically significant.

Results

Propofol prevented renal IRI

BUN and blood creatinine levels that are index of renal function in IR group were increased after reperfusion for 24 h compared to the sham group (BUN: 29.61±3.02 mg/dl to 63.45±8.20 mg/dl; creatinine: 49.52±8.71 μM to 110.89±10.86 μM). While both of the BUN and creatinine levels were significantly lower in the propofol-pretreated IR group than IR group (Fig. 1A and Fig. 1B). Moreover, there was no obvious difference in BUN or blood creatinine values between IR group and vehicle solution-pretreated IR group. These data indicate that propofol could protect renal function from IRI.

Renal tissue morphology is shown in Fig. 1C. In the sham-operated group, there appeared no morphological change. After IR, there were severe architectural disruptions, including tubular dilatation and brush border loss. In contrast, propofol treatment preserved the morphologic integrity of tubular structure without acute tubular necrosis.

Propofol reduced renal oxidative stress and lipid peroxidation caused by IR

Renal IR resulted in a significant decrease in SOD activity (Fig. 2A), which is an index of oxidative stress increase in MDA concentration (Fig. 2B), which is an index of lipid peroxidation and MPO activity (Fig. 2C), which is an index of oxidative stress in renal tissue compared to sham group. The propofol significantly reversed these changes caused by IR.
Propofol reduced mitochondrial stress and caspase-3 activation caused by IR

We counted the apoptotic cells in the kidney by TUNEL assay (Fig. 3A). TUNEL positive cells in rats suffered IRI (16.7±3.6) were significantly more than those of sham rats (0.7±0.1). However, fewer TUNEL positive cells were observable in propofol-pretreated group (6.0±1.7). These results indicate that propofol protected the kidney from renal tubular apoptosis.

As shown in Fig. 3B, after reperfusion for 24 h, the expression of pro-apoptotic protein Bax was increased, while the expression of anti-apoptotic protein Bcl-2 was decreased. The ratio of Bax/Bcl-2 increased about 13 folds under IR compared with sham group. After propofol pretreatment, the level of Bax was down-regulated while the level of Bcl-2 was up-regulated, the ratio of Bax/Bcl-2 was significantly reduced, suggesting that propofol could inhibit apoptosis via mitochondria-dependent pathway.

The ratio of cleaved-caspase-3/caspase-3 was increased in the IR group compared to the sham group, confirming that caspase-3 was involved in the IR induced apoptosis. As we
Fig. 3. Propofol decreased cell apoptosis caused by IR. (A) Representative images of TUNEL (green fluorescence) and nuclei staining (Hoechst, blue) of kidney (original magnification x400). (B) Protein expression levels after reperfusion for 24 h were detected by Western blot analysis. Blotting (up) and quantification of protein levels (down) are shown. Data are presented as mean ± SEM. n = 3; *p<0.05; **p<0.01 vs. sham group, #p<0.05; ##p<0.01 vs. IR group.

Fig. 4. Propofol protected kidney from IRI via endoplasmic reticulum stress pathway. Protein expression levels of GRP78, caspase-12 and CHOP in kidney after reperfusion for 24 h were detected by Western blot analysis. Blotting (up) and quantification of protein levels (down) are shown. Data are presented as mean ± SEM. n = 3; *p<0.05; **p<0.01 vs. sham group, #p<0.05; ##p<0.01; ###p<0.001 vs. IR group.

see, pretreatment with propofol inhibited the caspase-3 pathway. We also found treatment with propofol obviously inhibited the phosphorylation of p53 that was involved in IR induced cell apoptosis.
Propofol reduced ER stress caused by IR

In addition to mitochondrial pathway, we also detected the ER pathway marker GRP78 (Fig. 4). After IR, the expression of GRP78 was significantly increased by 5 folds compared to sham group, which meant that ER played a crucial role in IR. We also found another hallmarks CHOP and caspase-12 increased after IR. The levels of these proteins were decreased by propofol pretreatment, which indicated that propofol reduced IR induced ER stress.

Propofol improved the cell viability and reduced apoptosis in H/R-induced NRK-52E cells

Propofol did not show significant cytotoxicity under 100 μM in normoxic condition (Fig. 5A). As we see in Fig. 5B, the cell viability decreased to about 50% after H/R compared with the sham group, which was significantly improved by propofol pretreatment for an hour before exposing to H/R in a dose-dependent manner (4, 10, 25 μM).

We observed the apoptotic NRK-52E cells by TUNEL assay. TUNEL positive cells induced by H/R were obviously more than those cultured in normal condition (Fig. 5C). However, fewer TUNEL positive cells were visible in propofol pretreated H/R group than in untreated H/R group. Annexin-V FITC/PI staining was used to confirm the anti-apoptotic effect of propofol quantitatively (Fig. 5D). Compared with control group, the portion of annexin-V(+)/PI(+) cells in H/R group increased from 1.79±0.55% to 37.98±2.31% (Fig. 5E). However pretreatment with different concentrations of propofol for 1 h before H/R
**Fig. 6.** Effects of propofol on mitochondrial stress pathway in NRK-52E cells. Protein expression levels of NRK-52E cells after reoxygenation for 4 h were detected by Western blot analysis. Blotting (up) and quantification of protein levels (down) are shown. Data are presented as mean ± SEM. n = 3; **p<0.01 vs. sham group, ##p<0.01; ###p<0.001 vs. IR group.

**Fig. 7.** Effects of propofol on endoplasmic reticulum stress pathway in NRK-52E cells. Protein expression levels of NRK-52E cells after reoxygenation for 4 h and cultured in 2 μg/ml tunicamycin for 24 h were detected by Western blot analysis. Blotting (up) and quantification of protein levels (down) are shown. Data are presented as mean ± SEM. n = 3; ***p<0.001 vs. sham group, #p<0.05; ##p<0.01; ###p<0.001 vs. IR group.

Propofol reduced the apoptosis via mitochondria dependent pathway in NRK-52E cells
The proteins Bax, cleaved caspase-3 were up-regulated after reoxygenation for 4 h (Fig. 6), whereas Bcl-2 was down-regulated. Propofol significantly reduced ratios of Bax/Bcl-2 and cleaved caspase-3/caspase-3 in a dose-dependent manner to inhibit cell apoptosis. Similarly, the phosphorylation of p53 was significantly decreased by propofol pretreatment.

Propofol reduced the apoptosis via ER dependent pathway in NRK-52E cells
As shown in Fig. 7A, the levels of protein GRP78, CHOP and caspase-12 were increased significantly decreased the percentage of annexin-V(+)/PI(+) cells to 26.05±3.13% (10 μM), 16.34±1.71% (25 μM), confirming the anti-apoptotic effect of propofol.
pattern. To confirm our finding, we used tunicamycin as an ER stress model. After being cultured with 2 μg/ml tunicamycin for 24 h, GRP78, CHOP and caspase-12 protein levels in NRK-52E cells were increased and then reversibly suppressed by 4-PBA or propofol pretreatment, which indicates that propofol treatment could prevent the apoptosis via ER dependent pathway in NRK-52E cells.

Discussion

The aim of this study was to determine whether propofol could protect kidney from renal IRI by its anti-oxidative activity. Experimental results showed that propofol pretreatment could prevent renal injury, preserve renal function, and reduce renal oxidative stress and apoptosis in rat with renal IR. In vitro cell model confirmed that propofol protected cells from H/R caused apoptosis via inhibiting oxidative stress and its downstream pathways, such as mitochondrial stress and ER stress.

Oxidative stress is a major cause of renal IRI [40]. When ischemic tissues were reperfused, the plentiful molecular oxygen (O$_2$) reaches the ischemic tissues to form excessive free oxygen radicals, which resulted in extensive apoptosis of tubular epithelial cells and tissue damage [41]. Previous studies showed that reduction of oxidative stress can protect kidney from renal IR [42]. Zou et al. found that pioglitazone protected against renal ischemia-reperfusion injury by increasing the level of enzymatic activities of superoxide dismutase and enhancing antioxidant capacity [43]. Similar mechanisms were seen in the protective effects of curcumin and intermedin [44].

It was reported that propofol had anti-oxidative activity [45]. Propofol scavenges H$_2$O$_2$ and reduces the formation of lipid peroxides [46], which validates in heart protection [47, 48]. Hsu et al. discovered that propofol attenuates lipopolysaccharide-induced reactive oxygen species production through suppression of NADPH oxidase in human alveolar epithelial cells [49]. Our study suggests that propofol attenuated renal IRI via inhibiting oxidative stress by increasing SOD activity and decreasing MDA and MPO.

Both mitochondrial stress and ER stress are downstream pathways of oxidative stress [50]. To confirm that protective effect of propofol bases on the anti-oxidative activity, the proteins related to mitochondrial stress were analyzed in this study. The experimental results showed that propofol increased the expression of Bcl-2 and suppressed the expression of Bax. The decreased ratio of Bax/Bcl-2 made low release of cytochrome c from the mitochondria into the cytosol resulting in the inhibition of caspase-3, reducing cellular damage. Another study found that propofol treatment alleviated renal IRI by increasing the Bcl-2/Bax ratio and decreasing active caspase-3 expression [51]. But it was reported that propofol induced apoptosis of hippocampal neurons in vitro via down-regulation of NF-κB p65 and Bcl-2 and up-regulation of caspase-3. The controversial effect of propofol on apoptosis in different tissues may be due to the specificity of cell types or the conditions of propofol treatment [52].

To confirm the protective effect of propofol and its mechanism on renal IRI we found in vivo, in vitro NRK-52E cells were used to create a hypoxia-reoxygenation model. After treatment of propofol at different concentrations, the viability of the cells undergone with H/R was significantly increased. Propofol significantly reduced H/R-induced cell apoptosis in a dose-dependent manner. Decrease in Bax/Bcl-2 ratio and active caspase-3 expression confirmed that propofol prevented renal IRI via alleviating mitochondrial stress.

ER stress is another important pathway between oxidative stress and cellular apoptosis. Moderate ER stress is beneficial to cells [53]. Excessive ER stress induces cellular apoptosis and the perturbation of Ca$^{2+}$ homeostasis [54]. Previous studies reported the pivotal role of ER stress as a major contributor to increase the apoptosis and exacerbate cell damage after IR [55, 56]. It was found that IR led to an accumulation of CHOP protein, a marker of ER stress, in renal epithelial and endothelial cells, which caused renal IRI [57]. It was also reported that taurousodeoxycholic acid (TDUCA), an inhibitor of ER stress, could protect
renal function and reduce apoptosis of tubular epithelial cells. Its beneficial effects were associated with a reduction of GRP78, which was a central regulator of ER function [58]. Yu et al. also found that berberine protected human renal proximal tubular cells by inhibiting ER stress [59].

In our study, tunicamycin induced ER stress model was used to confirm that the protective effect of propofol via reducing ER stress. Tunicamycin, as a kind of nucleoside antibiotic that inhibits the carbohydrate chain process and interferes the new protein galactosylated modification in ER, is often used to induce cell ER stress [60]. In present study, tunicamycin caused up-regulation of the ER stress-related factors GRP78, CHOP and caspase-12, which were decreased by propofol in a dose-dependent manner. The results suggest that propofol modulates ER stress.

In our in vivo study, propofol was delivered at 20 mg/kg/h, this is equivalent to a propofol infusion of 25 ml/h in a 70 kg human, which is often sustainable and will not result in significant systemic hypotension. In our in vitro cell culture study, propofol was delivered directly at 4-25 μM concentrations. The clinically relevant concentration of propofol was 2–11 μg/ml (approximately 10–62 μM) [61, 62], so the concentration of 4-25 μM was selected for the experiments in our study, which makes our results more clinically relevant. The similar concentrations had been reported having protective effect on hypoxia-induced apoptosis in alveolar epithelial type II cells [63].

It was believed that other mechanisms are also involved in renal IRI, such as inflammation, intracellular MAPK pathways, activation of K$_{ATP}$ channel and heme oxygenase-1 (HO-1) [64-66]. Whether propofol directly or indirectly affects these pathways still need to study.

In conclusion, our in vivo and in vitro studies found that propofol protected kidney from renal IRI via inhibiting oxidative stress and its downstream pathways for the first time (Fig. 8), which suggests that propofol may be used as a promising therapeutic agent for preventing renal IRI in clinical surgeries. However, the mechanisms of renal IRI are complicated. The detail mechanism in which propofol inhibited oxidative stress and protect kidney still needs to further study.

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Disclosure Statement

The authors declare no conflict of interest.

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