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Effect of propofol on calcium homeostasis in hypoxia-reoxygenated neonatal rat cardiomyocytes

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ABSTRACT

Intracellular Ca^{2+} overload induced by hypoxia–reoxygenation alters Ca^{2+} homeostasis, which plays an important role in myocardial cell injury. Even though propofol is known as a radical scavenger with Ca^{2+} channel blocking properties, little is known about cardioprotective effect associated with Ca^{2+} homeostasis in cardiomyocytes. In the present study, we showed that propofol protects cardiomyocytes against hypoxia–reoxygenation injury. In propofol-treated cardiomyocytes, we observed a decrease in the expression of pro-apoptotic protein Bax, cytochrome *c*, caspase-3 activation and intracellular Ca^{2+} content. We also found that propofol treatment enhanced expression of anti-apoptotic protein Bcl-2 and activation of ERK concerned with survival. Propofol attenuated alterations of genes involving Ca^{2+}-regulatory mechanism and significantly modulated abnormal changes of SERCA2a genes in hypoxia-reoxygenated neonatal cardiomyocytes. These results suggest that propofol modulates the expression of genes involved in Ca^{2+} homeostasis, thereby producing cardioprotective effects through a reduction in apoptotic cell death.

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1. Introduction

Ischemia–reperfusion injury consists of inflammatory damage, caused by the accumulation of free radical oxygen species, and triggers stress signaling processes that eventually results in cell death (Fliss and Gattinger, 1996; Anaya-Prado and Toledo-Pereyra, 2002). Thus, approaches to lessen ischemia–reperfusion injury have been extensively studied, and the concept of ischemic preconditioning has been introduced as an effective treatment modality for cardioprotection against ischemia–reperfusion injury (Murry et al., 1986). Ischemic preconditioning is an endogenous phenomenon whereby repeated brief episodes of coronary artery occlusion protect the heart against further prolonged ischemia (Marber et al., 1993). The cardioprotective effects by ischemic preconditioning have been demonstrated by the reduction of myocardial infarct size, improvement of postischemic contractile function, and decreased prevalence of arrhythmias (Zaugg and Schaub, 2003). In addition, it has been shown that administration of pharmacological agents such as anesthetics can produce pharmacologic preconditioning against ischemia–reperfusion injury (Javadov et al., 2000; Schultz and Gross, 2001; Tanaka et al., 2004). Anesthetic preconditioning has been shown to promote protection against ischemia–reperfusion injury including ischemic preconditioning-like effect, blockade of Ca^{2+} overflow to the cytosolic space, an antioxidant-like effect, and interference in the neutrophil/plate-endothelium interaction (Kato and Foëx, 2002). Also, anesthetic agents prevent the accumulation of Ca^{2+} that occurs during myocardial hypoxia or ischemia, which can induce cell apoptosis (Hannon and Cody, 2002; An et al., 2006). Intracellular Ca^{2+} overload, as a consequence of dysregulation of Ca^{2+} homeostasis, has been suggested to explain the adverse effects of ischemia–reperfusion and hypoxia–reoxygenation, which leads to cardiomyopathy and heart failure (Tani, 1990). Therefore, the interruption of Ca^{2+} overload has been proposed as an important target for increasing the tolerance to ischemia–reperfusion and hypoxia–reoxygenation injury.

Propofol is an intravenous anesthetic agent with advantageous properties such as rapid emergence after cessation of infusion (De Ruijter et al., 2002). There have been several reports that propofol can protect hearts from damage during ischemia–reperfusion (Ko et al., 1997; Kokita et al., 1998; Kato and Foëx, 2002). It has been shown, mostly in isolated rat heart model, that propofol may mediate its cardioprotective effects by acting as a free radical scavenger (Green et al., 1994) or by blocking plasma membrane Ca^{2+} channels, which may prevent the Ca^{2+} overload (Buljubasic et al., 1996). In addition, propofol can inhibit the lipid peroxidation induced by oxidative stress (Musacchio et al., 1991) and the mitochondrial permeability transition pore, although the concentrations required are typically greater than

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those used clinically (Sztark et al., 1995; Javadov et al., 2000). Accordingly, many previous studies have demonstrated that propofol contributes to cardioprotection against ischemia–reperfusion injury, but the effects of propofol on cardiomyocytes in response to hypoxia–reoxygenation have not been investigated. We hypothesized that propopol-induced cardioprotection is mediated by maintaining Ca^{2+} homeostasis during ischemia–reperfusion or hypoxia–reoxygenation. In the present study, we tested the protective effects of propofol against hypoxia–reoxygenation injury by measuring the expression of proteins involved in Ca^{2+} homeostasis, apoptosis, survival signals, and cell viability in response to hypoxia–reoxygenation following pre-treatment with various concentrations of propofol.

2. Materials and methods

2.1. Animals and chemicals

Hearts of neonatal Sprague Dawley rats (1–2 days old) were used for isolation of cardiomyocytes. The animal experimental procedures were approved by the committee for the Care and Use of Laboratory Animals, Yonsei University College of Medicine.

2.2. Isolation of neonatal rat cardiomyocytes

Neonatal rat cardiomyocytes were isolated and purified by previously described methods (Hwang et al., 2004). Briefly, hearts of 1–2 day-old Sprague Dawley rat pups were dissected and then the ventricles were washed with Dulbecco's phosphate-buffered saline solution (PBS) (pH 7.4, Gibco BRL, USA) lacking Ca^{2+} and Mg^{2+}. Using micro-dissecting scissors, hearts were minced until the pieces were approximately 1 mm^3 and treated with 10 ml of collagenase I (0.8 mg/ml, 262 U/mg, Gibco BRL, USA) for 15 min at 37 °C. The supernatant was then removed and the tissues were treated with fresh collagenase I solution for an additional 15 min. The cells in the supernatant were transferred to a tube containing cell culture medium (α-MEM containing 10% fetal bovine serum, Gibco BRL, USA). The tubes were centrifuged at 1200 rpm for 4 min at room temperature, and the cell pellet was resuspended in 5 ml of cell culture medium. The above procedures were repeated 7–9 times until little tissue remained. Cell suspensions were collected and incubated in 100 mm tissue culture dishes for 1–3 h to reduce fibroblast contamination. The non-adherent cells were collected and seeded to achieve a final concentration of 5×10^5 cells/ml. After incubation for 4–6 h, the cells were rinsed twice with cell culture medium and 0.1 μM BrdU was added. Cells were then cultured with 10% (v/v) fetal bovine serum (FBS) in a CO_2 incubator at 37 °C for 3–5 days.

2.3. Induction of hypoxia

Culture dishes containing cardiomyocytes were subjected to hypoxic stress in an anaerobic chamber (ThermoForma, USA) maintained at 37 °C in which ambient oxygen was replaced by a mixture of 5% CO_2, 5% H_2 and 90% nitrogen. Three mesh-encased wafers of desiccant, palladium catalyst and activated charcoal further ensured anaerobic conditions by removing H_2O, H_2S, and H_2O_2. The cardiomyocytes were exposed to different concentrations of propofol (0.1–500 μM) and immediately replaced in the hypoxic chamber to maintain hypoxia. After 1 h exposure, culture dish was transferred to the CO_2 incubator and cells were incubated at 37 °C for 5 h. Propofol was diluted to various concentrations according to the described methods (Park and Lynch, 1992).

2.4. Cell viability assay

Cell viability was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma, USA) assay. Cardiomyocytes were plated in triplicate wells of 96-well plates at a density of 1×10^4 per well, and incubated for 24 h. The media on the samples was changed with degassed serum-free media and samples were pretreated with various concentrations of propofol (0.1–500 μM). Then, samples were subjected to hypoxic chamber for 1 h and exposed to reoxygenation at 37 °C for 5 h. After the incubation period, the MTT was added to each well to a final concentration of 0.5 mg/ml and the cells were incubated at 37 °C for 3 h to allow MTT reduction. Formazan crystals were dissolved by adding dimethylsulfoxide (DMSO) and the absorbance was measured at 570 nm with a spectrophotometer. In the following experiments, we used 1 μM propofol due to a decreased survival rate above this concentration.

2.5. Confocal microscopy and fluorescence measurements

The cytosolic free Ca^{2+} was measured by confocal microscopy. Neonatal rat cardiomyocytes were plated on glass coverslips coated with laminin (5 mg/cm^2) for 1 day in cell culture medium (α-MEM containing 10% fetal bovine serum, Gibco BRL, USA) and 0.1 μM BrdU. Following pretreatment with 1 μM propofol, cardiomyocytes were subjected to hypoxic chamber for 1 h and exposed to reoxygenation for 5 h. After incubation, the cells were washed with modified Tyrode's solution containing 0.265 g/l $CaCl_2$, 0.214 g/l $MgCl_2$, 0.2 g/l KCl, 8.0 g/l NaCl, 1 g/l glucose, 0.05 g/l NaH_2PO_4 and 1.0 g/l $NaHCO_3$. Cells were then loaded with 5 mM acetoxymethyl ester of Fluo-4 (Fluo-4 AM, Molecular Probes, USA) for 20 min by incubation in modified Tyrode's solution in the dark and at room temperature. Fluorescence images were obtained using an argon laser confocal microscope (Carl Zeiss Inc., USA). This fluorochrome is excited by the 488 nm line of an argon laser and emitted light is collected through a 510–560 nm bandpass filter. Relative changes of free intracellular Ca^{2+} were determined by measuring fluorescent intensity.

2.6. Immunoblot analysis

Cardiomyocytes pretreated with 1 μM propofol were subjected to hypoxic chamber for 1 h and exposed to reoxygenation for 5 h. Cells were washed once in PBS and lysed in a lysis buffer (Cell signaling, USA). Protein concentrations were determined using the Bradford protein assay kit (Bio-Rad, USA). Proteins were separated in a 12% SDS-polyacrylamide gel and transferred to PVDF membrane (Millipore Co., USA). After blocking the membrane with Tris-buffered saline-Tween 20 (TBS-T, 0.1% Tween 20), containing 5% non-fat dried milk, for 1 h at room temperature, membranes were washed twice with TBS-T and incubated with primary antibodies for 1 h at room temperature or overnight at 4°C. The following primary antibodies were used: rabbit anti-ERK, mouse anti-phospho ERK, mouse anti-Bcl-2, mouse anti-cytochrome *c* (Santa Cruz Biotechnology, USA), rabbit anti-Bax (Assay Designs, USA) and mouse anti-β-actin (Sigma, USA). The membrane was washed three times with TBS-T for 10 min, followed by incubation for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies. After extensive washing, the bands were detected by enhanced chemiluminescence (ECL) reagent (Santa Cruz Biotechnology, USA). β-actin gene was used as the standard for equal of the protein samples. The band intensities were quantified using a Photo-Image System (Molecular Dynamics, Sweden).

2.7. Detection of apoptotic cells by TUNEL staining

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) analysis was performed with a commercially available kit according to the manufacturer's instruction (Intergen, USA). Cultured cardiomyocytes were prepared as described previously on coverslips in 24-well plates. Briefly, the cells were fixed in 1% paraformaldehyde and pre-cooled ethanol: acetic acid (2:1), and equilibrated with an equilibration buffer. The samples were then treated with terminal deoxynucleotidyl transferase (TdT) and incubated in a humidified chamber for 1 h at 37 °C. After washing in PBS, anti-digoxigenin peroxidase conjugate was added, and incubation continued in a humidified chamber for 30 min at

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