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Original Article

Propofol prevents against oxygen glucose deprivation and reperfusion-induced autophagic cell death in H9c2 cells by increasing miR-30

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Abstract: Timely reperfusion saves a great amount of ischemic myocardium, but causes free radical injury. Autophagy renders cardiomyocytes more prone to the injury. Propofol is widely used in general anesthesia and sedation. However, effects of propofol on cardiac ischemia and reperfusion injury remain unknown. Current study aimed to investigate effects of propofol on oxygen glucose deprivation and reperfusion (OGD/R)-induced autophagic cell death in cardiomyoblasts and explore possible molecular mechanisms. H9c2 rat cardiomyoblasts were deprived of oxygen and glucose, and then reoxygenated in normal medium. Propofol (8, 16 and 32 μM) was added to cells at 1 h before OGD/R. MTT assay was utilized to evaluate the viability of cardiomyoblasts. Autophagy was detected by monodansylcadaverine (MDC) staining kit. Protein expression of cellular autophagy markers Beclin-1 and LC3B in cardiomyoblasts in the presence or absence of propofol was detected by Western Blot. The expression of miR-30 was detected by RT-PCR. MiR-30 mimics was transfected into cardiomyoblasts, and protein expression of Beclin-1 was examined in the presence or absence of propofol and miR-30. Propofol inhibited OGD/R-induced cell death. There was a clear increase in fluorescence intensity and number of autophagic vacuoles in OGD/R group compared to the control cells. Propofol treatment attenuated the increase in autophagic cells and significantly attenuated OGD/R-induced increased expression of Beclin-1 and LC3B dose-dependently. Propofol after OGD/R dramatically increased miR-30 expression in a dose-dependent manner, whereas OGD/R alone decreased the expression. MiR-30 mimics significantly attenuated the protein expression of Beclin-1 in OGD/R-treated cardiomyoblasts, and the inhibition was enhanced when combined with propofol. MiR-30 mimics also markedly decreased Beclin-1 expression in cardiomyoblasts that were not treated with OGD/R.

Keywords: H9c2 cells, propofol, OGD/R, miR-30

Introduction

Myocardial ischemic injury results from severe impairment of coronary blood supply. Myocardial infarcts evolve as rapid necrosis from subendocardium to subepicardium. Timely reperfusion saves a great amount of ischemic myocardium, and extends the window of myocardial viability. However, reperfusion of ischemic myocardium is frequently associated with microvascular injury, due to increased permeability of blood vessels that increases fluid filtration across the tissue. The restored blood flow reintroduces oxygen within cells, and activated endothelial cells following reperfusion produce reactive oxygen species that cause subsequent inflammatory responses [1]. Free radicals damage plasma membrane, cellular proteins and DNA, and damage to plasma membrane may in turn result in release of more free radicals [2].

The application of propofol includes starting and maintenance of general anesthesia, sedation for mechanically ventilated adults, procedural sedation, and status epilepticus if other medications have not worked [3, 4]. However, effects of propofol on ischemia and reperfusion injury in heart remain unknown. In current study, effects of propofol on myocardial ischemia and reperfusion injury were investigated utilizing oxygen glucose deprivation/reoxygenation (OGD/R) model.

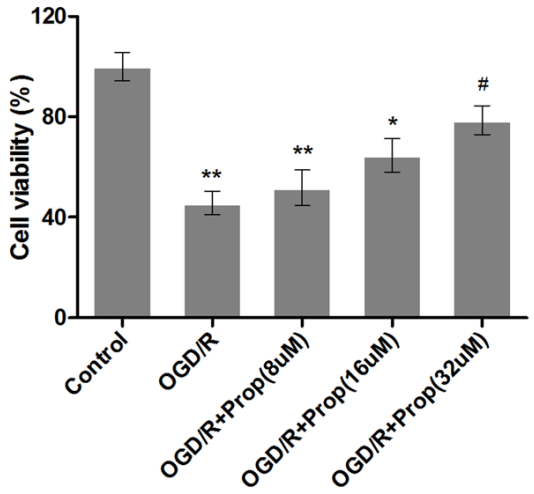

Figure 1. Propofol inhibited OGD/R-induced cell death in rat cardiomyoblasts. OGD/R was performed in rat cardiomyoblasts as described in Methods. Cells were pretreated with various concentrations of propofol (8, 16, and 32 μM) at 1 h before OGD/R. MTT assay was utilized to evaluate the viability of cardiomyoblasts. OGD/R induced cell death compared to the control group, whereas propofol inhibited OGD/R-induced cell death in a dose-dependent manner (mean ± SEM, n = 4/group). * and ** indicate that $P < 0.05$ and $P < 0.01$ when compared to the control group, respectively. # shows that $P < 0.05$ as compared to the OGD/R group. OGD/R: oxygen glucose deprivation/reoxygenation. Prop: propofol.

Although necrosis and apoptosis have been focused largely to examine mechanisms responsible for ischemia and reperfusion-induced cardiac abnormalities [5], a unique role of dysregulated autophagy in the heart has recently been highlighted. Autophagy may render cardiomyocytes more prone to ischemia and reperfusion injury, and long-term postinfarction cardiac remodeling [6, 7]. Beclin-1 and microtubule-associated proteins 1A/1B light chain 3B (MAP1LC3B, LC3B) are essential proteins associated with autophagy [8]. Therefore, we examined protein expression of Beclin-1 and LC3B in order to explore the effects of propofol on OGD/R-induced autophagic cell death.

MicroRNA-30 (miR-30) has recently been revealed to be associated with autophagy. Cardiomyocyte autophagy was enhanced, and miR-30 was down-regulated in cardiac tissue in a rat model of cardiac hypertrophy established by transverse abdominal aortic constriction [9]. 5-fluorouracil (5-FU) suppressed miR-30, and induced autophagic cell death and cell proliferation arrest in gastric carcinoma cells [10]. Up-regulation of miR-30 by high-fat diet was also reported to impair autophagy of endothelial cell [11]. In addition, H9c2 rat cardiomyoblast cell line has been widely used in studying various cardiac diseases and molecular mechanisms, such as myocardial ischemia [12], endotoxinemia [13], hyperglycemia [14], free radical-induced intracellular acidification [15], and pharmacological study [16].

Therefore, current study aimed to investigate effects of propofol on OGD/R-induced autophagic cell death in H9c2 cardiomyoblasts, and explore possible mechanisms by measuring expression of Beclin-1, LC3B, and miR-30.

Methods

Cell culture

H9c2 rat cardiomyoblast cell line was purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and maintained in Dulbecco's modified Eagle's medium (DMEM; Hyclone Laboratories Inc., Logan, UT, USA) supplemented with 4.5 g/L of glucose, 10% (v/v) fetal bovine serum (Invitrogen Inc., Grand Island, NY, USA), and 1% penicillin-streptomycin solution at 37°C under an atmosphere of 5% CO_2.

Oxygen glucose deprivation/reoxygenation (OGD/R) model

H9c2 rat cardiomyoblasts were exposed to hypoxic conditions (oxygen deprivation, 0.5% O_2) for 24 h in culture medium deprived of glucose and serum. After hypoxia, cells were reoxygenated under normoxic conditions (reoxygenation) for 24 h in normal medium. Propofol (Fresenius Kabi Inc., Bad Homburg, Germany) with different concentrations (8, 16, and 32 μM) was added to cells at 1 h before the hypoxia-oxygenation.

MTT assay

To assess cell viability, H9c2 cardiomyoblasts were added to 96-well plates. Cells in different groups were pretreated with various concentrations of propofol (8, 16, and 32 μM) at 1 h before the hypoxia-oxygenation. There were 3 wells on the plate for each group. Viability of cardiomyoblast was detected using MTT assay after OGD/R. Briefly, MTT solution was added to

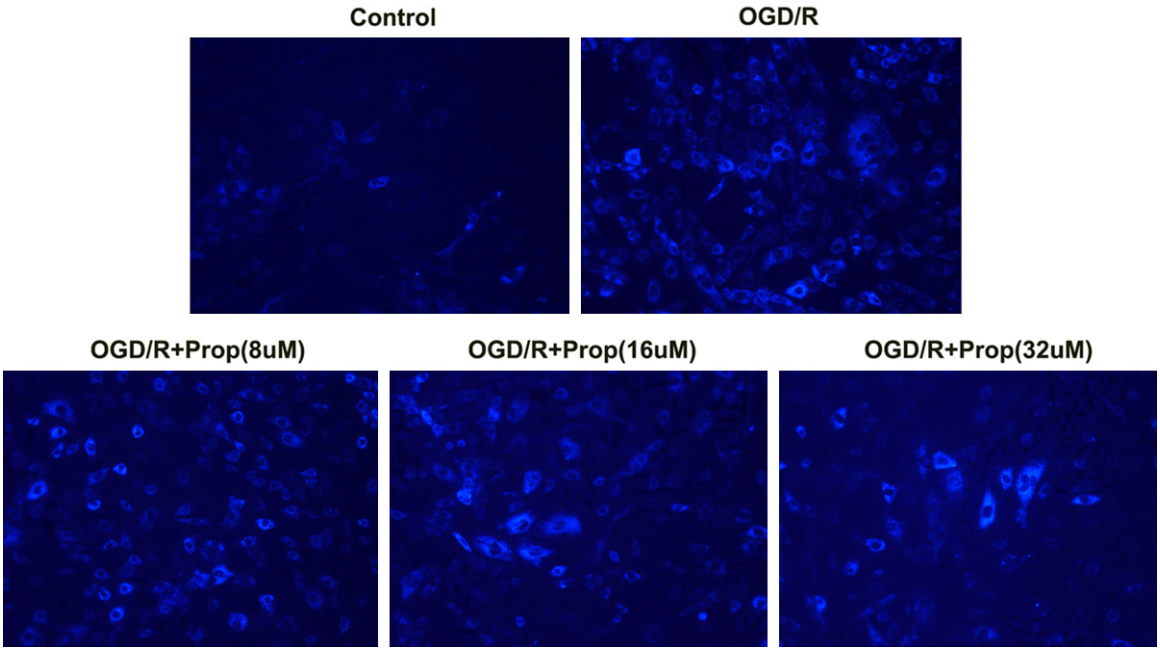

Figure 2. Propofol inhibited OGD/R-induced autophagy in cardiomyoblasts as indicated by MDC staining. Rat cardiomyoblasts in 5 groups were treated as mentioned in Methods. Autophagy was detected by MDC staining kit. There was a clear increase in fluorescence intensity and number of autophagic vacuoles in OGD/R group compared to the control cells, indicating that OGD/R increased the number of autophagic cells compared to control group. Propofol treatment attenuated the increase in autophagic cells in a dose-dependent manner. OGD/R: oxygen glucose deprivation/reoxygenation. Prop: propofol.

cell culture medium (final concentration = 500 mg/mL) at the end of the OGD treatment. The reaction was terminated by addition of 10% acidified sodium dodecyl sulfate (SDS, 100 mL) to the cell culture at 4 h after addition of MTT. The absorbance (A) value was measured at 570 nm utilizing a multiwall spectrophotometer (Bio-Rad Laboratories, Hercules, California). The percentage of cell viability (%) = A of experiment well/A of control well × 100%.

Detection of autophagy by monodansylcadaverine (MDC) staining

H9c2 cardiomyoblasts in 5 groups were treated as abovementioned in 24-well plates, and autophagy was detected by MDC staining kit (KeyGEN Biotech Inc., Nanjing, China). One hundred μL of MDC staining solution was added into each well, and incubated in dark for 30 min. Supernatant was then discarded, and cells were washed with washing buffer. Cells were observed under fluorescent microscope, and photos were taken.

Western blot

Protein expression of cleaved LC3B and Beclin-1 were detected by Western Blot. Protein of H9c2 cardiomyoblasts in different experimental groups was extracted and separated by 10% SDS-polyacrylamide gel during eletrophoresis at 120 V. Signals were transferred to polyvinylidene fluoride (PVDF) membrane at 100 V for 120 min. Membranes were blocked by 5% nonfat milk powder for 1 h. Membranes were then incubated with Anti-cleaved LC3B antibody (1:1000; Abcam Inc., Cambridge, MA, USA), or anti-Beclin-1 antibody (1:1000; Cell Signaling Technology Inc., Danvers, MA, USA) at 4°C overnight. Membrane were washed with tris-buffered saline and tween 20 (TBST), and incubated with goat anti-rabbit secondary antibody labeled with horseradish peroxidase (HRP) (1:3000; Invitrogen Inc., Grand Island, NY, USA) at room temperature for 1 h. Membranes were washed, and incubated shortly with electrochemi-luminescence (ECL) solution. Films were exposed in a dark room.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA of H9c2 cardiomyoblasts was extracted and purified by Trizol (Invitrogen Inc., Grand Island, NY, USA) according to manufacturer's instructions. A Universal cDNA synthesis kit

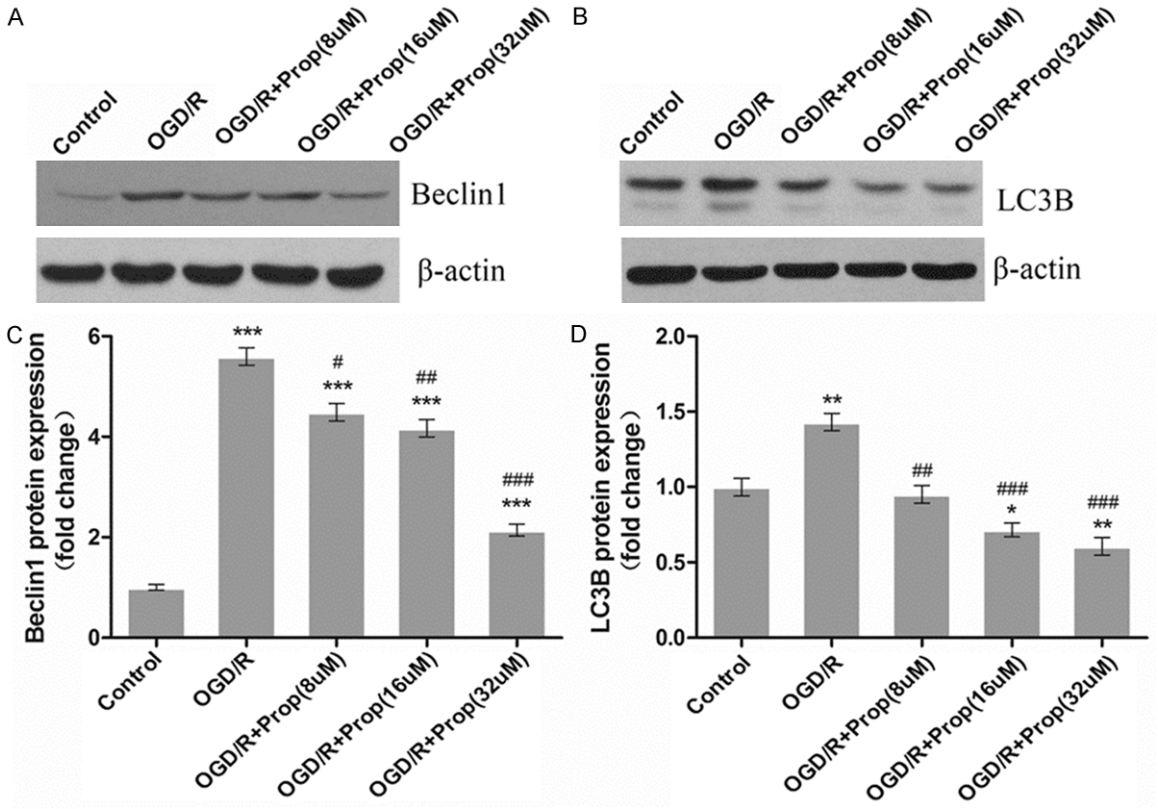

Figure 3. Propofol inhibited OGD/R-induced autophagic cell death in cardiomyoblasts. A. Representative blot image of Beclin-1 in cardiomyoblasts. B. Representative blot image of LC3B in cardiomyoblasts. C. Statistical analysis of blot images of Beclin-1. D. Statistical analysis of blot images of LC3B. OGD/R induced protein expression of Beclin-1 and LC3B compared to the control group. Protein expression of cellular autophagy markers Beclin-1 and LC3B in cardiomyoblasts was detected by Western Blot. Propofol significantly attenuated OGD/R-induced protein expression of Beclin-1 and LC3B dose-dependently (mean ± SEM, n = 4/group). *, **, and *** indicate that $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, when compared to the control group. #, ##, and ### represent that $P < 0.05$, $P < 0.01$, and $P < 0.001$ as compared to the OGD/R group, respectively. OGD/R: oxygen glucose deprivation/reoxygenation. Prop: propofol.

(Tiangene Biotech Inc., Beijing, China) was utilized for reverse transcription. Each reaction contained 1 μL of random hexamer primers (0.2 μg/μL) and 40 U M-MuLV reverse transcriptase (20 U/μL). No reverse transcriptase was added in the negative control. The U6 small nuclear RNA (snRNA) was used as the housekeeping small RNA reference gene. The specific primer for detection of miR-30 gene was: 5'-TGTAAACATCCTCGACTGGAAG-3'. MiRcute miRNA qPCR Detection kit (SYBR Green; Tiangene Biotech Inc., Beijing, China) was used for qPCR. PCR conditions were as follows: pre-denaturing at 94°C for 2 min; denaturing at 94°C for 20 s; annealing and polymerization at 60°C for 34 s; and 42 cycles. PCR was performed in an ABI Step one Plus qPCR System. The expression of miR-30 was determined as the ratio of relative optical density of target genes to U6 snRNA using comparative cycle time (Ct) method ($2^{-\Delta\Delta Ct}$ method) [17].

Transfection of miR-30 mimics

The original medium of rat cardiomyoblasts was changed into medium without serum or antibiotics. Mixture of Lipofectamine 2000 and miRNA was prepared. Negative control and miR-30 mimics were diluted. One hundred and twenty-five μL of OPTI-MEM was used to dilute RNA. In addition, 125 μL of OPTI-MEM was also used to dilute 10 μL of Lipofectamine 2000, and the mixture was incubated at room temperature for 5 min. The prepared RNA mixture and Lipofectamine 2000 mixture were then mixed, and incubated at room temperature for 20 min. The derived solution was added into cardiomyoblasts cultured with antibiotic-free

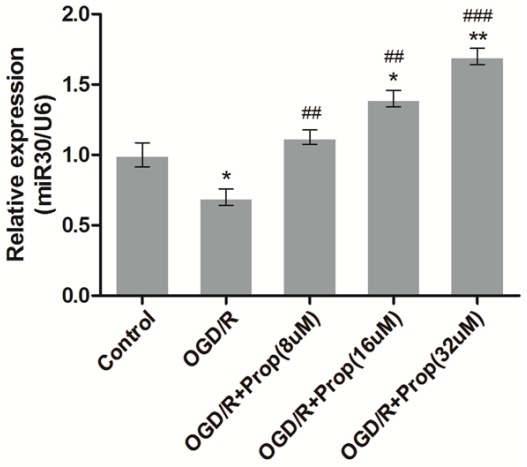

Figure 4. Propofol increased the expression of miR-30 in cardiomyoblasts after OGD/R. The expression of miR-30 in cardiomyoblasts was detected by RT-PCR. OGD/R decreased relative expression of miR-30 compared to the control group, and propofol dramatically increased the expression of miR-30 after OGD/R treatment in a dose-dependent manner compared to the OGD/R alone group (mean ± SEM, n = 4/group). * represent that $P < 0.05$ when compared to the control group. ## and ### indicate that $P < 0.01$ and $P < 0.001$ as compared to the OGD/R alone group, respectively. OGD/R: oxygen glucose deprivation/reoxygenation. Prop: propofol.

medium. Culture medium was changed into antibiotic-containing medium after transfection of 4 to 8 h.

Statistical analysis

The figures were created and statistical data were analyzed by GraphPad Prism 5.0 software (GraphPad Software Inc; La Jolla, CA, USA). Statistical results are presented as mean ± SEM. Differences among 3 or more groups were compared by analysis of variance (ANOVA), followed by post-hoc Tukey's test, for multiple comparisons. Differences between 2 groups were compared by student's t test. *P* values of less than or equal to 0.05 were considered statistically significant.

Results

Propofol inhibited OGD/R-induced cell death in rat cardiomyoblasts

OGD/R was performed in rat cardiomyoblasts as described in Methods. Cells were pretreated with various concentrations of propofol (8, 16, and 32 μM) at 1 h before the hypoxia-oxygenation. MTT assay was utilized to evaluate the viability of cardiomyoblasts. OGD/R induced cell death compared to the control group ($P < 0.01$; **Figure 1**). Propofol inhibited OGD/R-induced cell death in a dose-dependent manner. High dose of propofol (32 μM) increased cell viability significantly compared to OGD/R alone group ($P < 0.05$; **Figure 1**).

Propofol inhibited OGD/R-induced autophagic cell death in cardiomyoblasts

Rat cardiomyoblasts in 5 groups were treated as mentioned in Methods. Autophagy was detected by MDC staining kit. Protein expression of cellular autophagy markers Beclin-1 and LC3B in cardiomyoblasts was detected by Western Blot. There was a clear increase in fluorescence intensity and number of autophagic vacuoles in OGD/R group compared to the control cells, indicating that OGD/R increased the number of autophagic cells compared to control group. Propofol treatment attenuated the increase in autophagic cells in a dose-dependent manner (**Figure 2**). OGD/R induced protein expression of Beclin-1 ($P < 0.001$; **Figure 3A** and **3C**) and LC3B ($P < 0.05$; **Figure 3B** and **3D**) compared to the control group. Propofol significanlty attenuated OGD/R-induced protein expression of Beclin-1 and LC3B dose-dependently ($P < 0.05$; **Figure 3**). All doses of propofol were shown to markedly inhibit OGD/R-induced autophagic cell death.

Propofol increased the expression of miR-30 in OGD/R-treated cardiomyoblasts

The expression of miR-30 in cardiomyoblasts was detected by RT-PCR. OGD/R decreased relative expression of miR-30 compared to the control group ($P < 0.05$; **Figure 4**). Propofol dramatically increased the expression of miR-30 in OGD/R-treated cardiomyoblasts in a dose-dependent manner, as compared to the OGD/R alone group ($P < 0.01$; **Figure 4**).

MiR-30 mimics decreased the protein expression of Beclin-1 in cardiomyoblasts

MiR-30 mimics was transfected into cardiomyoblasts, and protein expression of Beclin-1 in different groups was examined by Western Blot. OGD/R increased protein expression of Beclin-1 in cardiomyoblasts. Both high-dose propofol (32 μM) and miR-30 mimics significantly attenuated the protein expression of

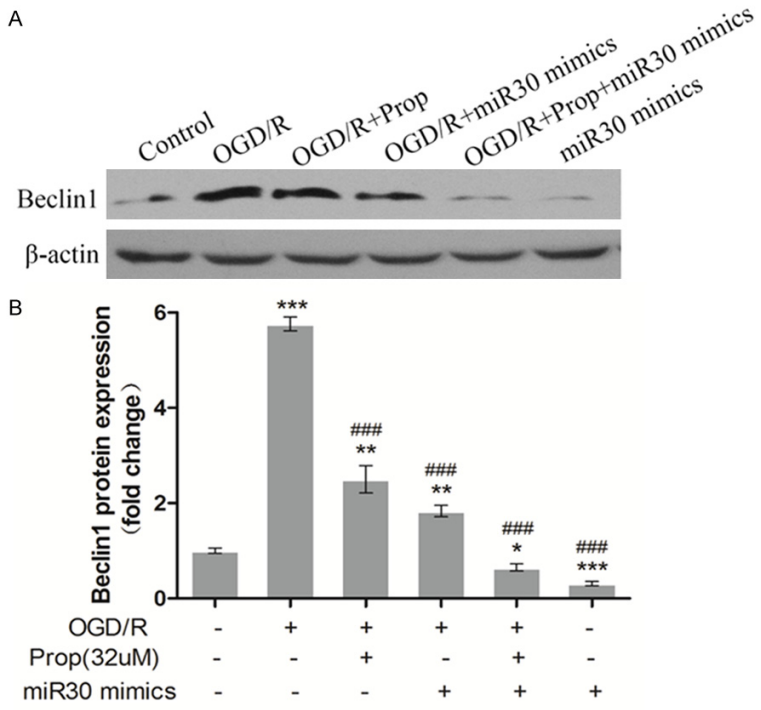

Figure 5. MiR-30 mimics decreased the protein expression of Beclin-1 in cardiomyoblasts. A. Representative blot image of Beclin-1 in cardiomyoblasts. B. Statistical analysis of blot images of Beclin-1. MiR-30 mimics was transfected into cardiomyoblasts, and protein expression of Beclin-1 indifferent groups was examined by Western Blot. OGD/R increased protein expression of Beclin-1 in cardiomyoblasts. Both high-dose propofol (32 μM) and miR-30 mimics significantly attenuated the protein expression of Beclin-1 in OGD/R-treated cardiomyoblasts. The inhibition was enhanced when propofol and miR-30 mimics were combined together. MiR-30 mimics alone also markedly decreased protein expression of Beclin-1 in cardiomyoblasts that were not treated with OGD/R when compared to the control group (mean ± SEM, n = 4/group). *, **, and *** show that $P < 0.05$, $P < 0.01$, and $P < 0.001$ as compared to the control group, respectively. ### indicate that $P < 0.001$ when compared to the OGD/R alone group. OGD/R: oxygen glucose deprivation/reoxygenation. Prop: propofol.

Beclin-1 in OGD/R-treated cardiomyoblasts ($P < 0.001$; **Figure 5**). When propofol and miR-30 mimics were combined together, the inhibition was enhanced ($P < 0.001$; **Figure 5**). In addition, miR-30 mimics alone also markedly decreased protein expression of Beclin-1 in cardiomyoblasts that were not treated with OGD/R when compared to the control group ($P < 0.001$; **Figure 5**).

Discussion

We demonstrated that propofol inhibited OGD/R-induced cell death, and significantly attenuated OGD/R-induced increased expression of autophagy markers Beclin-1 and LC3B dose-dependently. Propofol after OGD/R dramatically increased miR-30 expression, and miR-30 mimics attenuated protein expression of Beclin-1 significantly. MiR-30 mimics also markedly decreased Beclin-1 expression in cardiomyoblasts that were not treated with OGD/R.

Autophagy is a tightly regulated and lysosome-dependent catabolic process. It is responsible for turnover of long-lived proteins and intracellular structures that are damaged or malfunctioning [18, 19]. This evolutionally conserved bulk degradation process is turned on when cells experience stress, including nutrient and energy as seen in myocardial ischemia.

Beclin-1 and LC3B are essential components in the process of autophagy. Beclin-1 and LC3B were reported to be increased during reperfusion in fibrillated mouse hearts [20]. Beclin-1 affects every major step in autophagic pathways, from autophagosome formation to maturation of autophagosome/endosome [21]. Many of the effects have been shown to be achieved by activating specific Beclin 1-binding proteins, such as autophagic inducers and inhibitors. Cofactors, including Bif-1, high mobility group box (HMGB) 1 and survivin promoted formation of Beclin 1-Vps34-Vps15 core complexes and induce autophagy. In contrast, Bcl-2 or Bcl-XL was reported to inhibit BH3 domain of Beclin-1, which reduced autophagy [21]. LC3B is a subunit of neuronal microtubule-associated proteins 1A and 1B. LC3B mediates autophagy, and has been revealed to activate extrinsic apoptotic pathway during cigarette smoking-induced emphysema by interacting with Caveolin-1 and Fas [22].

We unveiled in current study that propofol significantly attenuated OGD/R-induced increased expression of autophagy markers Beclin-1 and

LC3B in cardiomyoblasts. This indicates that propofol is protective against autophagy during nutrient deprivation and reperfusion of cardiac cells. To reveal the underlying mechanisms, we measured expression of miR-30, whose down-regulation was shown to enhance autophagy in cardiac hypertrophy and gastric carcinoma [9, 10]. We revealed that miR-30 mimics attenuated autophagy marker Beclin-1 in cardiac cells that in the presence or absence of nutrition deprivation and reperfusion. Propofol dramatically increased the expression of miR-30, which may subsequently inhibit Beclin-1 expression and autophagic pathways.

In conclusion, we demonstrated for the first time that propofol prevents against oxygen glucose deprivation and reperfusion-induced autophagic cell death in H9c2 rat cardiomyoblasts by increasing the expression of miR-30. Although more extensive molecular pathways need to be explored about effects of propofol on the heart, propofol may represent a promising agent protecting cardiac ischemia and reperfusion injury.

Disclosure of conflict of interest

None.

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