Remifentanil protects against myocardial ischemia/reperfusion injury via miR-205-mediated regulation of PINK1

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ABSTRACT — Myocardial ischemia/reperfusion (I/R) injury could lead to severe cardiovascular ischemic disease, including myocardial infarction and contractile dysfunction. Remifentanil demonstrated protective effect on myocardial I/R injury. The underlying pathophysiological mechanism was then investigated in this study. In the current study, primary cardiomyocytes were isolated from rats, and then preconditioned with remifentanil. Rats, tail vein injected with miR-205 antagomir, were subjected to infusion of remifentanil, and then subjected to regional ischemia followed by reperfusion. The results demonstrated that cell viability of hypoxia/reoxygenation-induced cardiomyocytes was increased post remifentanil, while the apoptosis was decreased accompanied with reduced cleaved caspase-3 expression. Hypoxia/reoxygenation treatment increased miR-205 and decreased PINK1 (PTEN induced putative kinase 1) expression. However, preconditioning with remifentanil reduced miR-205 and enhanced PINK1. Moreover, over-expression of miR-205 decreased PINK1 expression and counteracted the effects of remifentanil-induced increase of cell viability and decrease of cell apoptosis in hypoxia/reoxygenation-induced cardiomyocytes. Injection with miR-205 antagomir improved remifentanil-induced decrease of infarct size and LDH (lactic acid dehydrogenase) activity in rat model with I/R injury. In conclusion, miR-205 might participate in the protective effect of remifentanil in rat myocardial I/R injury via regulation of PINK1, providing a potential target for amelioration of cardiovascular ischemic disease.

Key words: miR-205, PINK1, Remifentanil, I/R injury

INTRODUCTION

Myocardial ischemia, with the blockage of blood and oxygen supply to heart, could lead to serious complications, such as myocardial infarction (Toldo et al., 2018). Congestive heart failure and malignant arrhythmia caused by myocardial infarction could result in cardiovascular diseases, that have a high incidence and mortality globally (Jiao et al., 2017). Reperfusion of occluded coronary artery is regarded as the “gold standard” for the treatment of myocardial ischemia (E et al., 2019). However, the adverse effect of reperfusion could cause myocardial ischemia/reperfusion (I/R) injury, such as systolic function decline (Dorweiler et al., 2007). Therefore, to improve myocardial ischemia, it is imperative to identify strategies to ameliorate myocardial I/R injury during reperfusion.

Remifentanil is a newly synthesized piperidine derivative and widely used as component of anesthesia, that has been shown to protect the uterus (Atalay et al., 2015) or ovary (Atalay et al., 2016) against ischemia-reperfusion injury. Pretreatment with remifentanil could confer car-
dioprotection through opioid receptor (Yu et al., 2007). During coronary artery bypass surgery, anesthesia regimens with remifentanil reduced biomarkers of myocardial damage, including creatine kinase and cardiac troponin I (Wong et al., 2010). A study has shown that intravenous infusion of remifentanil could effectively repress hypoxia-reoxygenation-induced injury in cardiomyocytes and reduce the myocardial infarction area in rats in a dosedependent pattern (Dou et al., 2016). Moreover, remifentanil was reported to attenuate oxidative injury in cardiomyocytes (Lei et al., 2018) and activate anti-apoptotic pathways to sustain survival of myocardium in ischemia-reperfused rats (Kim et al., 2009). However, the underlying pathophysiological mechanism of remifentanil-mediated protective effect against myocardial I/R injury remains unclear.

Previous studies have demonstrated that the cardioprotective effect of diazoxide (Zhang et al., 2020a) or dexmedetomidine (Zhang et al., 2020b) during myocardial I/R injury or hypoxia-reoxygenation injury relied on regulation of miR-10a or IncRNA H19/miR-29b-3p axis, respectively. Administration of the anesthetic, propofol, during reperfusion alleviated myocardial I/R injury with reduced cardiomyocyte apoptosis and infarct size through up-regulation of miR-451 (Li et al., 2019). MiR-205 was up-regulated in the plasma and heart of mice post imatinib mesylate and doxorubicin treatment (Hanousková et al., 2019), and down-regulation of miR-205 could reduce myocardial apoptosis during chronic heart failure (Xuan et al., 2017). Therefore, this study hypothesized that remifentanil might prevent myocardial I/R injury through regulation of miR-205.

The alteration of miR-205 level was evaluated in hypoxia-reoxygenation-induced cardiomyocyte and rat model with myocardial I/R injury post remifentanil. The effects of miR-205 on myocardial apoptosis and infarction size induced by hypoxia-reoxygenation and I/R were also determined in this study.

**MATERIALS AND METHODS**

**Animals**

Neonatal rats (1 to 2 days) and Sprague-Dawley rats (6-8 weeks old, 300-350 g weight) were purchased from Experimental Animal Centre of Soochow University (Suzhou, China). All animal experiments were approved by the Ethics Committee of The Affiliated Cardiovascular Hospital of Qindao University for the use of animals and conducted in accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines.

**Cell isolation and culture**

Neonatal rats were anesthetized with 40 mg/kg pentobarbital, and the hearts were harvested and then cut into 2 mm³ pieces. The pieces were digested with 0.1% collagenase type II (Sigma Aldrich, St. Louis, MO, USA), and the supernatants of the digestion were collected by centrifugation at 1500 x g for 10 min. The supernatants were cultured in DMEM-F12 medium containing 10% fetal bovine serum (Beyotime Institute of Biotechnology, Haimen, China). Cardiomyocytes were separated from the fibroblasts via differential wall adhesion method, and cultured in DMEM-F12 medium containing 10% fetal bovine serum and 0.1 mM 5-bromodeoxyuridine in a 37°C incubator.

**Hypoxia-reoxygenation injury model**

The isolated primary cardiomyocytes were plated and pretreated with remifentanil (1, 5 or 10 μM) for 10 min. Thirty minutes later, cells were incubated with 4 mM Na₂S₂O₄ for 1 hr. After removing Na₂S₂O₄, cells were then cultured in the normal DMEM-F12 medium for another 12 hr to generate hypoxia-reoxygenation injury model. For over-expression or knockdown of miR-205, the isolated primary cardiomyocytes were transfected with 50 nM miR-205 mimic or inhibitor (GenePharm, Shanghai, China) via Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Two days later, cells were conducted with or without hypoxia-reoxygenation injury model.

**Cell viability and apoptosis assays**

MTT was used to detect cell viability. Primary cardiomyocytes with indicated treatment and transfection were seeded in plates, and incubated with 20 μL MTT reagent (Sigma Aldrich) for 4 hr. The medium was removed and cells were incubated with dimethyl sulfoxide, and the absorbance at 490 nm was measured by microplate spectrophotometer (Thermo Scientific, Waltham, MA, USA). TUNEL was used to detect cell apoptosis. Primary cardiomyocytes with indicated treatment and transfection were fixed and then stained with TUNEL and DAPI (Roche, Mannheim, Germany). A microscope (Zeiss Axio Observer Z1, Zeiss, Tokyo, Japan) with software Zen 2011 was used to capture images and detect TUNEL-positive cells.

**Luciferase reporter assay**

Sequences of wild type or mutant PINK1 3' UTR (165 bp) were cloned into pMIR-GLO™ Luciferase vector (Promega, Madison, WI, USA) and named as PINK1-WT and PINK1-MUT. HEK293T was co-transfected with miR-205 mimic or miR-NC (negative control), miR-205
inhibitor of NC inh and PINK1-WT or PINK1-MUT. Forty-eight hours later, cells were conducted with a Dual-Luciferase Reporter Assay system (Promega).

**Myocardial I/R injury model**

Sixty Sprague–Dawley rats were randomly separated into six groups: sham, acute myocardial infarction (AMI), AMI with remifentanil preconditioning (RPC), AMI + RPC with NC antagonim, AMI + RPC with miR-205 antagonim and AMI with miR-205 antagonim. For AMI models, rats were anesthetized with 80 mg/kg pentobarbital. A 16-gauge cannula, connected to a ventilator (Harvard Rodent Ventilator Model 683, Holliston, MA, USA) was intubated into the trachea of rats. A 24-gauge catheter, connecting to pressure transducer, was cannulated into right carotid artery. Drug administration was performed through the 24-gauge catheter installed in the left jugular vein. The chest was opened and the heart was exposed. The first branch of the anterior descending coronary artery was placed with a 6-0 prolene loop, and a snare for reversible occlusion was formed via thread through the ends of the suture. For AMI with RPC group, rats were administered three times with 20 μg/kg per minute remifentanil for five minutes. For antagonim groups, rats were tail vein injected with 80 mg/kg miR-205 antagonim or NC antagonim (GenePharm) for 3 days. Rats were then conducted with the AMI model and remifentanil administration. For AMI models, hearts of rats were subjected to regional ischemia for 30 min, followed by reperfusion for 2 hr. Rats in the sham group were conducted with the surgery without the regional ischemia and reperfusion.

**Measurement of infarct size**

The hearts were excised and then transferred to Langendorff apparatus after the reperfusion. The hearts were perfused with normal saline, and then injected with 0.25% Evan blue dye to stain the perfused region in hearts. Hearts were sliced into 2-mm slices and stained with 2% triphenyl-tetrazolium chloride (Sigma Aldrich) for 20 min. After immersion in 10% formalin, the infarct size in hearts was determined by computerized planimetry technique (SigmaScan 4.0, Systat Software, Richmond, CA, USA).

**Measurement of lactate dehydrogenase**

At the end of reperfusion, the blood sample was collected from the carotid artery of rats. Samples were centrifuged at 5000 x g for 10 min to isolate serum. Activity of lactate dehydrogenase in the serum was then measured LDH assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

**qRT-PCR**

Total RNAs, extracted from primary cardiomyocytes or heart tissues were conducted with complementary DNA synthesis. FastStart Essential DNA Green Master kit (Roche Life Sciences, Indianapolis, IN, USA) was used for qRT-PCR analysis of miR-205 and U6 with primers: miR-205 (forward; 5’-CTTGGTC-CTTCATTCCACCGGA-3’ and reverse; 5’-TGCCGC-CTGAACTTCATCC-3’) and U6 (forward: 5’-CTG-GTTAGTACTTGGACGAGAC-3’ and reverse: 5’-GTGCAGGGTGCGAGGT-3’).

**Western blot analysis**

Lysates of primary cardiomyocytes or heart tissues were separated by electrophoresis and transferred to polyvinylidene difluoride membranes (EMD Millipore, Bedford, MA, USA). After blocking in 5% skim milk, membranes were incubated with primary antibodies against Pink1 (1:2000; Cell Signaling Technology, Danvers, MA, USA), cleaved caspase-3 (1:2500; Cell Signaling Technology) and β-actin (1:3000; Cell Signaling Technology). The membranes were incubated with horseradish peroxidase-linked secondary antibody (1:5000; Cell Signaling Technology). ChemiDoc MP system (Bio-Rad, Hercules, CA, USA) was used to detect signals, and Image J software was used for densitometric analysis.

**Statistical analysis**

Data were presented as mean ± standard deviation, and conducted with SPSS 11.5 statistical software. Statistical analysis was performed via Student’s t-test or one-way analysis of variance. The *p* < 0.05 was considered significant.

**RESULTS**

**Preconditioning with remifentanil suppressed hypoxia/reoxygenation-induced cell apoptosis of cardiomyocytes**

To establish *in vitro* cell model of myocardial I/R injury, primary cardiomyocytes were isolated from rats and then conducted with hypoxia/reoxygenation treatment. Hypoxia/reoxygenation treatment decreased cell viability (Fig. 1A) and promoted cell apoptosis (Fig. 1B) of cardiomyocytes. However, cardiomyocytes preconditioned with different concentrations of remifentanil (1, 5 or 10 μM) attenuated hypoxia/reoxygenation-induced decrease of cell viability (Fig. 1A) and increase of cell...
apoptosis (Fig. 1B), suggesting that preconditioning with remifentanil suppressed hypoxia/reoxygenation-induced cell apoptosis of cardiomyocytes. Hypoxia/reoxygenation-induced enhancement of cleaved caspase-3 was downregulated by remifentanil preconditioning (Fig. 1C), confirming the suppressive effect of remifentanil on hypoxia/reoxygenation-induced cell apoptosis of cardiomyocytes.

Preconditioning with remifentanil reduced hypoxia/reoxygenation-induced increase of miR-205 in cardiomyocytes

Transfection efficiency of miR-205 mimic or inhibitor into cardiomyocytes is shown in Fig. 2A. To unravel involvement of miR-205 in myocardial I/R injury, expression level of miR-205 in hypoxia/reoxygenation-induced cardiomyocytes was determined by qRT-PCR analysis. The results showed that miR-205 was upregulated in hypoxia/reoxygenation-induced cardiomyocytes (Fig. 2B). Moreover, preconditioning with remifentanil reduced hypoxia/reoxygenation-induced increase of miR-205 in cardiomyocytes (Fig. 2B), showing that miR-205 might participate in myocardial I/R injury.

MiR-205 was involved in protective effect of remifentanil preconditioning against hypoxia/reoxygenation-induced injury

To investigate the role of miR-205 in myocardial I/R injury, cardiomyocytes were transfected with miR-205 mimic or inhibitor, and then conducted with remifentanil preconditioning and hypoxia/reoxygenation treatment. Result showed that transfection with miR-205 mimic or inhibitor did not affect cell viability of cardiomyocytes without hypoxia/reoxygenation treatment (Fig. 3A). However, knockdown of miR-205 increased cell viability of hypoxia/reoxygenation-induced cardiomyocytes preconditioned with remifentanil (Fig. 3B), but repressed the cell apoptosis (Fig. 3C) and decreased protein expression of cleaved caspase-3 (Fig. 3D). Moreover, over-expression of miR-205 demonstrated reversed effects on cell viability (Fig. 3B), apoptosis (Fig. 3C) and cleaved caspase-3 expression (Fig. 3D) in hypoxia/reoxygenation-induced cardiomyocytes preconditioned with remifentanil, indicating that remifentanil preconditioning might reduce miR-205 to protect against hypoxia/reoxygenation-induced injury in cardiomyocytes.
Fig. 2. Preconditioning with remifentanil reduced hypoxia/reoxygenation-induced increase of miR-205 in cardiomyocytes. (A) Transfection efficiency of miR-205 mimic or inhibitor into cardiomyocytes was identified by qRT-PCR. (B) Remifentanil preconditioning decreased expression of miR-205 in hypoxia/reoxygenation-induced cardiomyocytes. N = 3. *p < 0.05; **p < 0.01. H/R: hypoxia/reoxygenation, RPC-1, -5, -10: 1, 5, 10 μM remifentanil.

Fig. 3. MiR-205 was involved in protective effect of remifentanil preconditioning against hypoxia/reoxygenation-induced injury. (A) Transfection with miR-205 mimic or inhibitor did not affect cell viability of cardiomyocytes without hypoxia/reoxygenation treatment. N = 3. (B) Knockdown of miR-205 increased cell viability of hypoxia/reoxygenation-induced cardiomyocytes preconditioned with remifentanil, and over-expression of miR-205 decreased the cell viability. N = 3. (C) Knockdown of miR-205 repressed the cell apoptosis of hypoxia/reoxygenation-induced cardiomyocytes preconditioned with remifentanil, and over-expression of miR-205 promoted the cell apoptosis. N = 3. (D) Knockdown of miR-205 decreased protein expression of cleaved caspase-3 in hypoxia/reoxygenation-induced cardiomyocytes preconditioned with remifentanil, and over-expression of miR-205 increased the expression. N = 3. *p < 0.05; **p < 0.01. H/R: hypoxia/reoxygenation.
MiR-205 suppressed PINK1 expression

Data from Targetscan (http://www.targetscan.org/vert_72/) showed that miR-205 could bind to 3'UTR of PINK1 (Fig. 4A). Luciferase activity assay revealed that over-expression of miR-205 decreased luciferase activity of PINK1-WT, while knockdown of miR-205 increased the activity (Fig. 4B). However, mutation at the binding site between miR-205 and PINK1 abolished the effect of miR-205 on luciferase activity of PINK1-MUT (Fig. 4B), suggesting that miR-205 bind to PINK1. Transfection with miR-205 mimic decreased protein expression of PINK1 in cardiomyocytes, and PINK1 was increased in cardiomyocytes transfected with miR-205 inhibitor (Fig. 4C). Protein expression of PINK1 was reduced in hypoxia/reoxygenation-induced cardiomyocytes (Fig. 4D), while preconditioning with remifentanil...
increased PINK1 (Fig. 4D), demonstrating that remifentanil preconditioning might regulate miR-205/PINK1 axis to protect against hypoxia/reoxygenation-induced injury in cardiomyocytes.

Knockdown of miR-205 enhanced protective effect of remifentanil preconditioning against myocardial I/R injury

To establish in vivo model of myocardial I/R injury, rats were conducted with regional ischemia and reperfusion treatment. Rats with myocardial I/R injury model (AMI) showed higher expression of miR-205 compared with the sham group (Fig. 5A), while tail vein injection with miR-205 antagonist decreased miR-205 expression in AMI rats (Fig. 5A). However, preadministration of remifentanil decreased miR-205 expression (Fig. 5A), and injection with miR-205 antagonist aggravated remifentanil-induced decrease of miR-205 expression (Fig. 5A). Lower expression of PINK1 and higher expression of cleaved caspase were detected in AMI rats compared to the sham group (Fig. 5B), while injection with miR-205 antagonist increased PINK1 and decreased cleaved caspase expression in AMI rats (Fig. 5B). Moreover, preadministration of remifentanil increased PINK1 and decreased cleaved caspase expression (Fig. 5B), and injection with miR-205 antagonist increased PINK1 and decreased cleaved caspase expression (Fig. 5B). Regional ischemia and reperfusion treatment induced myocardial I/R injury in rats with increased infarct size (Fig. 5C) and LDH activity (Fig. 5D). However, injection with miR-205 antagonist ameliorated the injury through decrease of infarct size (Fig. 5C) and LDH activity (Fig. 5D). Moreover, preadministration of remifentanil decreased infarct size (Fig. 5C) and LDH activity (Fig. 5D), and injection with miR-205 antagonist aggravated remifentanil-induced
decrease of infarct size (Fig. 5C) and LDH activity (Fig. 5D). These results revealed that knockdown of miR-205 enhanced the protective effect of remifentanil preconditioning against myocardial I/R injury.

**DISCUSSION**

Anesthesia, such as with sevoflurane and propofol, has been implicated in cardioprotective effect against myocardial I/R injury (Lotz et al., 2020). Sevoflurane anesthesia could repress miR-135b-5p to reduce cardiomyocyte necrosis and apoptosis during myocardial I/R injury (Xie et al., 2017). Since remifentanil was widely used as anesthesia, and demonstrated protective effect against myocardial I/R injury (Zhang et al., 2004). MiRNAs involved in remifentanil-mediated myocardial I/R injury were then evaluated in this study to unravel the underlying mechanism.

Hypoxia/reoxygenation could induce cell damage and inflammation in cardiomyocytes, and represent an in vitro cell model of myocardial I/R injury (Gao et al., 2017). This study firstly isolated primary cardiomyocytes from neonatal rats, and then treated the cells with hypoxia/reoxygenation. Cell viability of cardiomyocytes was decreased while apoptosis was increased post hypoxia/reoxygenation treatment, confirming the cytotoxic effect. However, in line with previous study that preconditioning with remifentanil could attenuate hypoxia/reoxygenation-induced injury in cardiomyocytes (Dou et al., 2016), this study also revealed that remifentanil preconditioning increased cell viability of hypoxia/reoxygenation-induced cardiomyocytes and decreased the apoptosis via reduce of cleaved caspase-3.

Data from qRT-PCR analysis demonstrated higher expression of miR-205 in hypoxia/reoxygenation-induced cardiomyocytes and hearts post regional ischemia and reperfusion treatment, suggesting potential relation between miR-205 and myocardial I/R injury. A previous study has shown that miR-205 was decreased in plasma and heart samples of imatinib mesylate mice or doxorubicin-induced cardiotoxicity in mice (Hanoušková et al., 2019), and the decreased miR-205 protects cardiomyocytes via repression of myocardial apoptosis (Xuan et al., 2017). Here, knockdown of miR-205 increased cell viability of hypoxia/reoxygenation-induced cardiomyocytes preconditioned with remifentanil while repressed the cell apoptosis and decreased protein expression of cleaved caspase-3. Moreover, injection with miR-205 antagonir aggravated remifentanil-induced decrease of infarct size and LDH activity in rat model with myocardial I/R injury, indicating that remifentanil preconditioning might decrease miR-205 to protect against myocardial I/R injury. Moreover, remifentanil could reduce inflammation and oxidative stress to attenuate myocardial injury (Chen et al., 2020), inflammatory response and oxidative stress contribute to myocardial I/R injury (Liu et al., 2020). Since miR-205 was involved in high glucose-induced inflammation and oxidative stress in mesangial cells (Chen et al., 2019), remifentanil might also decrease miR-205 to regulate inflammation and oxidative stress in cardiomyocytes and demonstrate protective effect against myocardial I/R injury.

PINK1 was reported to regulate mitochondrial dysfunction and oxidative stress, and participate in cardiac dysfunction and heart failure (Billia et al., 2011). PINK1 was reduced in hypoxia/reoxygenation-induced H9c2 cells, and forced PINK1 expression could alleviate myocardial I/R injury through regulation of mitochondrial dysfunction (Li et al., 2017). Our results also demonstrated that remifentanil preconditioning enhanced PINK1 via reduce of miR-205 to repress hypoxia/reoxygenation-induced cell damage and myocardial I/R injury. The direct binding ability between miR-205 and PINK1 was validated by luciferase activity assay. PINK1 was validated as target gene of miR-421-mediated mitochondrial fragmentation during myocardial infarction (Wang et al., 2015), as well as target gene of miR-27a/b (MiR-27a and miR-27b regulate autophagic clearance of damaged mitochondria by targeting PTEN-induced putative kinase 1 (PINK1)). In addition, circular RNA ACR could suppress autophagy through up-regulation of PINK1 to attenuate myocardial I/R injury (Zhou et al., 2019). Whether miR-205/PINK1 axis could regulate mitochondrial dysfunction and autophagy to participate in remifentanil-mediated myocardial I/R injury should be further investigated. Administration with PINK1 interference should also be performed in rat model with myocardial I/R injury post remifentanil to strength functional role of miR-205/PINK1 axis in remifentanil-mediated myocardial I/R injury.

In summary, this study provided evidence that remifentanil preconditioning could reduce miR-205 to enhance PINK1, repress hypoxia/reoxygenation-induced cell injury in cardiomyocytes and myocardial I/R injury in rats. Although the downstream pathway remains elusive, results in this study shed new light on the development of potential target for ischemic cardiac injury.

**Conflict of interest**— The authors declare that there is no conflict of interest.
miR-205/PINK1 axis in remifentanil-mediated I/R injury

REFERENCES


