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MiR-34a-5p mediates sevoflurane preconditioning induced inhibition of hypoxia/reoxygenation injury through STX1A in cardiomyocytes

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ABSTRACT

Anesthetic preconditioning is a cellular protective approach whereby exposure to a volatile anesthetic renders cardio injury. Sevoflurane preconditioning has been shown to exhibit cardio protective effect on hypoxia/reoxygenation (H/R) injury, but the underlying mechanism is unclear. Syntaxin 1A (STX1A), an important regulator in cardio disease, was predicted to be the target gene of microRNA-34a-5p (miR-34a-5p). The current research was designed to delineate the role of miR-34a-5p in regulating sevoflurane preconditioning in cardiomyocytes injury. In this study, the results demonstrated that the expression of STX1A was significantly increased, while miR-34a-5p was dramatically decreased in sev-preconditioning H9c2 cells as compared with cells only under H/R stimulation. Moreover, miR-34a-5p regulated the protective effect of sev-preconditioning in injured H9c2 cells by mediating cell proliferation and cell apoptosis. Additionally, the luciferase report confirmed the targeting reaction between STX1A and miR-34a-5p. Taken together, our study suggested that miR-34a-5p regulated sev-preconditioning induced inhibition of hypoxia/reoxygenation injury through mediating STX1A, provided a potential therapeutic target for anesthetic protection in cardio disease.

1. Introduction

Heart diseases is a major cause of morbidity and mortality worldwide [1]. The ischemia-reperfusion injury (IRI) is common in patients with myocardial ischemia after treatment [2]. To improve the clinical outcome in patients suffer from myocardial IRI, innovative treatment and new therapeutic strategies to alleviate IRI are needed. The volatile anesthetics were usually used in general anesthesia for patients undergo surgery, including heart surgery [3]. Anesthetic preconditioning (APC) is a cellular protective mechanism whereby exposure to a volatile anesthetic renders tissues more resistant to a subsequent ischemic insult [4]. This benefit is well established in models of myocardial protection. The process can elicit a biphasic pattern of cardio-protection. Sevoflurane is a widely used inhalational anesthetic in clinical practice [5]. It has been demonstrated that preconditioning with sevoflurane could induce ischemic tolerance both in vitro and in vivo [6]. However, the underlying mechanisms of sevoflurane preconditioning in cardio-protection are needed to elucidate.

Syntaxin 1A (STX1A), a membrane protein, is widely expressed in brain, endocrine system, heart as well as other organs [7]. It has been reported that STX1A was able to regulate myocardial ischemia-reperfusion related signaling pathways such as K_{ATP} and calcium channels [8]. Additionally, STX1A is significantly increased in rats with ischemia as compared with healthy control [9]. A recent study has indicated that STX1A mediated isoflurane induced alleviation of hypoxia-reoxygenation injury in rat cardiomyocytes. These findings suggested a potential role of STX1A in cardiac IRI [10]. However, the effect and underlying mechanism of STX1A in sevoflurane preconditioning regulated cardiac IRI has not been investigated.

In recent years, microRNAs (miRNAs) have emerged as an important target for many diseases, and regulated numerous physiological and pathophysiological processes [11]. MiRNAs are non-coding and small RNAs consisting of approximately 22 nucleotides that modulate gene expression by targeting the 3′-untranslated region (UTR), leading to translation inhibition [12]. Therefore, miRNAs regulate diverse cellular processes including cell proliferation, cell apoptosis, and cell differentiation [13]. Nowadays, increasing evidences have correlated miRNA functions with cardiovascular diseases [14]. Using bioinformatics software targetscan, we predicted the microRNAs that can react with STX1A. MiR-34a-5p has been reported to be down-regulated and function as tumor-suppressor in several cancers [15,16]. But the effect of miR-34a-5p in anesthesia is still not clear.

In the present study, H9c2 cells were used to establish a hypoxia/reoxygenation (H/R) induced myocardial cell injury model. By culturing cells in a sevoflurane environment using anesthesia machine, we found that the expression of STX1A was significantly increased, while

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miR-34a-5p was decreased in sev-preconditioning H9c2 cells as compared with cells only under H/R stimulation. MiR-34a-5p regulated the proliferation and apoptosis of sev- preconditioning mediated H/R induced H9c2 cell injury. Taken together, our results suggested that miR-34a-5p may provide novel insight into the protective effect of sevoflurane on cardio injury.

2. Materials and methods

2.1. Cell culture and treatment

Myocardial cell H9c2 (ATCC® CRL-1446™) was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). H9c2 cells were normally cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA) plus 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C with 5% CO2. The H9c2 cells were first divided into three groups: Control group, normal H9c2 cells; H/R group (hypoxia/reoxygenation group), H9c2 cells was cultured in 95% N_2 and 5% CO_2 for 6 h, and then the culture medium was replaced with fresh medium containing 10% FBS, after which the cells were cultured in 95% O_2 and 5% CO_2 for another 6 h; seV-PC group (sevoflurane preconditioning group), H9c2 cells cultured in 95% O_2 and 5% CO_2 with 2% sevoflurane (Sigma, St. Louis, MO, USA) for 1 h, then treated as H/R group.

2.2. Western blot

A total of 25 μg of proteins was loaded and separated through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electro-transferred to nitrocellulose membranes (Amersham, Little Chalfont, UK). Then, the membranes were blocked with 3% nonfat milk for 1 h at 37 °C. After washing with Tris-buffered saline with Tween (TBST) three times, the membranes were incubated with primary antibodies against STX1A, Bax, Caspase-3, Caspase-9, Bcl-2, and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C overnight. Then, the peroxidase-conjugated secondary antibody (1:1,000, Boster Corporation, Wuhan, Hubei, China) was added and incubated for 1 h at room temperature. The band density of each gene was normalized to the corresponding density of β-actin and visualized using the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, Buckinghamshire,UK).

2.3. RNA extraction and quantitative real-time PCR (qRT-PCR)

The target miRNA for STX1A was predicted by targetscan (http://www.targetscan.org/vert_71/). Total RNA was extracted from H9c2 cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The purity and concentration of total RNA were determined by an Ultraviolet Spectrophotometer (Eppendorf, German). cDNAs were synthesized using the OneStep PrimeScript miRNA cDNA Synthesis Kit (Takara Biotechnol-ogy, Dalian, China). Briefly, 20 μL reactions containing reactions containing 50 ng of total RNA, 10 μL of 2 × SYBR Green PCR MasterMix, 6.25 U of AMV reverse transcriptase, 10 U of RNase inhibitor and 0.1 mM of primers were subjected to one cycle of 95 °C for10 min and then 40 cycles of 95 °C for 15 s, 56 °C for 30 s and 72 °C for 45 s. The expression of miRNA was normalized to the U6 RNA. Data were analyzedby the △Ct method and expressed as fold-changes.

2.4. Cell transfection

MiRNA mimics and inhibitors specific for miR-34a-5p (Sangon, Shanghai, China) were used to increase or silence the expression of miR-34a-5p in H9c2s, respectively. For miRNA transfection, miR-34a-5p mimic, mimic control, miR-34a-5p inhibitor, inhibitor control was transfected into H9c2 cells for a final concentration of 50 nM using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Then the cells were treated as the sev-PC group. The transfection efficiency was assessed by qRT-PCR.

2.5. Cell proliferation assay

The cell proliferation of H9c2 was measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After the addition of growth medium containing 10% MTT (Sigma, St. Louis, MO, USA), the cells were seeded in a 96-well plate and cultured at 37 °C overnight in 5% CO_2. Then, the formazan crystals were dissolved with dimethylsulfoxide (DMSO). Optical density was determined using a micro-culture plate reader (Becton Dickinson, San Diego, USA) at 490 nm.

2.6. Cell apoptosis analysis

Cell apoptosis rate was determined using the flow cytometry method. Cells were harvested, washed with PBS three times and then fixed with 75% ethanol overnight at 4 °C. After incubation with RNase at 37 °C for 30 min, cells were stained with propidium iodide for 30 min. In total, 10^4 events were examined by a FACSCalibur flow cytometer (Becton-Dickinson, Shanghai, China). The histograms were analyzed by Cell-Quest software (Becton-Dickinson, Mountain View, CA, USA).

2.7. Dual-luciferase reporter assay

A 600 bp sequence from the 3′-UTR of STX1A containing a putative miR-34a-5p binding site was amplified by PCR using the cDNA synthesized from H9c2 cells. The sequence for the mutation within the miR-34a-5p binding site was amplified by the point mutation method using the KOD-Plus mutagenesis kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions. The sequences were then sub-cloned into the pmirGLO Luciferase reporter vector (Promega, Madison, WI, USA) with XbaI and NotI, and the recombinant plasmids were assessed by DNA sequencing. For the detection of luciferase activity, 10 ng of the recombinant plasmids were co-transfected with 50 nM of miR-34a-5p mimics into H9c2 cells.

2.8. Statistical analysis

All results in this study were presented as the mean ± SD from a minimum of three replicates. All statistical analyses were evaluated by SPSS version 15.0 statistical software. Student's t test was used when comparing only two groups; one-way ANOVA was used when more than two groups were compared. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Sevoflurane preconditioning decreases miR-34a-5p expression in H/R stimulated cardiomyocytes

To investigate the underlying mechanism of sevoflurane preconditioning in hypoxia/reoxygenation injury, we first measured the expression of STX1A. Western blot result demonstrated that the expression of STX1A was slightly increased in H/R group compared with control group, and further significantly increased in sev-PC group compared with H/R group (Fig. 1A and C). To explore the role of miRNA in sevoflurane preconditioning regulated hypoxia/reoxygenation injury, we used bioinformatics software TargetScan to predict the target miRNA of STX1A. The result suggested that STX1A may be directly regulated by miR-34a-5p (Fig. 1B). Moreover, the expression of miR-34a-5p was detected by qRT-PCR. The results demonstrated that miR-34a-5p was significantly decreased in sev-PC group compared with