Down-regulation of lncRNA KCNQ1OT1 protects against myocardial ischemia/reperfusion injury following acute myocardial infarction

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ABSTRACT
This study aimed to investigate the protective effects of long non-coding RNA KCNQ1OT1 against myocardial ischemia/reperfusion (I/R) injury following acute myocardial infarction, as well as its regulatory mechanism. We used the cardiac muscle H9c2 cells under condition of oxygen glucose deprivation followed by reperfusion (OGD/R) to induce myocardial I/R injury. Then H9c2 cells were transfected with si-NC, si-KCNQ1OT1, pc-NC, pc-KCNQ1OT1, si-AdipoR1 and si-AdipoR2, respectively. The myocardial cell viability and apoptosis were respectively detected. In addition, the expression levels of inflammatory factors, apoptosis-related proteins and p38 MAPK/NF-κB pathway-related proteins were detected. Besides, an inhibitor of p38 MAPK/NF-κB pathway SB203580 was used to treat cells to verify the relationship between KCNQ1OT1 and p38 MAPK/NF-κB pathway. The expression of KCNQ1OT1 was significantly up-regulated in OGD/R-induced myocardial H9C2 cells. The OGD/R-induced decreased cell viability and AdipoR1 expression could be reversed after suppression of KCNQ1OT1. In addition, suppression of KCNQ1OT1 reduced OGD/R-induced increased expressions of TNF-α, IL-6 and IL-1β and OGD/R-induced increased cell apoptosis, which were reversed after knockdown of AdipoR1. Moreover, SB203580, an inhibitor of p38 MAPK/NF-κB signal pathway, could further enhance the inhibitory effects of KCNQ1OT1 suppression on the expression of p-p38, TNF-α, IL-6, IL-1β and p-NF-κB in OGD/R-induced myocardial H9C2 cells. Suppression of KCNQ1OT1 may prevent myocardial I/R injury following acute myocardial infarction via regulating AdipoR1 and involving in p38 MAPK/NF-κB signal pathway.

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1. Introduction
Acute myocardial infarction remains one of the most common lethal diseases in the world [1,2]. It is characterized by the interruption of blood supply to a part of the heart causing damage to the heart muscle [3]. Patients with acute myocardial infarction always harbor multiple complex coronary plaques related to adverse clinical outcomes [4]. Ischemia and reperfusion (I/R) injury is a common cause of acute myocardial infarction [5–7] because I/R injury can activate inflammatory response via prompting a release of cytokines, oxygen free radicals and other pro-inflammatory [8]. Several therapeutic strategies for preventing myocardial I/R injury are capable of improving clinical outcomes in patients with acute myocardial infarction [9,10]. Therefore, elucidation of key mechanism preventing myocardial I/R injury has great significance for the development of effective therapeutic therapy for acute myocardial infarction.

Long noncoding RNAs (lncRNAs) are endogenous cellular RNAs that are mRNA-like transcripts ranging in length from 200 nt to 100 kb. Due to lack of significant open reading frames, lncRNAs do not function as templates for protein synthesis, but several lncRNAs have been identified to play pathological roles in many cardiovascular diseases [11–13]. For instance, lncRNA autophagy promoting factor (APF) can regulate myocardial infarction and may serve as a
potential target for myocardial infarction [14]. LncRNAs Zinc finger antisense 1 (ZFAS1) and Cdr1 antisense (CDR1AS) are dysregulated in patients with acute myocardial infarction and may be novel biomarkers of acute myocardial infarction [15]. Notably, KCNQ1OT1 is 91.5 kb ncRNA, which maps to the 1 Mb Kcnq1 imprinted cluster located at the distal end of mouse chromosome 7 [16,17]. It has been reported that KCNQ1OT1 levels are higher in patients with myocardial infarction than in healthy volunteers and may improve the prediction of outcome [18]. However, whether dysregulation of lncRNA KCNQ1OT1 has protective effects against myocardial ischemia/reperfusion injury following acute myocardial infarction has not been fully investigated.

In the present study, we used the cardiac muscle H9c2 cells under condition of oxygen glucose deprivation followed by reperfusion (OGD/R) to induce myocardial I/R injury in vitro. The effects of overexpression or suppression of KCNQ1OT1 on cell viability, inflammatory response and cell apoptosis were investigated. Moreover, the regulatory relationship between KCNQ1OT1 and adiponectin receptors (AdipoR1 and AdipoR2) was investigated, as well as the regulatory relationship between KCNQ1OT1 and p38 MAPK/NF-κB pathway. Our study aimed to investigate the protective effects of KCNQ1OT1 against OGD/R-induced myocardial I/R injury, as well as its regulatory mechanism. All of our efforts are expected to provide a new insight for the therapy of acute myocardial infarction.

2. Materials and methods

2.1. Cell culture

The cardiac muscle cell line H9c2 was purchased from ATCC (CRL-1446, Manassas, VA). H9c2 cells were then cultured in Dulbecco’s Modified Eagle Medium (DMEM) at 37 °C in an incubator with 95% air and 5% CO2.

2.2. Establishment of a model of myocardial I/R injury

To induce myocardial I/R injury in vitro, H9c2 cells underwent a condition of oxygen glucose deprivation followed by reperfusion (OGD/R). In brief, H9c2 cells were seeded into 35 mm plates and continued to incubate for 24 h. The cells were then cultured with glucose-free DMEM and incubated in an oxygen-free atmosphere (95% N2 and 5% CO2, 37 °C) for 4 h. Afterwards, the cells were changed to a normal culture medium (4.5 mg/mL glucose) and normal atmosphere (95% air and 5% CO2, 37 °C) for another 24 h.

2.3. Cell transfection

Vector pc-KCNQ1OT1 for overexpression of KCNQ1OT1 was constructed by inserting the coding oligonucleotides of KCNQ1OT1 into pcDNA3.1 vector (Invitrogen, Shanghai, China). pcDNA3.1 vector (pc-NC) was considered as the control. Small interference RNAs (siRNAs) targeting KCNQ1OT1 (si-KCNQ1OT1), AdipoR1 (si-AdipoR1) and AdipoR2 (si-AdipoR2) and their corresponding control siRNAs (si-NC) were designed and synthesized by Ribobio (Guangdong, China). For cell transfection, OGD/R-induced myocardial H9c2 cells were cultured in the plates for 24 h and then transfected with pc-KCNQ1OT1, pc-KCNQ1OT1, si-AdipoR1, si-AdipoR1 and their corresponding controls using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). Cells continued to incubate for another 48 h before subsequent experiments.

2.4. Cell treatment

To verify the relationship between KCNQ1OT1 and p38 MAPK/NF-κB signal pathway in preventing OGD/R-induced myocardial injury, the OGD/R-induced myocardial H9c2 cells were exposed to 10 μM of SB203580 (ab120243, Abcam, Cambridge, MA), an inhibitor of p38 MAPK/NF-κB signal pathway, throughout OGD/R procedures.

2.5. MTT assay

Using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2Htetrazolium bromide (MTT) colorimetric assay, cell viability was assessed. In brief, different transfected cells at logarithmic stage were grown into a 96-well plate. At 24, 48, 72 and 96 h of transfection, 20 μL MTT was added into each well to incubate cells for another 4 h. Then 150 μL dimethylsulfoxide (DMSO) was added into each well to dissolve the formazan precipitates for 10 min. The absorbance (490 nm) was measured under an absorption spectrophotometer (Olympus, Japan). Each experiment was repeated 3 times.

2.6. Detection of cell apoptosis

Using Annexin V-Fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA), cell apoptosis was evaluated by means of flow cytometry. In brief, cells were harvested after transfection of 48 h, and then double staining with FITC-Annexin V and propidium iodide (PI) using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences). Within 1 h, the flow cytometric analysis was subsequently conducted using the BD LSRII Flow Cytometer System (BD Biosciences) equipped with FACSDiva Software. The apoptotic cells (annexin V-positive and PI-negative) were sent out and analyzed with a CellQuest 3.0 software (BD Biosciences).

2.7. RNA isolation and qRT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). After detecting the quality of total RNA by SMA 400 UV-VIS (Merion, Shanghai, China), reverse-transcription reactions into cDNA was performed using the PrimeScript RT reagent Kit (Takara, Dalian, China). qRT-PCR was then performed using a SYBR Green PCR kit (Toyobo, Osaka, Japan) on the ABI 7500 PCR System (Applied Biosystems). Primers used for targets amplification were displayed as follows: KCNQ1OT1: forward 5′-TGGTAGGATTGTGGTGTAGG-3′; reverse 5′-CAACCTCCCCACTACC-3′; AdipoR1: forward 5′-AGTCTCGTATAAGTCTGGGAGG-3′; reverse 5′-CACATCTACGGGATGACTCTCA-3′; AdipoR2: forward 5′-TCTCTATATTGAAATACGCCCGGA-3′; reverse 5′-CATGATGGGAATGATTGGAG-3′. Phosphoglyceraldehyde dehydrogenase (GAPDH) was considered as the internal control. Each reaction was conducted in triplicate and the relative expression levels of targets were determined according to threshold cycle (Ct) values with the 2ΔΔCt method.

2.8. Western Blotting

The total protein was extracted from cells after being lysed with radioligand precipitation (RIPA, Sangon Biotech, China) containing PMSF (phenylmethylsufonyl fluoride, Sigma, USA). The protein concentration was then measured with the Bradford assay (Thermo, Hercules, CA). Equal amount of total protein was loaded on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto nitrocellulose membranes (Millipore). After blocking with 5% non-fat dried milk for 1 h, the membranes incubated with primary antibody against AdipoR1 (1:1000; Abcam, Cambridge, MA), AdipoR2 (1:1000; LifeSpan...
Biosciences, Seattle, WA), TNF-α (1:1000; Cell Signaling Technology, Beverly, MA), IL-6 (1:1000; Cell Signaling Technology), IL-1β (1:1000; Cell Signaling Technology), Bcl-2 (1:1000; Cell Signaling Technology), Bax (1:1000; Cell Signaling Technology), cleaved caspase-3 (1:1000; Cell Signaling Technology), pro-caspase-3 (1:1000; Cell Signaling Technology), p38 (1:1000; Cell Signaling Technology), p-p38 (1:1000; Cell Signaling Technology), NF-κB-65 (1:1000; Cell Signaling Technology), p-NF-κB-65 (1:1000; Cell Signaling Technology), JAK2 (1:1000; Cell Signaling Technology), p-JAK2 (1:1000; Cell Signaling Technology) and GAPDH (1:1000; Cell Signaling Technology) overnight at 4°C, followed by the incubation of appropriate secondary antibodies (Odyssey, LI-COR, 1:5000 dilution) for 2 h. GAPDH was used as the internal control. The bands were finally detected with a chromogenic substrate and visualized with the enhanced chemiluminescence (ECL) method. The intensity of each protein band was quantified with Image Lab™ Software (Bio-Rad, Shanghai, China).

2.9. Statistical analysis

The experimental data were presented as the mean ± SEM. The differences between groups were analyzed using a one-way analysis of variance (ANOVA). Post-hoc Tukey test was performed to measure the comparison between 2 groups. Statistical analyses were performed using SPSS 19.0 (SPSS Inc., Chicago, IL). A statistically significant result was obtained with a value of P < 0.05.

3. Results

3.1. Suppression of KCNQ1OT1 may prevent OGD/R-induced myocardial I/R injury via regulating AdipoR1

As shown in Fig. 1A, the expression of KCNQ1OT1 was significantly up-regulated in OGD/R-induced myocardial H9C2 cells (P < 0.01). Thus, OGD/R-induced myocardial H9C2 cells were transfected with si-KCNQ1OT1 and pc-KCNQ1OT1 to suppress and overexpress KCNQ1OT1. Expected results were obtained that, compared with OGD/R-induced myocardial H9C2 cells, the expression of KCNQ1OT1 was significantly decreased in si-KCNQ1OT1 transfected OGD/R-induced myocardial H9C2 cells and significantly increased in pc-KCNQ1OT1 transfected OGD/R-induced myocardial H9C2 cells (P < 0.05, Fig. 1B), indicating that KCNQ1OT1 was successfully suppressed and overexpressed in OGD/R-induced myocardial H9C2 cells. Furthermore, the effects of KCNQ1OT1 suppression and overexpression on cell viability were investigated. As shown in Fig. 1C, in comparison with normal cells, cell viability significantly decreased after OGD/R treatment, which could be reversed after suppression of KCNQ1OT1. Besides, the effects of KCNQ1OT1 suppression and overexpression on the expression of adiponectin receptors (AdipoR1 and AdipoR2) in OGD/R-induced H9c2 cells were explored (Fig. 1D and E). In comparison with control cells, AdipoR1 and AdipoR2 were all significantly down-regulated in OGD/R-induced H9c2 cells (P < 0.05), while the expression of AdipoR1 was significantly up-regulated after suppression of KCNQ1OT1 (P < 0.05), and there was no significant changes in AdipoR1 expression. These data indicate that suppression of KCNQ1OT1 may prevent OGD/R-induced myocardial I/R injury via regulating AdipoR1.

3.2. Suppression of KCNQ1OT1 reduced inflammation in OGD/R-induced myocardial H9C2 cells via regulating AdipoR1

To further verify whether suppression of KCNQ1OT1 prevented OGD/R-induced myocardial I/R injury via regulating AdipoR1 but not AdipoR2, we knocked down the expression of AdipoR1 and AdipoR2 by transfection with si-AdipoR1 and si-AdipoR2 to OGD/R-induced myocardial H9C2 cells, respectively. In comparison with their corresponding controls, the expression levels of AdipoR1 (Fig. 2A) and AdipoR2 (Fig. 2B) were significantly decreased in OGD/R-induced myocardial H9C2 cells (P < 0.05), indicating that

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Fig. 1. Effects of KCNQ1OT1 in OGD/R-induced myocardial H9C2 cells. A: The expression of KCNQ1OT1 in OGD/R-induced myocardial H9C2 cells. B: The expression of KCNQ1OT1 in different transfected groups. C: Cell viability in different transfected groups. D and E: The expression of AdipoR1 and AdipoR2 in different transfected groups. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
AdipoR1 and AdipoR2 were successfully knocked down. Furthermore, the expression levels of inflammatory factors in different transfected groups were determined, including TNF-α, IL-6 and IL-1β. As shown in Fig. 2C, compared with control group, the expressions of TNF-α, IL-6 and IL-1β were all significantly increased after OGD/R treatment (P < 0.05). Moreover, we found that...
suppression of KCNQ1OT1 reduced the increased expressions of TNF-α, IL-6 and IL-1β in OGD/R-induced myocardial H9C2 cells (P < 0.05). Besides, the effects of KCNQ1OT suppression on the expression levels of these inflammatory factors in OGD/R-induced myocardial H9C2 cells were reversed after knockdown of AdipoR1 (P < 0.05), but not AdipoR2. These data confirmed that suppression of KCNQ1OT1 reduced inflammation in OGD/R-induced myocardial H9C2 cells via regulating AdipoR1.

### 3.3. Suppression of KCNQ1OT1 inhibited the apoptosis of OGD/R-induced myocardial H9C2 cells via regulating AdipoR1

The effects of suppression of KCNQ1OT1 on the apoptosis in OGD/R-induced myocardial H9C2 cells were detected. The results of flow cytometry showed that suppression of KCNQ1OT1 decreased the OGD/R-induced increased percentage of apoptotic cells, which could be reversed after knockdown of AdipoR1 (P < 0.05, Fig. 3A). Furthermore, we detected the expressions of apoptosis-related proteins. The results showed that the expression levels of Bcl-2...
and pro-caspase-3 were significantly decreased in OGD/R-induced myocardial H9C2 cells after OGD/R treatment, while the expression levels of Bax and cleaved caspase-3 were markedly increased (P < 0.05, Fig. 3B). In addition, the expression changes of these apoptosis-related proteins in OGD/R-induced myocardial H9C2 cells were significantly reversed after suppression of KCNQ1OT1 (P < 0.05, Fig. 3B). Besides, knockdown of AdipoR1 could further reverse the effects of KCNQ1OT1 suppression on the expression of these proteins (P < 0.05, Fig. 3B). These data confirmed that suppression of KCNQ1OT1 inhibited the apoptosis of OGD/R-induced myocardial H9C2 cells via regulating AdipoR1.

3.4. Suppression of KCNQ1OT1 prevents OGD/R-induced myocardial I/R injury by regulating p38 MAPK/NF-κB signal pathway

To further elucidate the regulatory mechanism of KCNQ1OT1 in preventing myocardial I/R injury following acute myocardial infarction, the relationship between KCNQ1OT1 and p38 MAPK/NF-κB signal pathway was investigated. As displayed in Fig. 3C, suppression of KCNQ1OT1 significantly down-regulated the OGD/R-induced increased expression of p-p38 and p-NF-κB in OGD/R-induced myocardial H9C2 cells (P < 0.05). Moreover, silencing of AdipoR1 markedly reversed the effects of suppression of KCNQ1OT1 on the expressions of p-p38 and p-NF-κB (P < 0.05).

Fig. 4. Suppression of KCNQ1OT1 may prevent OGD/R-induced myocardial I/R injury by regulating p38 MAPK/NF-κB signal pathway. A: The expression levels of p-p38 in different transfected groups. B: The expression levels of TNF-α, IL-6, IL-1β and p-NF-κB in different transfected groups. C: Flow cytometry showed the percentage of apoptotic cells of different transfected groups. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
However, the expression of p-JAK2 in OGD/R-induced myocardial H9C2 cells had no any change after suppression of KCNQ1OT1, Adipor1 and AdipoR2 (P > 0.05).

To further verify whether suppression of KCNQ1OT1 prevented OGD/R-induced myocardial I/R injury via regulating p38 MAPK/NF-κB signal pathway, we used 10 μM of SB203580, an inhibitor of p38 MAPK/NF-κB signal pathway, to treat OGD/R-induced myocardial H9C2 cells. We found that SB203580 treatment resulted in a significantly decrease of p-p38 expression levels in OGD/R-induced myocardial H9C2 cells. Moreover, SB203580 treatment could further enhance the inhibitory effects of KCNQ1OT1 suppression on the expression of p-p38 (P < 0.05, Fig. 4A). Moreover, SB203580 treatment markedly decreased the expression levels of TNF-α, IL-6, IL-1β and p-NF-κB in OGD/R-induced myocardial H9C2 cells (P < 0.01, Fig. 4B). KCNQ1OT1 suppression was also significantly decreased the expressions of these inflammatory factors, and SB203580 could further decrease their expressions (P < 0.01, Fig. 4B). However, SB203580 had no significantly effects on the apoptosis of OGD/R-induced myocardial H9C2 cells (Fig. 4C).

4. Discussion

In the present study, we investigated the effects of KCNQ1OT1 on preventing OGD/R-induced myocardial I/R injury in myocardial H9C2 cells. The results showed that KCNQ1OT1 was significantly up-regulated in OGD/R-induced myocardial H9C2 cells. Suppression of KCNQ1OT1 increased OGD/R-induced decreased cell viability, reduced OGD/R-induced increased expressions of TNF-α, IL-6 and IL-1β, and inhibited OGD/R-induced increased cell apoptosis, which were reversed after knockdown of AdipoR1. Moreover, suppression of KCNQ1OT1 significantly down-regulated the OGD/R-induced increased expression of p-p38 and p-NF-κB, which were also reversed after knockdown of AdipoR1. Besides, an inhibitor of p38 MAPK/NF-κB signal pathway SB203580 could further enhance the inhibitory effects of KCNQ1OT1 suppression on the expression of p-p38, TNF-α, IL-6, IL-1β and p-NF-κB in OGD/R-induced myocardial H9C2 cells. These data imply that KCNQ1OT1 may play key role of in preventing myocardial I/R injury following acute myocardial infarction.

Interestingly, our results showed that the expression of AdipoR1 was significantly up-regulated in OGD/R-induced myocardial H9C2 cells after suppression of KCNQ1OT1. In previous studies, low serum adiponectin is considered as a risk factor for acute myocardial infarction [19,20]. AdipoR1 is a key receptor of adiponectin to mediate the cardioprotective effects of adiponectin [21–23]. Wang et al. confirmed that adiponectin plays cardioprotective effects on myocardial I/R injury through mediating by AdipoR1 [24]. Guo et al. also demonstrated that electroacupuncture induced protective effects against cerebral I/R injury in diabetic mice by AdipoR1-mediated phosphorylation of GSK-3β [25]. On the other hand, inflammation and myocardial apoptosis are key pathological processes involved in myocardial I/R injury. It is reported that oligophrenin1 plays protective roles against myocardial I/R injury via modulating inflammation and myocardial apoptosis [26]. DAPT (a γγ'-secretase inhibitor) can protect against I/R injury via reducing inflammation and apoptosis [27]. In our study, suppression of KCNQ1OT1 increased OGD/R-induced decreased cell viability, reduced OGD/R-induced increased expressions of TNF-α, IL-6 and IL-1β, and inhibited OGD/R-induced increased cell apoptosis, which were reversed after knockdown of AdipoR1. The role of KCNQ1OT1 in preventing myocardial I/R injury and the relationship between KCNQ1OT1 with AdipoR1 has not been fully investigated, our results prompt us to speculate that KCNQ1OT1 may protect against OGD/R-induced myocardial I/R injury via modulating inflammation and apoptosis mediating by AdipoR1.

To further elucidate the regulatory mechanism of KCNQ1OT1 in preventing myocardial I/R injury, the regulatory relationship between KCNQ1OT1 and p38 MAPK/NF-κB pathway was investigated. Activation of p38 in microglia results in the increased levels of inflammatory factors, like TNF-α, IL-1β, and IL-6 [28]. p38 MAPK can abrogate the protective effects of insulin against myocardial I/R injury [29]. Doxorubicin-induced inflammation is reported to be ascribed to the activation of p38 MAPK/NF-κB pathway in H9C2 cardiac cells [30,31]. Licochalcone D exerts its cardioprotective effect against myocardial I/R injury in rat hearts via blockage of the p38 MAPK and NF-κB/p65 pathways [32]. Besides, p38 MAPK inhibitors is shown to have protective effect on myocardial I/R injury and apoptosis signaling pathway [33]. In our study, suppression of KCNQ1OT1 significantly down-regulated the OGD/R-induced increased expression of p-p38 and p-NF-κB. Also, an inhibitor of p38 MAPK/NF-κB signal pathway SB203580 could further enhance the inhibitory effects of KCNQ1OT1 suppression on the expression of p-p38, TNF-α, IL-6, IL-1β and p-NF-κB in OGD/R-induced myocardial H9C2 cells. It is thus intriguing to speculate that KCNQ1OT1 may reduce inflammation and exert protective effects against myocardial I/R injury via regulating p38 MAPK/NF-κB signal pathway.

In conclusion, our findings indicate that suppression of KCNQ1OT1 may prevent myocardial I/R injury following acute myocardial infarction via regulating AdipoR1 and involving in p38 MAPK/NF-κB signal pathway. KCNQ1OT1 may serve as a potential biomarker or therapeutic target for acute myocardial infarction. Further studies are still needed to verify our findings and hypothesis.

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Conflicts of interest

None.

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