

LncRNA NONRATT021972 involved the pathophysiologic processes mediated by P2X₇ receptors in stellate ganglia after myocardial ischemic injury

Lifang Zou¹ · Guihua Tu¹ · Wei Xie² · Shiyao Wen² · Qiuyu Xie² · Shuangmei Liu¹ · Guilin Li¹ · Yun Gao¹ · Hong Xu¹ · Shouyu Wang¹ · Yun Xue¹ · Bing Wu¹ · Qiulan Lv¹ · Mofeng Ying¹ · Xi Zhang¹ · Shangdong Liang¹

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Abstract Adenosine triphosphate (ATP) acts on P2X receptors to initiate signal transmission. P2X₇ receptors play a role in the pathophysiological process of myocardial ischemic injury. Long noncoding RNAs (lncRNAs) participate in numerous biological functions independent of protein translation. LncRNAs are implicated in nervous system diseases. This study investigated the effects of NONRATT021972 small interference RNA (siRNA) on the pathophysiologic processes mediated by P2X₇ receptors in stellate ganglia (SG) after myocardial ischemic injury. Our results demonstrated that the expression of NONRATT021972 in SG was significantly higher in the myocardial ischemic (MI) group than in the control group. Treatment of MI rats with NONRATT021972 siRNA, the P2X₇ antagonist brilliant blue G (BBG), or P2X₇ siRNA improved the histology of injured ischemic cardiac tissues and decreased the elevated concentrations of serum myocardial enzymes, creatine kinase (CK), CK isoform MB (CK-MB), lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) compared to the MI rats. NONRATT021972 siRNA, BBG, or P2X₇ siRNA treatment in MI rats decreased the expression levels of P2X₇ immunoreactivity, P2X₇ messenger RNA (mRNA), and P2X₇ protein, interleukin-6 (IL-6), tumor necrosis factor- α (TNF- α), and phosphorylated p38 mitogen-activated protein kinase (p38 MAPK) in the SG compared to

MI rats. NONRATT021972 siRNA treatment prevented the pathophysiologic processes mediated by P2X₇ receptors in the SG after myocardial ischemic injury.

Keywords Stellate ganglia · Adenosine triphosphate · P2X₇ receptor · Myocardial ischemia · Long noncoding RNA

Introduction

Long noncoding RNAs (lncRNAs) are transcripts greater than 200 nucleotides in length [1, 2]. LncRNAs were initially considered to be byproducts of RNA polymerase II transcription without a biological function. However, the role of increasing numbers of lncRNAs in a variety of biological functions independent of protein translation was demonstrated [3, 4]. LncRNAs participate in posttranscriptional gene regulation via the control of protein synthesis, RNA maturation and transport, and transcriptional gene silencing via regulation of chromatin structure [3–6]. LncRNAs, unlike proteins, act in cis at the site of transcription (act locally to regulate the expression of nearby genes) or trans (distally to regulate gene expression across multiple chromosomes) [7]. LncRNAs allow cells to address regional-, locus-, or allele-specific transcriptions [8]. LncRNAs impact the formation of nuclear domains at the regional level and the transcriptional status of an entire chromosome. LncRNAs also participate in the interaction of two different chromosomal regions. LncRNAs regulate the chromatin state and the activity of a chromosomal locus or specific gene at a locus-specific level [7]. LncRNAs are implicated in the processes of many pathological conditions [9].

The native agonist extracellular adenosine 5'-triphosphate (ATP) acts at P2X receptors on the plasma membrane. P2X ionotropic ligand-gated ion channel receptors are classified

Lifang Zou and Guihua Tu Joint first authors

✉ Shangdong Liang
liangsd@hotmail.com

¹ Department of Physiology, Medical School of Nanchang University, Nanchang 330006, People's Republic of China

² Undergraduate student of grade 2012, Medical School of Nanchang University, Nanchang 330006, People's Republic of China

into P2X₁–P2X₇ [10–13]. P2X receptors participate in the regulation of cardiovascular function and disease [14, 15]. P2X₇ receptors play important roles in the transmission of inflammatory and nociceptive signals [11, 12, 16–18]. Cervical sympathetic ganglia are involved in ischemic heart diseases [19–29]. The ATP that is released from injured cells following myocardial ischemic injury activates P2X receptors in sympathetic afferent and efferent nerves [11, 19–21, 23, 26–31]. Our previous studies implicated P2X₇ receptors in cervical sympathetic ganglia in myocardial ischemia [19, 23, 27]. lncRNAs are involved in normal development and disease [32]. However, the roles of lncRNAs in sympathetic ganglia are poorly characterized. NONRATT021972 is an lncRNA [<http://www.noncode.org>; 33]. This research investigated the effects of lncRNA NONRATT021972 small interference RNA (siRNA) on the pathophysiologic processes mediated by P2X₇ receptors in the stellate ganglia (SG) after myocardial ischemic injury.

Materials and methods

Sprague-Dawley (SD) rats weighing 180–220 g were used in all experiments. The Animal Care and Use Committee of Medical College of Nanchang University reviewed and approved the use of animals. Experiments were performed in accordance with the guidelines of the US National Institutes of Health for the care and use of animals for experimental procedures. Rats were randomly divided into seven groups (eight rats per group): control group (Con group), sham group, myocardial ischemic group (MI group), myocardial ischemic rats treated with the P2X₇ receptor antagonist brilliant blue G (BBG) group (MI + BBG group), myocardial ischemic group rats treated with NONRATT021972 siRNA group (MI + NONRATT021972 si), myocardial ischemic rats treated with P2X₇ siRNA group (MI + P2X₇ si), and myocardial ischemic rats transfected with scrambled siRNA group (MI + SC si group). The myocardial ischemic model was established via occlusion of the left coronary artery (LCA) [18]. Briefly, rats were anesthetized with 10 % chloral hydrate (0.3 mL/100 g body weight) via intraperitoneal injection. Rats were mechanically ventilated, and a thoracotomy was performed. A 5-0 suture on a small, curved needle was passed through the myocardium beneath the LCA. The suture was pulled tightly against a vinyl tube to ligate the blood vessel. The LCA was not ligated in the sham surgery, but the cardiac pericardium was damaged.

Electrocardiogram (ECG) changes were continuously monitored post-surgery. The relative voltage of the negative peak of the S-wave against the QQ line was defined as the ST segment. Myocardial ischemia was confirmed as an observed abnormal Q wave and ST-segment displacement in a lead II ECG. Rats in the MI + BBG group received intraperitoneal

BBG (30 mg/kg, dissolved in saline at a final concentration of 0.2 mg/mL) daily for 30 days beginning 24 h after surgery [34]. Rats in the MI + NONRATT021972 si, MI + P2X₇ si, and MI + SC si groups received NONRATT021972 si, P2X₇ si, and scrambled siRNA, respectively, via sublingual injections.

The following small interfering RNA target sequences were used:

P2X ₇ siRNA	GTGCAGTGAATGAGTACTA.
NONRATT021972	TGTGAATCATGGAAATATC.

Reverse transcription-polymerase chain reaction (RT-PCR)

Serum RNA from patients with coronary heart disease or control healthy subjects was tested using RT-PCR. Total RNA was isolated from sera using the RNApure Circulating Reagent (CW BIO, Beijing, cat.no.CW2281), with some modifications. Briefly, 300 µL of serum was added to three volumes of RNApure Circulating Reagent, mixed thoroughly by vortexing and left at room temperature for 5 min. A 1/5 volume of chloroform was added, shaken vigorously for 30 s, incubated for 5 min at room temperature, and centrifuged for 20 min at 12,000×g at 4 °C. The upper aqueous phase was transferred to a new collection tube, and 1 volume of isopropanol was added, mixed thoroughly for 30 min at room temperature and centrifuged for 20 min at 12,000×g at 4 °C. The precipitate was washed twice with 1 mL of 75 % ethanol which is diluted with diethyl pyrocarbonate (DEPC) water. RNA was eluted by 20 µL of RNase-free water. RNA quality was profiled using a NanoDrop 2000 (Thermo scientific, USA).

Total RNA (1000 ng) was used as a template for reverse transcription using the Revert Aid First Strand cDNA Synthesis Kit (Thermo, USA). PCR amplification of NONRATT021972 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, control) was performed according to our previous method using oligonucleotides as described previously [35]. The following primers were used: NONRATT021972, sense 5'-CGGGATCCAGGCCTGCTGAA AATGACTGAGTATAAAC-3' and antisense 5'-CCG CTCGAGTTCATAGTCACCATAACTATTTTTATTACAT-TAC-3'; and GAPDH sense 5'-CAGGGCTGCTTTTAACT CTGGT-3' and antisense 5'-GATTTTGGAGGGA TCTCGCT-3'. The length of the PCR product was 250 bp for NONRATT021972 and 199 bp for GAPDH. PCR conditions of NONRATT021972 and GAPDH included a hotstart at 94 °C for 3 min, 45-s denaturation (94 °C), 45-s annealing (57 °C), 45-s extension (72 °C) for 36 or 32 amplification cycles, and a 5-min final extension at 72 °C. PCR products were separated on 1.5 % agarose gels and stained with ethidium bromide (EB) using standard protocols. These results are expressed as the ratio of the NONRATT021972 band

intensity/GAPDH band intensity. Data were analyzed using Image-Pro Plus software.

Quantitative real-time PCR

Total RNA was isolated from SG using the TRIzol Total RNA Reagent (Beijing Tiangen Biotech Co.). The reverse transcription reaction was completed using a RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Glen Bernie, USA) following the manufacturer's instructions. The primers were designed using Primer Express 3.0 software (Applied Biosystems), and the following sequences were used: P2X₇, sense 5'-CTTCGGCGTGCCTTTG-3' and antisense 5'-AGGACAGGGTGGATCCAATG-3'; NONRATT021972, sense 5'-TAGGATGAGTACCAGTCAGGT-3' and antisense 5'-TTTTTGGTTTTTGGACAGGG-3'; and β-actin, sense 5'-GCTCTTTTCCAGCCTTCCTT-3' and antisense 5'-CTTCTGCATCCTGTCAGCAA-3'. Quantitative PCR was performed using the SYBR® Green MasterMix in an ABI PRISM® 7500 Sequence Detection System (Applied Biosystems Inc., Foster City, CA). The thermal cycling parameters were 95 °C for 30 s, followed by 40 cycles of amplifications at 95 °C for 5 s, 60 °C for 30 s [36]. Amplification specificity was determined using a melting curve, and the results were processed using software within the ABI7500 PCR instrument.

ISH

Messenger RNA (mRNA) expression was assessed using in situ hybridization (ISH) [20]. Rats were anesthetized, and the SG were dissected immediately. SG were fixed in 4 % paraformaldehyde (PFA) for 2 h at room temperature and transferred to 15 % sucrose in 4 % PFA overnight. Tissues were sectioned at 15 μm. Samples were stored in 4 % PFA in a cryostat at 4 °C. DEPC water was used for all solutions and appliances of ISH. An ISH kit for NONRATT021972 was used. Sections were treated with 0.05 % H₂O₂, followed by digestion with pepsin at 37 °C for 1–2 min. The reaction was terminated with 0.5 mol phosphate-buffered saline (PBS), and sections were washed with PBS for 15 min. Sections were incubated in a prehybridization buffer for 2 h at 37 °C and hybridization buffer overnight at 37 °C. Sections were washed with a gradient of SSC (2× SSC 17.6 g sodium chloride, 8.8 g sodium citrate in 1000 mL distilled water) thoroughly, 2× SSC for 10 min, 0.5× SSC for 15 min, and 0.2× SSC for 15 min to remove background signals. Sections were treated with a biotinylated digoxin antibody at 37 °C for 2 h. Sections were thoroughly washed with PBS and incubated with streptavidin-biotin complex for 30 min and biotinylated peroxidase for 30 min at 37 °C. The color reaction was developed using a DAB substrate, and sections were dehydrated and mounted using neutral gum.

HE staining of myocardial tissue

Myocardial tissues were washed in PBS, fixed with 4 % PFA for 24 h and dehydrated in 30 % sucrose overnight at 4 °C. Myocardial tissues were cut into 14-μm-thick sections using a cryostat. The sections were stained with hematoxylin for 20 s, washed with tap water for 10 min, and dehydrated in 70, 80, and 90 % ethanol for 2 min each. The sections were stained with eosin for 15 s and dehydrated in 95, 95, 100, and 100 % ethanol for 2 min each.

Immunohistochemistry

The rats were killed 30 days after surgery. The heart and SG tissues were dissected immediately and washed in PBS. Tissue samples were fixed in 4 % PFA for 24 h, and the ganglia were dehydrated in 30 % sucrose overnight at 4 °C. The ganglia were cut into 12-μm-thick sections on a cryostat. Sections were washed with PBS three times and incubated in 3 % H₂O₂ for 7 min to block endogenous peroxidase activity. The sections were washed in PBS three times and incubated in 5 % bovine serum albumin (BSA) for 2 h at 37 °C. Sections were rinsed three times in PBS and incubated with a rabbit anti-P2X₇ antibody (1:100; Alomone Labs, Jerusalem, Israel), rabbit anti-tumor necrosis factor-α (TNF-α) antibody (1:100 dilution, Millipore International Inc., USA), and rabbit anti-interleukin-6 (IL-6) antibody (1:100 dilution, Abcam International Inc., USA), diluted in PBS overnight at 4 °C. Sections were rinsed three times in PBS and incubated with a biotinylated goat anti-rabbit secondary antibody (Beijing Zhongshan Biotech, Co.) for 1 h at 37 °C. The preparations were washed in PBS and incubated with streptavidin-horseradish peroxidase (Beijing Zhongshan Biotech, Co.) for 30 min at room temperature. Staining was developed using diaminobenzidine chromogen for 2 min, and the slides were washed with distilled water and cover-slipped. The sections were mounted and examined under a microscope (Olympus TH4-200, Japan). Changes in integrated optical density (IOD) for ganglionic neurons were analyzed using Image Pro-Plus software.

ELISA measurement of serum cardiac enzymes and cytokine levels

Blood samples were collected via orbit vein 30 days after surgery. Samples were maintained at room temperature for 30 min and centrifuged at 3000 rpm for 10 min. Sera were collected and stored at -20 °C. Levels of cardiac enzymes in sera, including CK CK-MB, LDH, and AST, were measured using an automatic electrochemiluminescence immunoassay analyzer (Roche COBAS E601, USA) [37]. Serum cytokine levels, including TNF-α and IL-6, were quantified using an enzyme-linked immunosorbent assay (ELISA) kit [20] following the manufacturer's protocol (Senxiong Co., Shanghai,

China). The reactions were read using an ELISA reader (Rayto, RT-6000, USA) at 450 nm.

Western blotting

Protein expression levels were determined using Western blotting [20, 37, 38]. Rats were killed 30 days after surgery. Ganglia tissues were dissected and flushed with ice-cold PBS. Isolated tissues were homogenized via mechanical disruption in lysis buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.1 % dodecyl sodium sulfate, 1 % Nonidet P-40, 0.5 % sodium deoxycholate, 100 µg/mL phenylmethylsulfonyl fluoride, and 1 µg/mL aprotinin). The homogenate was incubated on ice for 40 min and centrifuged at 12,000 rpm for 10 min. The supernatant was collected, and protein concentration was quantified using the Lowry method. Samples were diluted with loading buffer (100 mM Tris-Cl, 200 mM dithiothreitol, 4 % sodium dodecylsulfate, 0.2 % bromophenol blue, and 20 % glycerol) and heated at 95 °C for 5 min. Equal amounts of total protein (20 µg) were separated using SDS-polyacrylamide gel electrophoresis in a Bio-Rad electrophoresis and blotting system. Separated proteins were transferred to PVDF membranes using the same system. The membranes were blocked with 5 % nonfat dry milk in a solution of 25 mM Tris-buffered saline (pH 7.2) plus 0.1 % Tween 20 (TBST) for 2 h at room temperature and incubated with a rabbit anti-P2X₇ antibody (1:1000 dilutions, Alomone Labs, Jerusalem, Israel), mouse monoclonal anti-β-actin antibody (1:800 dilution, Advanced Immunochemicals, Long Beach, CA), rabbit anti-p-P38 antibody (1:1000 dilutions), and rabbit anti-P38 antibody (1:1000), at 4 °C overnight. Membranes were washed in TBST and incubated with HRP-conjugated IgG (1:5000 dilutions, Beijing Zhongshan Biotechnology Co.) for 1 h at room temperature. Membranes were washed in TBST, and protein bands were developed using an enhanced chemiluminescence (ECL) kit (Shanghai Pufei Biotechnology Co.) according to the manufacturer's protocol. Chemiluminescent signals were collected on autoradiography films. Band intensity was quantified using Image Pro-Plus software. The relative band intensity of target proteins was normalized against the intensity of respective β-actin bands as internal controls.

Statistical analysis

Data were analyzed using SPSS 11.5 software. Numerical values are reported as the means±SE. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Fisher's post hoc test for multiple comparisons. A $p < 0.05$ was considered statistically significant.

Results

Changes in NONRATT021972 expression in SG

NONRATT021972 expression in SG was examined using real-time PCR, and NONRATT021972 expression was significantly higher in the MI group than the control group ($p < 0.01$) (Fig. 1a). ISH demonstrated that NONRATT021972 expression in SG was significantly higher in the MI group than the control group ($p < 0.01$) (Fig. 1b). Serum NONRATT021972 concentrations were higher in patients with coronary heart disease compared to control healthy subjects ($p < 0.05$, $n = 8$ for each group, Fig. 1c).

The effects of NONRATT021972 siRNA on myocardial structure and serum myocardial enzymes

Hematoxylin-eosin (HE) staining measured structural changes in myocardial tissue. The HE stain of cardiac tissue in MI rats revealed solidified necrosis of myocardial fibers, nuclei fragmentation and disappearance, an irregular coarse granular cytoplasm, interstitial edema, and a small amount of neutrophil infiltration. Myocardial fibers were pink in the Con and Sham groups, and the cytoplasm and nucleus were clear. The cell membrane exhibited good integrity, and the cellular arrangement was orderly with moderate intercellular spacing. Solidified necrosis of myocardial fibers, nuclei fragmentation, and disappearance in MI rats treated with BBG, NONRATT021972si, or P2X₇ si were ameliorated in comparison with those in MI rats. The injury changes in cardiac tissues in the MI + BBG, MI + NONRATT021972si, and MI + P2X₇ si groups improved (Fig. 2).

Changes in myocardial enzymes were measured using an automatic electrochemiluminescence immunoassay analyzer. Serum concentrations of CK-MB, CK, LDH, and AST were significantly higher in the MI group than the Con, Sham, MI + BBG, MI + NONRATT021972 si, and MI + P2X₇ si groups ($n = 8$) ($p < 0.01$, $F_{(CK-MB)(5,42)} = 3.76$, $F_{(CK)(5,42)} = 10.23$, $F_{(LDH)(5,42)} = 7.14$, $F_{(AST)(5,42)} = 5.34$). There were no differences between the Con, Sham, MI + BBG, MI + NONRATT021972 si, and MI + P2X₇ si groups ($p > 0.05$, $F_{(CK-MB)(4,35)} = 1.03$, $F_{(CK)(4,35)} = 1.46$, $F_{(LDH)(4,35)} = 1.57$, $F_{(AST)(4,35)} = 2.04$). No differences were observed between the same MI and MI + SC si groups ($p > 0.05$) (Table 1).

The effects of NONRATT021972 siRNA on the expression of P2X₇ mRNA, immunoreactivity, and protein in SG

The expression level of P2X₇ mRNA in SG was measured using quantitative real-time PCR. P2X₇ mRNA expression in the MI and MI + SC si groups was significantly higher than the Con and Sham groups. P2X₇ mRNA expression in the MI + BBG, MI + NONRATT021972 si, and MI + P2X₇ si groups

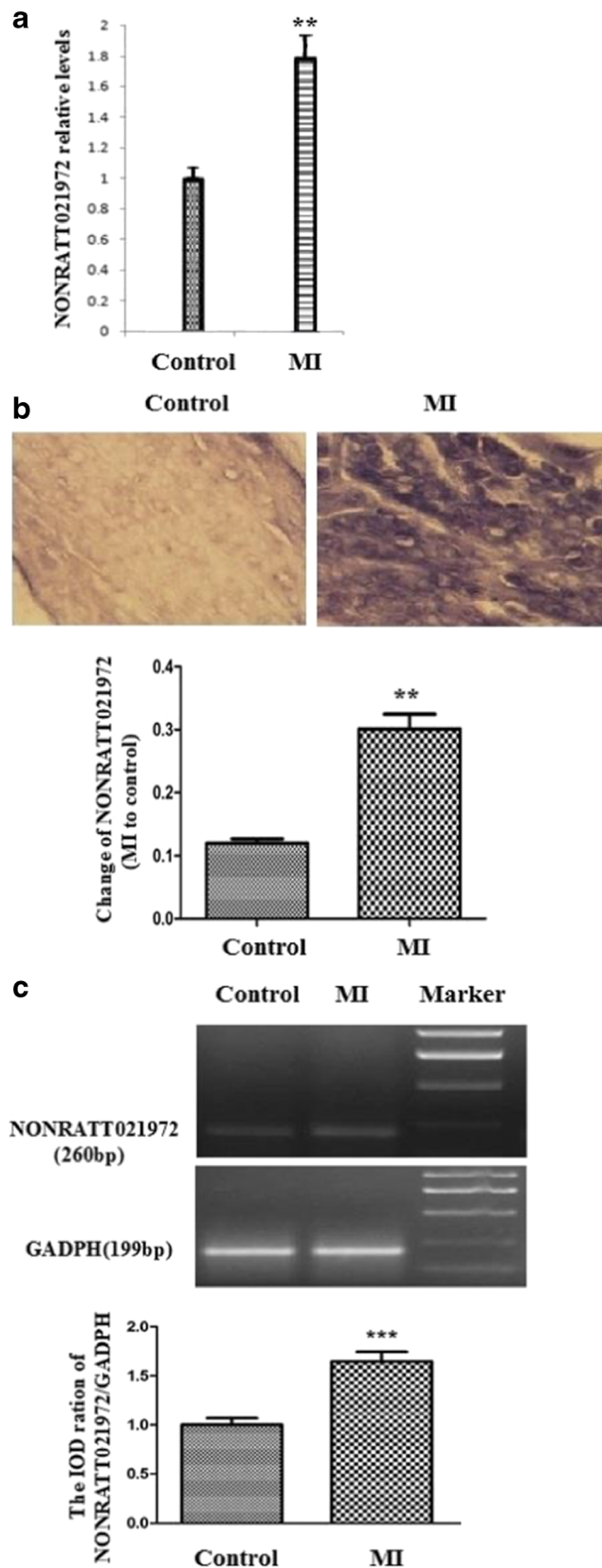


Fig. 1 Expression levels of NONRATT021972 in SG. **a** The histogram of relative mRNA levels. The expression levels of NONRATT021972 in SG were significantly higher in the MI group than the Con group ($p < 0.01$). $\bar{x} \pm s$, $n = 3$. $**p < 0.01$ compared to the Con group. **b** The expression of NONRATT021972 in SG is significantly higher in the MI group than the control group assessed using ISH ($p < 0.01$). **c** Serum NONRATT021972 concentrations in patients with coronary heart disease were higher compared to control healthy subjects as assessed using RT-PCR ($p < 0.05$, $n = 8$ for each group)

MI + BBG, MI + NONRATT021972 si, and MI + P2X₇ si groups ($p > 0.05$, $F(4,10) = 2.19$). No difference was found between the MI and MI + SC si groups ($p > 0.05$) (Fig. 3a).

P2X₇ expression in SG neurons was examined using immunohistochemistry. The intensity of P2X₇ immunoreactivity in the MI group was obviously increased compared to the Con, Sham, MI + BBG, MI + NONRATT021972 si, and MI + P2X₇ si groups ($p < 0.01$, $F_{(5,54)} = 29.43$). No difference was found between the Con, Sham, MI + BBG, MI + NONRATT021972 si, and MI + P2X₇ si groups ($p > 0.05$, $F_{(4,45)} = 2.12$). No difference was found between the MI and MI + SC si groups ($p > 0.05$). The selective P2X₇ antagonist BBG, NONRATT021972 siRNA, and P2X₇ siRNA inhibited the upregulated expression of P2X₇ receptors in SG after myocardial ischemic injury (Fig. 3b).

P2X₇ protein expression (normalized to β -actin internal controls) was investigated using Western blotting. The IOD in the MI group was obviously increased compared to the Con, Sham, MI + BBG, MI + NONRATT021972 si, and MI + P2X₇ si groups ($p < 0.05$, $F_{(5,30)} = 47.66$). No difference was found between the Con, Sham, MI + BBG, MI + NONRATT021972 si, and MI + P2X₇ si groups ($p > 0.05$, $F_{(4,25)} = 1.91$). There was also no difference between the MI group and MI + SCsi group ($p > 0.05$) (Fig. 3c).

The effects of NONRATT021972 siRNA on serum concentrations of IL-6 or TNF- α , TNF- α expression, or IL-6 immunoreactivity in SG

IL-6 and TNF- α concentrations in blood serum of rats were measured using ELISA. Serum concentration of IL-6 and TNF- α in the MI group was significantly higher than the Con group, Sham group, MI + BBG group, MI + NONRATT021972 si group, and MI + P2X₇ si group ($p < 0.01$, $F_{(IL-6)(5,30)} = 6.54$, $F_{(TNF-\alpha)(5,30)} = 4.72$). There were no differences between the Con, Sham, MI + BBG, MI + NONRATT021972 si, and MI + P2X₇ si groups ($p > 0.05$, $F_{(IL-6)(4,25)} = 2.27$, $F_{(TNF-\alpha)(4,25)} = 2.18$). There was no difference between the MI group and MI + SC si group ($p > 0.05$) (Fig. 4a).

TNF- α expression was detected using immunohistochemistry. IOD was used to quantify the relative expression levels. TNF- α immunoreactivity was significantly higher in the MI group than the Con, Sham, MI + BBG, MI +

decreased significantly compared to the MI group ($p < 0.01$, $F_{(5,12)} = 11.23$). No difference was found between the Con, Sham,

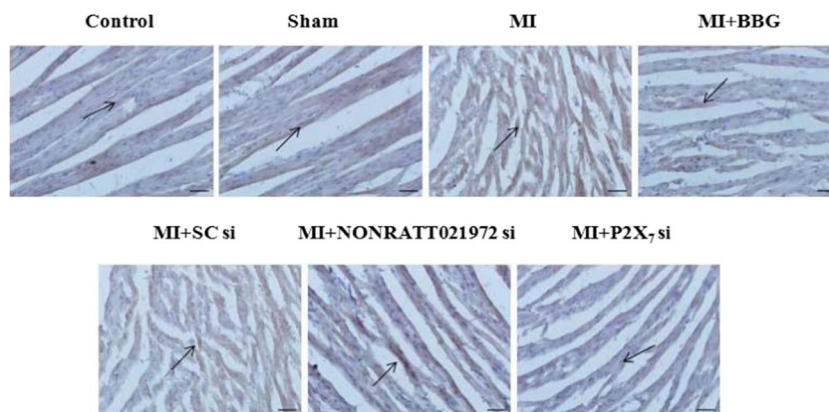


Fig. 2 Changes in HE staining in cardiac tissues of each group of rats. HE stains of cardiac tissue in myocardial ischemic rats show solidified necrosis of myocardial fibers, nuclei fragmentation and disappearance, an irregular coarse granular cytoplasm, interstitial edema, and a small amount of neutrophil infiltration. In the Con group and Sham group, the myocardial fibers were pink, and the cytoplasm and nucleus were clear. The cell membrane exhibited integrity, and cellular arrangement was

regular with moderate intercellular space. Solidified necrosis of myocardial fibers, nuclei fragmentation, and disappearance in MI rats treated with BBG, NONRATT021972si, or P2X₇ si were ameliorated in comparison with those in MI rats. The injury changes in cardiac tissues in the MI+BBG, MI+NONRATT021972si, and MI+P2X₇ si groups improved. Scale bar at 50 μm

NONRATT021972 si, and MI + P2X₇ si groups ($p < 0.01$, $F_{(5,54)} = 37.36$). No significant differences were observed between the Con, Sham, MI + BBG, MI + NONRATT021972 si, and MI + P2X₇ si groups ($p > 0.05$, $F_{(4,45)} = 1.94$) (Fig. 4b).

IL-6 expression in SG was detected using immunohistochemistry. IL-6 immunoreactivity was significantly higher in the MI group than the Con, Sham, MI + BBG, MI + NONRATT021972 si, and MI + P2X₇ si groups ($p < 0.01$, $F_{(5,54)} = 43.57$). No significant differences were observed between the Con, Sham, MI + BBG, MI + NONRATT021972 si, and MI + P2X₇ si groups ($p > 0.05$, $F_{(4,45)} = 2.76$) (Fig. 4c).

The effects of NONRATT021972 siRNA on p38 MAPK and p-P38 MAPK expression in SG using Western blot

The levels of P38 MAPK and p-P38 MAPK in the SG were analyzed by western blotting. IOD ratio of P38 MAPK to β-

actin was not significantly different between the MI group and control group ($p > 0.05$) (Fig. 5b). However, the IOD ratio of p-P38 MAPK to P38 MAPK ($n = 8$) in MI group was higher than that in the control group ($p < 0.05$, $n = 8$ for each group) (Fig. 5a, c). The results indicated that the role of P38 MAPK phosphorylation in the SG was related to the P2X₇ receptor-mediated myocardial ischemic injury in MI rats.

In addition, we examined whether the administration of NONRATT021972 siRNA could affect the phosphorylation of P38 MAPK in the MI SG. The IOD ratio of p-P38 MAPK to P38 MAPK in the MI rats treated with NONRATT021972 siRNA in the MI group was obviously increased compared to the Con, Sham, MI + BBG, MI + NONRATT021972 si, and MI + P2X₇ si groups ($p < 0.01$, $F_{(5,30)} = 53.43$, $n = 8$ for each group) (Fig. 5a, c). No difference was found between the Con, Sham, MI + BBG, MI + NONRATT021972 si, and MI + P2X₇ si groups ($p > 0.05$, $F_{(4,25)} = 2.45$). There were no

Table 1 The concentration of CK-MB, CK, LDH, and AST in the blood serum of the each group rats

Groups	CK-MB (U/L)	CK (U/L)	LDH (U/L)	AST (U/L)
Con	41.61±3.82	327.15±30.24	290.27±21.14	78.78±8.34
Sham	29.8±2.53	259.57±25.36	311.43±27.50	84.15±8.20
MI	97.50±8.45**	1852.44±180.50**	689.16±68.34**	197.25±14.67**
MI + BBG	43.13±6.58	261.51±28.33	321.41±36.62	54.88±5.94
MI + SCsi	85.45±6.35**	2103.47±213.48**	723.89±72.58**	223.63±21.59**
MI + NONRATT021972si	32.53±2.76	294.18±23.66	307.46±30.84	57.22±53.75
MI + P2X ₇ si	35.11±4.21	347.43±35.89	340.24±35.54	74.19±71.01

The serum concentration of CK-MB, CK, LDH, and AST in MI group was significantly higher than those in Con group, Sham group, MI + BBG group, MI+NONRATT021972 si group, or MI + P2X₇ si group ($p < 0.01$). There were no differences among Con, Sham, MI + BBG, MI+NONRATT021972 si, and MI + P2X₇ si groups ($p > 0.05$). It was the same between MI group and MI + SC si group ($p > 0.05$). $\bar{x} \pm s$, $n = 8$

** $p < 0.01$ compared with Con group

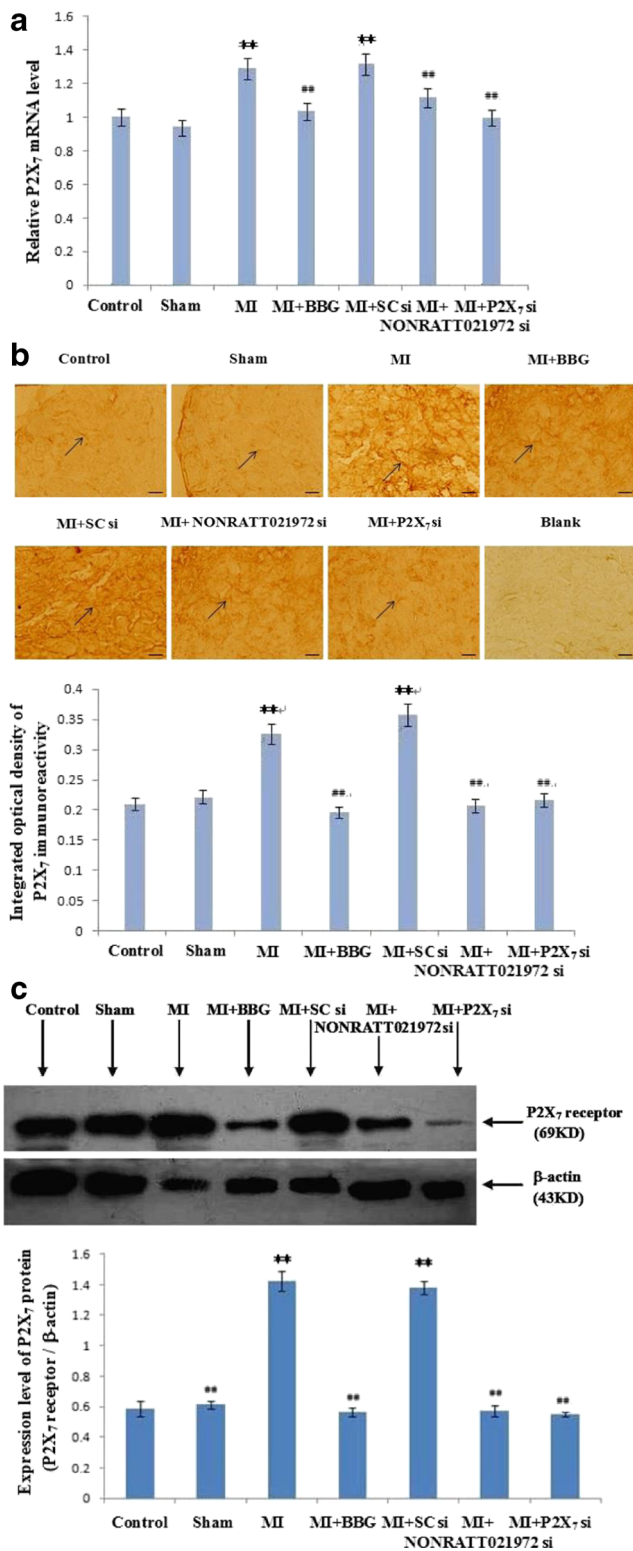


Fig. 3 The expression levels of P2X₇ receptor mRNA, immunohistochemistry, and protein in SG of each group. **a** The expression levels of P2X₇ receptor mRNA in SG were measured using quantitative real-time PCR. Histogram shows the relative mRNA levels. $\bar{x} \pm s$, $n=3$. ** $p<0.01$ compared to the Con group; ### $p<0.01$ vs. the MI group. **b** Representative photos of P2X₇ immunoreactivity in SG were measured using immunohistochemistry. Histogram shows the IOD of the results. *Arrows* indicate immunostained neurons. $\bar{x} \pm s$, $n=10$. ** $p<0.01$ compared to the Con group; ### $p<0.01$ vs. the MI group. *Scale bars* at 20 μm . **c** Representative photos of P2X₇ protein immunostaining in SG were measured using Western blots. Histogram shows the IOD of the results. $\bar{x} \pm s$, $n=6$. ** $p<0.01$, compared to the Con group; ### $p<0.01$ vs. the MI group

MAPK in the SG of MI rats to alleviate P2X₇ receptor-mediated myocardial ischemic injury.

Discussion

Cervical sympathetic ganglia innervate cardiac tissues and regulate myocardial function [39–42]. The altered expression of lncRNAs is involved in the onset and progression of many diseases [7, 9, 43]. Genetic knockouts of some lncRNAs caused developmental defects in mice [44]. Our in situ hybridization experiment demonstrated NONRATT021972 expression in SG. NONRATT021972 expression in SG was significantly higher in the MI group than the control group. Serum NONRATT021972 concentration levels were also higher in patients with coronary heart disease than control healthy subjects. This data suggest that the upregulation of NONRATT021972 in SG was related to the pathological process of the myocardial ischemia.

Ligation of the left anterior descending coronary artery causes myocardial hypoxia and induces myocardial ischemic injury. Myocardial ischemic damage increases myocardial cell permeability, which results in elevated myocardial enzymes in peripheral blood [18, 23, 37]. Myocardial enzymes, such as CK-MB, CK, LDH, and AST, are myocardial injury markers [18, 23, 37]. HE staining in this study revealed heart lesions after MI. Serum CK-MB, CK, LDH, and AST concentrations increased in the MI group compared to the control and sham groups. MI rats treated with NONRATT021972 siRNA, the P2X₇ antagonist BBG, or P2X₇ siRNA exhibited improved histological injury of ischemic cardiac tissues compared MI rats. NONRATT021972 siRNA treatment in MI rats significantly inhibited the ischemic injury-induced elevations in serum CK-MB, CK, LDH, and AST concentrations. Suppression of lncRNA function in vivo attains therapeutic aims [44, 45]. Therefore, NONRATT021972 siRNA treatment may relieve myocardial ischemic injury.

Myocardial ischemia-induced increase in ATP release acts on P2X receptors, which aggravates the myocardial ischemic injury [18, 20, 23, 27, 30, 38]. P2X₇ receptors are involved in nervous system function and diseases [11, 16, 17, 46]. P2X₇

differences in the IOD ratio of P38 MAPK to β -actin between the MI group and MI + scramble siRNA group ($p>0.05$, $n=8$ for each group) (Fig. 5a, b). Taken together, these results suggest that NONRATT021972 siRNA treatment may contribute to decreasing the phosphorylation and activation of P38

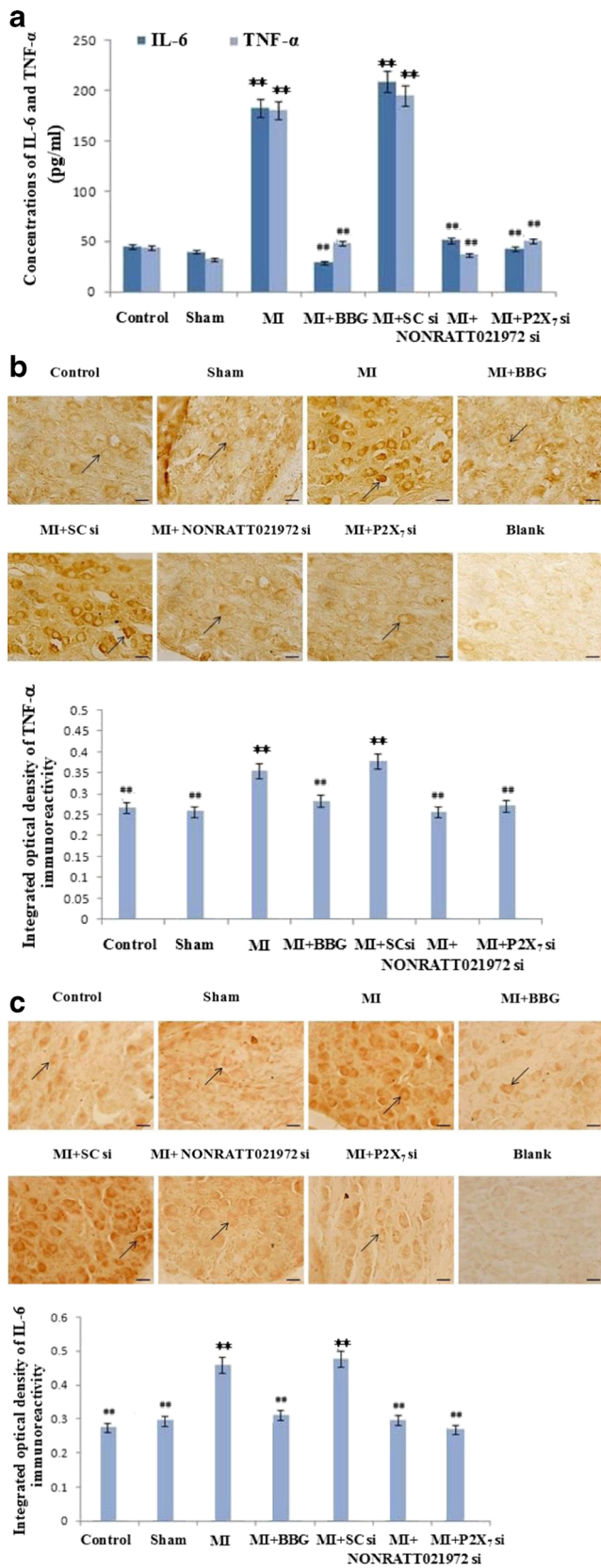


Fig. 4 The concentrations of IL-6 and TNF- α in blood serum and the expression of TNF- α and IL-6 protein immunoreactivity in SG of each group. **a** Serum concentrations of IL-6 and TNF- α were measured using ELISA. Serum concentrations of IL-6 and TNF- α in the MI group were significantly higher than the Con group, Sham group, MI + BBG group, MI + NONRATT021972 si group, and MI + P2X₇ si group ($p < 0.01$). There were no differences between Con, Sham, MI + BBG, MI + NONRATT021972 si, and MI + P2X₇ si groups ($p > 0.05$). There was no difference between the MI group and MI + SC si group ($p > 0.05$). $\bar{x} \pm s$, $n = 6$. ** $p < 0.01$, compared to the Con group; ## $p < 0.01$ vs. the MI group. **b, c** TNF- α expression and IL-6 protein immunoreactivity were detected using immunohistochemistry. Representative photos of TNF- α (**b**) and IL-6 (**c**) protein immunoreactivity in SG. Histogram shows the IOD of TNF- α (**b**) and IL-6 (**c**) protein immunoreactivity in SG. The expression levels of TNF- α (**b**) and IL-6 (**c**) immunoreactivity in the MI group were significantly higher than the Con, Sham, MI + BBG, MI + NONRATT021972 si, and MI + P2X₇ si groups ($p < 0.01$). No significant differences were observed between the Con, Sham, MI + BBG, MI + NONRATT021972 si, and MI + P2X₇ si groups ($p > 0.05$). Arrows indicate the immunostained neurons. $\bar{x} \pm s$, $n = 10$. ** $p < 0.01$, compared to the Con group; ## $p < 0.01$ vs. the MI group. Scale bars at 20 μ m

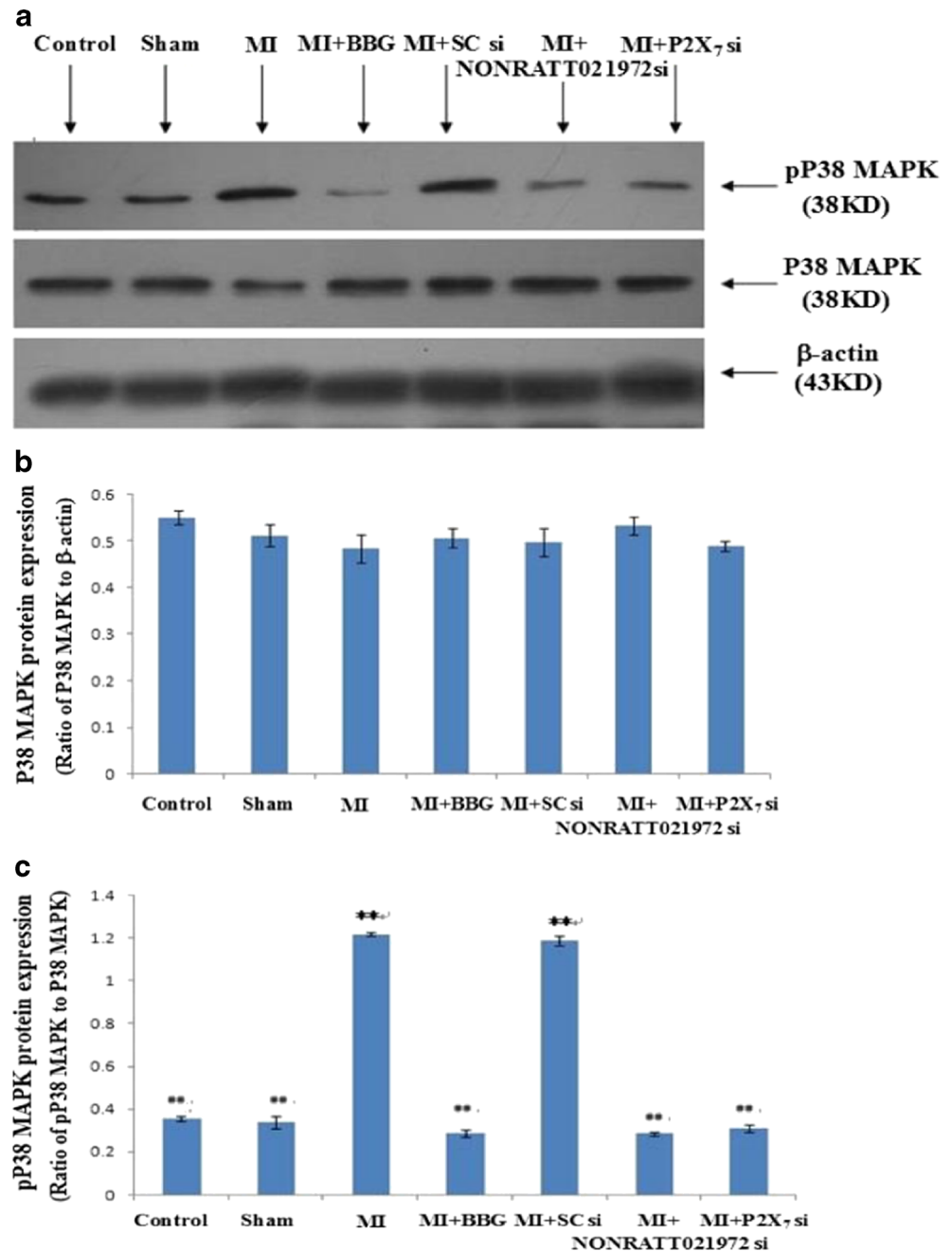
immunoreactivity, mRNA, and protein expression was much higher in MI rats than the control and sham groups. Treatment of MI rats with NONRATT021972 siRNA, BBG, or P2X₇ siRNA reduced the expression levels of P2X₇ immunoreactivity, mRNA, and protein compared to their myocardial ischemic counterparts. These results suggest that NONRATT021972 siRNA treatment acts on P2X₇ receptors to ameliorate myocardial ischemic injury.

Inflammatory mediators augment the nociceptive responses stimulated by ATP [11, 47], and inflammation plays a central role in acute coronary syndrome [18, 48, 49]. Inflammation is the major pathogenetic mechanism associated with myocardial ischemic damage [18, 23, 48–50]. The pro-inflammatory cytokine TNF- α and IL-6 are involved in the pathogenesis of heart ischemic injury [23, 51, 52]. The activation of P2X₇ receptors increases the release of inflammatory mediators and cytokines [53, 54]. IL-6 and TNF- α were significantly increased in the MI rats compared to control and sham rats. NONRATT021972 siRNA, BBG, or P2X₇ siRNA significantly decreased the enhanced expression of IL-6 and TNF- α after MI injury. The pathogenesis of myocardial ischemia is associated with the balance of anti- and pro-inflammatory cytokines [48, 50]. P2X₇ receptors play a significant role in inflammatory diseases [11, 16, 17, 46, 47]. Pharmacological inhibition of P2X₇ receptors attenuates inflammatory diseases [11, 46, 47, 55, 56]. NONRATT021972 siRNA may inhibit P2X₇ receptor activation to relieve myocardial ischemic inflammatory injury.

MAPKs, such as extracellular signal-regulated kinase (ERK), p38 MAPK, and c-Jun N-terminal kinase (JNK), are serine/threonine kinases [36, 57–59]. Activation of P2X₇ receptors activates p38 MAPK [36, 57–59]. Phosphorylation of

receptors are expressed in cervical sympathetic ganglia [17, 19, 23, 27]. Our results demonstrated that P2X₇

Fig. 5 The expression of p38 MAPK and p-P38 MAPK protein in SG of each group using Western blots. **a** Representative photos of p38 MAPK and p-P38 MAPK protein expression in SG. **b** Histogram shows the ratio of P38 MAPK to β -actin. **c** Histogram shows the ratio of p38 MAPK to P38 MAPK. The IOD of p-P38 MAPK expression in the MI group was obviously increased compared to the Con, Sham, MI + BBG, MI + NONRATT021972 si, and MI + P2X₇ si groups ($p < 0.01$). No difference was found between the Con, Sham, MI + BBG, MI + NONRATT021972 si, and MI + P2X₇ si groups ($p > 0.05$). There was also no difference between the MI group and M + SC si group ($p > 0.05$). $\bar{x} \pm s$, $n = 6$. ** $p < 0.01$, compared to the Con group; ### $p < 0.01$ vs. the MI group



p38MAPK kinase causes activation [36, 57–60]. P2X₇ receptor blocker decreased the ATP-provoked activation of p38 MAPK [36, 57, 59]. NONRATT021972 siRNA, BBG, or P2X₇ siRNA administration in MI rats in the present study decreased phosphorylated p38 MAPK in SG and improved myocardial ischemic injury. Our data suggest that the improvement of myocardial ischemic injury by P2X₇ receptor inhibition was ascribed to the reduction of the p38 MAPK-related signaling pathway. Therefore, gene silencing of NONRATT021972 should inhibit P2X₇ activation, reduce p38 MAPK phosphorylation, and prevent the pathological processes of myocardial ischemic injury.

In conclusion, NONRATT021972 expression in SG was significantly higher in the MI group than in the control group. Treatment of MI rats with NONRATT021972 siRNA, BBG, or P2X₇ siRNA improved the histological injuries of ischemic cardiac tissue and decreased the elevated serum myocardial enzyme concentrations compared to MI rats. The MI rats treated with NONRATT021972 siRNA, BBG, or P2X₇ siRNA reduced the expression of P2X₇ immunoreactivity, mRNA, protein, TNF- α , IL-6, and phosphorylated p38 MAPK compared to their myocardial ischemic counterparts. Therefore, NONRATT021972 siRNA treatment inhibited the upregulation of P2X₇ receptors in the SG and prevented the

pathophysiologic processes mediated by P2X₇ receptors in the SG after myocardial ischemic injury.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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