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ORIGINAL ARTICLE

Sensory–sympathetic coupling in superior cervical ganglia after myocardial ischemic injury facilitates sympathoexcitatory action via $P2X_7$ receptor

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Abstract P2X receptors participate in cardiovascular regulation and disease. After myocardial ischemic injury, sensory–sympathetic coupling between rat cervical DRG nerves and superior cervical ganglia (SCG) facilitated sympathoexcitatory action via $P2X_7$ receptor. The results showed that after myocardial ischemic injury, the systolic blood pressure, heart rate, serum cardiac enzymes, IL-6, and TNF-α were increased, while the levels of $P2X_7$ mRNA and protein in SCG were also upregulated. However, these alterations diminished after treatment of myocardial ischemic (MI) rats with the $P2X_7$ antagonist oxATP. After siRNA $P2X_7$ in MI rats, the systolic blood pressure, heart rate, serum cardiac enzymes, the expression levels of the satellite glial cell (SGC) or $P2X_7$ were significantly lower than those in MI group. The phosphorylation of ERK 1/2 in SCG participated in the molecular mechanism of the sympathoexcitatory action induced by the myocardial ischemic injury. Retrograde tracing test revealed the sprouting of CGRP or SP sensory nerves (the markers of sensory afferent fibers) from DRG to SCG neurons. The upregulated $P2X_7$ receptor promoted the activation of SGCs in SCG, resulting in the formation of sensory–sympathetic coupling which facilitated the sympathoexcitatory action. $P2X_7$ antagonist oxATP could inhibit the activation of SGCs and interrupt the formation of sensory–sympathetic coupling in SCG after the myocardial ischemic injury. Our findings may benefit the treatment of coronary heart disease and other cardiovascular diseases.

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Keywords $P2X_7$ receptor · Superior cervical ganglia · Dorsal root ganglia · Myocardial ischemic injury · Sensory–sympathetic coupling

Abbreviations

ATP	Adenosine triphosphate
CGRP	Calcitonin gene-related peptide
CK	Creatine kinase
CK-MB	Creatine kinase isoform MB
cTn-I	Cardiac troponin I
DRG	Dorsal root ganglia
ECG	Electrocardiogram
ELISA	Enzyme-linked immunosorbent assay
ERK1/2	Extracellular signal-regulated protein kinases
GS	Glutamine synthetase
HRP	Horseradish peroxidase
IOD	Integrated optical density
IL-6	Interleukin-6
ISH	In situ hybridization
LCA	Left coronary artery
LDH	Lactate dehydrogenase
MI	Myocardial ischemic
NeuN	Neuronal Nuclei
oxATP	Oxidized ATP (ATP with the 2′- and 3′-hydroxyl moieties oxidized to aldehydes by periodate treatment)
PCR	Polymerase chain reaction
p-ERK1/2	Phosphorylated extracellular signal-regulated protein kinases
SP	Substance P
TH	Tyrosine hydroxylase

TNF-α	Tumor necrosis factor-α
SGCs	Satellite glial cells
SCG	Superior cervical ganglia
siRNA	Small interference RNA

Introduction

Sympathoexcitatory action in cervical sympathetic ganglia is induced by cardiac afferent activation [4, 25, 43]. Sympathoexcitatory action can exaggerate myocardial ischemia further by increasing myocardial oxygen consumption while maintaining cardiac function transiently by enhancing contractility [25, 44]. Surgical interventions of sympathetic afferent pathways abolish or relieve angina pectoris [43]. Myocardial ischemia can produce a variety of chemical substances, which act on the cardiac afferent nerves. Adenine triphosphate (ATP) is released from the sympathetic ganglia [10, 11, 48] and is involved in signal transmission via acting on P2X receptors [10, 11]. The $P2X_7$ receptor is a member of P2X gene family, which encodes membrane glycoproteins that function as ATP-gated cation channels [11, 12, 56]. P2X receptors participate in cardiovascular regulation and disease [15, 30, 47].

Expression of P2X receptors is found in the cervical sympathetic ganglia [1, 20, 27, 28, 30, 38, 54, 58, 59]. Previous works in our laboratory showed that acute myocardial ischemic (MI) injury induced up-expressions of $P2X_3$ and $P2X_{2/3}$ receptors in superior cervical ganglia (SCG) and increased blood pressure and heart rate via sympathoexcitatory reflex [27, 28, 30, 38, 58, 59]. We also found that $P2X_3$ and $P2X_{2/3}$ receptors might be involved in the signal transmission of MI injury since A-317491, an antagonist of these receptors, could inhibit these events [27, 28, 30, 38, 50, 51, 58, 59]. $P2X_7$ receptor plays an important role in functions and diseases of the nervous system [13, 40, 41]. ATP and $P2X_7$ receptor are involved in the responses of injury [3, 8, 9, 11, 18, 41]. $P2X_7$ blockade reduced brain damage after ischemia [3].This study investigated the effects of $P2X_7$ receptor on the increased sympathoexcitatory action in SCG caused by MI injury and examined if there is a specific connection between SCG and the cervical dorsal root ganglion (DRG) afferents which would exaggerate the sympathoexcitatory reflex due to activation of $P2X_7$ receptor during MI injury.

Materials and methods

Animals and myocardial ischemic rat model

Sprague–Dawley rats weighing 180–230 g were used in all experiments. Use of the animals was approved by the Animal Care and Use Committee of Medical College of Nanchang University. Myocardial ischemic rat model was used in our studies [27, 28, 50, 51]. Rats were randomly divided (with six to eight rats in each group) into sham operation group (sham group), control rats treated with the $P2X_7$ receptor antagonist oxATP group (con+oxATP group), myocardial ischemic group (MI group), and oxATP-treated myocardial ischemic rats group (MI+oxATP group).

MI injury was established by ligating the left anterior descending coronary artery [22]. The rats were anesthetized with 10 % chloral hydrate (0.3 ml/100 g). Mechanical ventilation and thoracotomy were undergone in MI rat model. In rats with left coronary artery (LCA) occlusion, a 5–0 suture on a small, curved needle was passed through the myocardium beneath the LCA. Subsequently, both suture ends were passed through a small vinyl tube to make a snare. The suture was pulled tightly against the vinyl tube and ligated to occlude the LCA. After the surgery, the chest was closed and electrocardiogram (ECG) changes were continuously monitored. The relative voltage of negative peak of the S-wave against the QQ line was defined as the ST segment. The abnormal Q wave or ST-segment displacement in a lead II ECG was clearly observed in MI rats. The rats in the sham group were not ligated via LCA, but only probed up the cardiac pericardium 24 h after surgery. Rats in MI+oxATP group and con+oxATP group were treated with oxATP (1 mg/kg day) by intragastric administration (i.g.) for 20 days. Rats in sham group and MI group were given the same volume of normal saline (i.g.) for 20 days. OxATP was dissolved in saline and the final concentration was 0.2 mg/ml.

Measurement of blood pressure, cardiac enzymes, and cytokines

Blood pressure and heart rate were measured with an indirect tail-cuff method (Softron BP-98A, Softron Co, Tokyo, Japan) [28]. Twenty days after myocardial ischemia,the rats were anesthetized with 10 % chloral hydrate. Blood samples were obtained from carotid arterial cannulation. After statically left for 30 min, blood samples were centrifuged at 1,000 rpm for 15 min, and the serum was collected. Serum enzyme levels of lactate dehydrogenase (LDH), creatine kinase (CK), CK isoform MB (CK-MB), and cardiac troponin I (cTn-I) were measured with automatic electrochemiluminescence immunoassay analyzer (Roche COBAS E601, USA) [50].

Serum tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) levels were quantified with enzyme-linked immunosorbent assay (ELISA) by utilizing commercially available antibodies, according to the protocol provided by the supplier (Senxiong company, Shanghai, China). The reactions were recorded with an ELISA reader (Rayto, RT-6000, USA) at 450 nm. The concentrations of TNF-α and IL-6 were determined based on a standard curve [28].

Immunohistochemistry and double-label immunofluorescence

The expression levels of receptors or makers were measured by immunohistochemistry [28, 50, 51]. Co-expression was observed by double-label immunofluorescence [27, 49]. Twenty days after myocardial ischemia,the rats in four groups were anesthetized. Superior cervical ganglia (SCG) and cervical dorsal root ganglia (DRG) were dissected immediately and washed in phosphate-buffered saline (PBS), fixed in 4 % PFA for 24 h at 4 °C, and transferred to 20 % sucrose in 4 % PFA overnight. Tissues were sectioned at 12 μm thick and stored at −20 °C until immunohistochemical processing. The expression levels of $P2X_7$ receptor, $P2X_3$ receptor, glutamine synthetase (GS), neuronal nuclei (NeuN), calcitonin gene-related peptide (CGRP), substance P (SP) or tyrosine hydroxylase (TH) were measured by immunohistochemistry. After washed by PBS for three times, the sections were incubated in 3 % H_2O_2 for 10 min to block the endogenous peroxidase. Following rinses in PBS, sections were incubated with 5 % BSA for 60 min, then the sections were incubated with rabbit anti-$P2X_7$ (1:100; Alomone Labs, Jerusalem, Israel) [8], rabbit anti-$P2X_3$ (1:2,500, Millipore International, Inc, USA), mouse anti- GS (1:100 dilution; Abcam Inc., USA), mouse anti-NeuN (1:200 dilution, Millipore International, Inc, USA), rabbit monoclonal anti-CGRP (1:500 dilution, bioss, CO), rabbit monoclonal anti-SP (1:500 dilution, bioss, CO) or mouse anti-TH (1:1,000 dilution; Abcam International, Inc. USA) diluted in PBS for overnight at 4 °C. Sections were rinsed and incubated with biotinylated goat anti-rabbit secondary antibody or goat anti-mouse secondary antibody (1:100 dilution; Beijing Zhongshan Biotech. CO.) for 1 h at room temperature. The sections were washed in PBS and added streptavidin-horseradish peroxidase (HRP; Beijing Zhongshan Biotech. CO.) for 30 min. After development of the diaminobenzidine chromogen for 2 min, the slides were washed with distilled water and cover-slipped. Image scanning analysis system (Image-Pro Plus) was used to analyze the changes in integrated optical density (IOD) of $P2X_7$, $P2X_3$, GS, NeuN, CGRP, SP and TH. In the double-label immunofluorescence, the differences are those we used 3 % Triton-X100 (Beijing Zhongshan Biotech. CO.) instead of 3 % H_2O_2 and the sections were incubated with mixed primary antibodies. Fluorescein- or rhodamine-conjugated goat anti-rabbit and goat anti-mouse secondary antibodies (1:100 dilution; Beijing Zhongshan Biotech. CO.) were also used for 1 h at room temperature. Other sections for "ABC staining" were incubated with biotinylated secondary antibodies (Beijing Zhongshan Biotech. CO.) for 45 min at room temperature, processed with avidin-coupled horseradish peroxidase, and developed with diamino-benzidine. The sections of double-label immunofluorescence were incubated with fluorescent goat anti-rabbit FITC and goat anti-mouse TRITC secondary antibody (1:100 dilution; Beijing Zhongshan Biotech. CO.). Stained slides were mounted, coverslipped, and examined under Olympus microscope (Olympus TH4-200, Japan). The changes of IOD for ganglia were analyzed by Image Pro-Plus software. To verify the specificity of immunoreactivity of primary antibodies, 10 % normal goat serum in PBS was substituted for the primary antibody as a negative control.

Retrograde tracing labeling of horseradish peroxidase

Horseradish peroxidase (HRP) is a retrograde tracer. Retrograde tracing of HRP [31] in the cardiac afferent endings was used to observe the retrograde neuronal labeling from the cardiac afferent endings to SCG. The rats were anesthetized with 10 % chloral hydrate (0.3 ml/100 g). Mechanical ventilation and thoracotomy were undergone in the experimental rats. HRP (15 μl, diluted in 0.1 M PBS, 10 mg/ml) was injected into cardiac apex and conus arteriosus at five to seven points by a microinjector. Penicillin (2×10^5 U) was injected in the muscle of experimental rats. After 7 days, the SCG was isolated to carry out the test of labelled immunofluorescence.

HRP in SCG was used to observe the retrograde neuronal labeling from the cervical DRG (C1-T2) to SCG. The rats were anesthetized and cervical skin was cut open. Left SCG was softly fixed. HRP (15 μl, diluted in 0.1 M PBS, 10 mg/ml) was injected into SCG by a microinjector. Penicillin (2×10^5 U) was injected in the muscle of experimental rats. After 7 days, the cervical DRG (C1-T2) were isolated for the examination of labelled immunofluorescence.

SCG and DRG were isolated from the experimental rats. Mouse anti-HRP antibody (1:100, dilute with 0.1 M PBS) was mixed with the secondary antibody (1:200 Rhodamine (or TRITC)-conjugated AffiniPure Goat anti- mouse lgG) to carry out the double-label immunofluorescence test.

In situ hybridization

The mRNA expression was assessed by in situ hybridization (ISH) [28, 50]. The rats of four groups were anaesthetized. The ganglia were dissected immediately and fixed in 4 % paraformaldehyde (PFA) for 24 h at 4 °C, and then transferred to 20 % sucrose in 4 % PFA overnight. Tissues were sectioned at 12 μm thick and stored in refrigerator at −20 °C.

Diethyl pyrocarbonate water was used for all solutions and appliances necessary for ISH. Sections were treated with 0.05 % H_2O_2, followed by digestion with pepsin at 37 °C for 1–2 min, terminated with 0.5 mol/L phosphate-buffered saline (PBS), and washed for 15 min. The sections were incubated in prehybridization for 4–6 h at 37 °C and then in hybridization overnight at 37 °C. The in situ hybridization kit for $P2X_7$ receptors (Wuhan Boster Co.) was used. $P2X_7$ mRNA probe sequences are (1)5′-AATTA CGGCA

CCATC AAGTG GATCT TGCAC ATGAC-3′; (2)5′-TACTG GGACT GCAAC CTGGA CAGCT GGTCC CATCG-3′; (3)5′-TTTGT GGACG AGCCC CACAT TTGGA TGGTG GACCA-3′. The sections were washed with gradient SSC (2× SSC 17.6 g sodium chloride, 8.8 g sodium citrate in 1,000 ml distilled water) thoroughly, 2× SSC for 10 min, 0.5× SSC for 15 min, and 0.2× SSC for 15 min to remove the background signals and followed by treatment of biotinylated digoxim antibody at 37 °C for 2 h. After strongly washed with PBS, the sections were incubated with SABC-POD for 30 min and with biotinylated peroxidase (Beijing Zhongshan Biotech. CO.) for 30 min at 37 °C. The sections were developed in DAB substrate (Beijing Zhongshan Biotech. CO.) then dehydrated and mounted with neutral gum.

Western blotting

The protein expression was determined by Western blotting [28, 51]. The rats were anesthetized with 10 % chloral hydrate (0.3 ml/100 g) and sacrificed. The ganglia were isolated and flushed with ice-cold phosphate-buffered saline (PBS). Ganglia were homogenized by mechanical disruption in lysis buffer (50 mmol/L TrisCl, pH 8.0, 150 mmol/L NaCl, 0.1 % dodecyl sodium sulfate, 1 % Nonidet P-40, 0.5 % sodium deoxycholate, 100 μg/mL phenylmethylsulfonyl fluoride, and 1 μg/mL Aprotinin) and incubated on ice for 40 min. Homogenate was then centrifuged at 12,000 rpm for 10 min, and supernatant was collected. Using Lowry method, the quantity of total protein was determined in the supernatant. After diluted with sample buffer (100 mmol/L TrisCl, 200 mmol/L dithiothreitol, 4 % sodium deodecylsulfate (SDS), 0.2 % Bromophenol Blue, 20 % glycerol) and heated to 95 °C for 5 min, samples containing equal amounts of protein (20 μg) were separated by SDS-polyacrylamide gel electrophoresis by using Bio-Rad system and 10 % gel. In the wake of electrophoretic transfer onto a PVDF membrane using the same system, the membrane was blocked with 5 % non-fat dry milk in 25 mmol/L Tris-buffered saline, pH 7.2, plus 0.1 % Tween 20 (TBST) for 3 h at room temperature, followed by incubation with primary antisera (rabbit anti-$P2X_7$ 1:200; Alomone Labs, Jerusalem, Israel) in 5 % non-fat dry milk for overnight at 4 °C. Recovering to room temperature on the second day, the membrane was washed in TBST, and incubated with the secondary antibody, horseradish peroxidase (HRP)-conjugated goat anti- rabbit IgG (1:1,000, Beijing Zhongshan Biotech. CO.) in 5 % non-fat dry milk for 1 h at room temperature. After a final wash in TBST and then using the enhanced chemiluminescence kit (Shanghai Pufei Biotech. CO.), chemiluminescent signals were collected on autoradiography film. The quantity of band intensity was determined using AlphaImager 2200 software. The primary antibodies and dilutions used were as follows: rabbit polyclonal anti-$P2X_7$ (1:200; Alomone Labs, Jerusalem, Israel) and monoclonal β-actin (1:1,000; Advanced Immunochemicals, VB Long Beach, CA). Band densities were normalized to each β-actin internal control.

The expression of ERK1/2 and p-ERK1/2 protein was examined by the same protocol used for $P2X_7$ protein. The primary antibodies and dilutions used were as follows: rabbit polyclonal anti-phospho-p44/42 MAPK (ERK1/2, 1:1,000, Cell Signal CO.) and rabbit polyclonal anti-p44/42 MAPK (ERK1/2, 1:1000, Cell Signal CO.)

$P2X_7$ receptor small interference RNA treatment

The small interference RNA (siRNA) specific for rat $P2X_7$ was purchased from Invitrogen (Carlsbad, CA). siRNA

Table 1 Effects of oxATP or knockdown $P2X_7$ on systolic blood pressure, diastolic blood pressure, heart rate

Group	Systolic blood pressure (mmHg)	Diastolic blood pressure (mmHg)	Heart rate (/min)
A			
Sham (*n*=8)	110.04±9.53	72.35±7.35	341.88±35.43
con+oxATP (*n*=8)	108. 65±10.72	73.43±8.56	334.70±36.90
MI (*n*=8)	128.00±13.54*	91.06±9.14*	426.72±44.21*
MI+oxATP (*n*=8)	111.16±10.87	73.65±9.87	398.72±39.81**
B			
Sham (*n*=8)	110.17±10.56	76.66±7.14	352.24±21.38
MI (*n*=8)	133.40±8.37***	97.89±8.23***	438.42±30.53***
MI+$P2X_7$ siRNA (*n*=8)	111.16±7.62	77.27±6.98	349.78±25.74
MI+Scramble siRNA (*n*=8)	131.93±10.65***	100.81±7.35***	428.20±28.89***

Data are presented as means ± SE

*$p<0.05$, compared with sham group, oxATP control group, and myocardial ischemic rats treated with oxATP group; **$p<0.05$, compared with sham and oxATP control group; ***$p<0.05$, compared with sham group and myocardial ischemic rats treated with $P2X_7$ siRNA group.

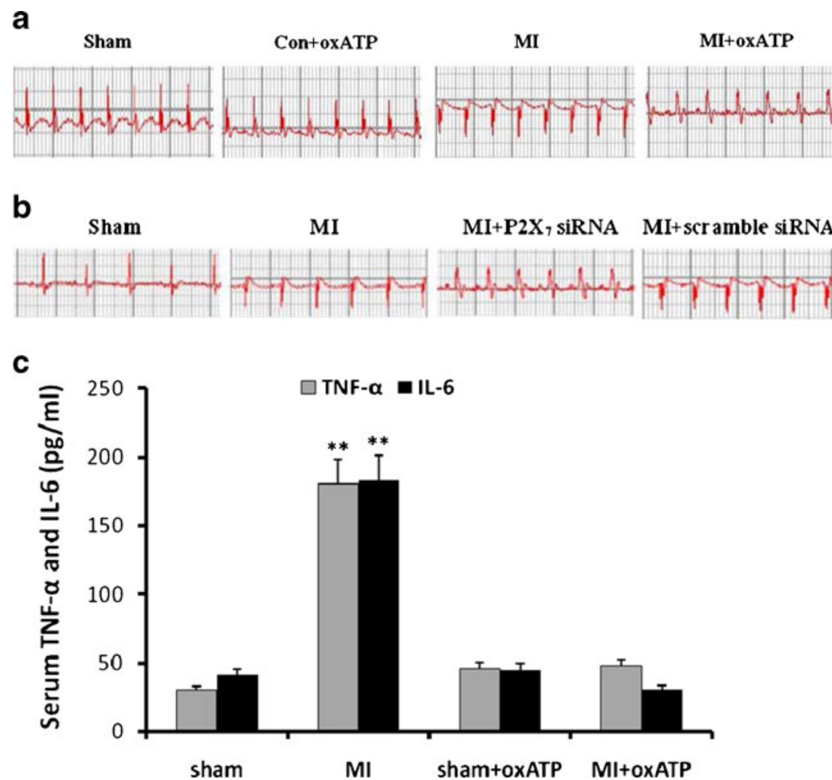


Fig. 1 Representative traces of ECG in the different groups of rats. Representative traces of ECG were measured 20 days after myocardial ischemia. **a** The abnormal Q wave appeared obviously in MI rats (n=8) compared with sham (n=8) and con+oxATP rats (n=8). In MI+oxATP group (n=8), abnormal Q wave induced by MI injury was improved. **b** After MI rats treated with siRNA $P2X_7$, abnormal Q wave induced by MI injury was improved in comparison with that in MI and MI+scramble siRNA rats. **c** The serum concentration of TNF-α and IL-6 was measured by ELISA. The concentrations of TNF-α and IL-6 in MI group were higher than those in sham group, con+oxATP group, MI+oxATP group (p<0.01). No significant difference was found in the concentration of TNF-α or IL-6 among sham group, con+oxATP group, and MI+oxATP group (p>0.05). The n value is 8 in all groups. Results are mean ± SE. **p<0.05 vs sham group, con+oxATP group, and MI+oxATP group

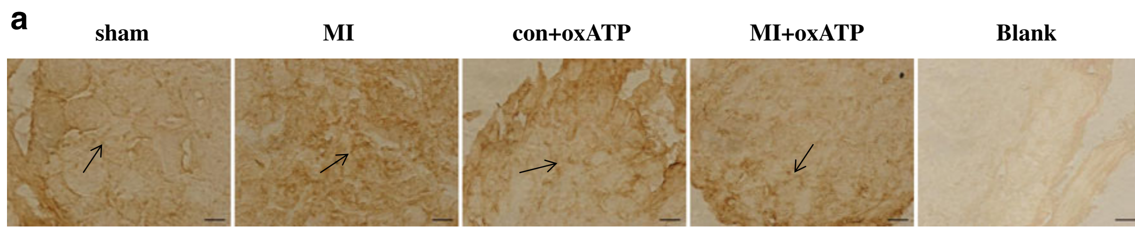
oligonucleotides targeted specifically to rat $P2X_7$ were used in this experiment [35]. $P2X_7$-receptor knockdown was achieved by RNA interference (RNAi) using an Entranster™ in vivo Transfection Reagent [57]. The siRNA was diluted with 80 μL RNA-free water, then added 80 μL 10 % glucose, we got the mixture A. Mixture B was obtained by mixing 80 μL Transfection Reagent and 80 μL 10 % glucose. B was added into A immediately,

Table 2 Effects of oxATP or knockdown $P2X_7$ on the serum concentration of CK-MB, CK, LDH, and cTn-I

Group	CK-MB (u/l)	CK (u/l)	LDH (u/l)	cTn-I (ng/ml)
A				
Sham (n=8)	57.34±5.843	533.65±52.12	321.85±31.24	0.058±0.005
con+oxATP (n=8)	31.48±3.98	400.35±40.65	234.90±20.96	0.048±0.005
MI (n=8)	82.20±8.76*	2231.95±135.04*	936.85±91.49*	0.186±0.009*
MI+oxATP (n=8)	68.10±7.66**	874.25±91.60**	487.80±50.76**	0.084±0.007**
B				
Sham (n=8)	32.80±2.53	559.57±55.36	311.43±37.50	0.051±0. 005
MI (n=8)	87.50±8.45***	2252.44±220.50***	889.16±88.34***	0.185±0.02***
MI+$P2X_7$ siRNA (n=8)	35.11±4.21	547.43±55.89	340.24±35.54	0.049±0.005
MI+Scramble siRNA (n=8)	85.45±6.35***	2103.47±217.48***	823.89±82.58***	0.163±0.01***

Data are presented as means ± SE

*p<0.05, compared with sham group, oxATP control group and myocardial ischemic rats treated with oxATP group; **p<0.05, compared with sham and oxATP control group; ***p<0.05, compared with sham group and myocardial ischemic rats treated with $P2X_7$ siRNA group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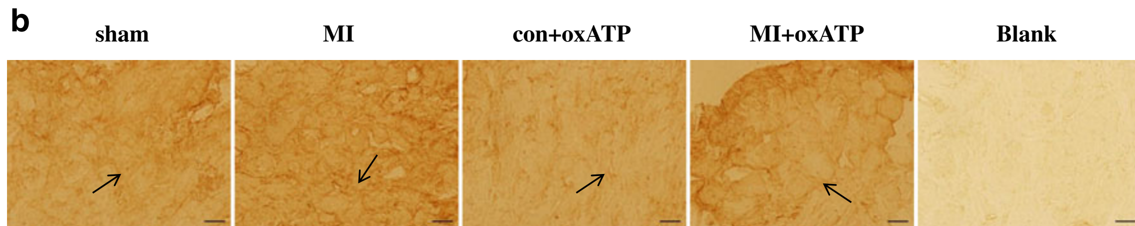
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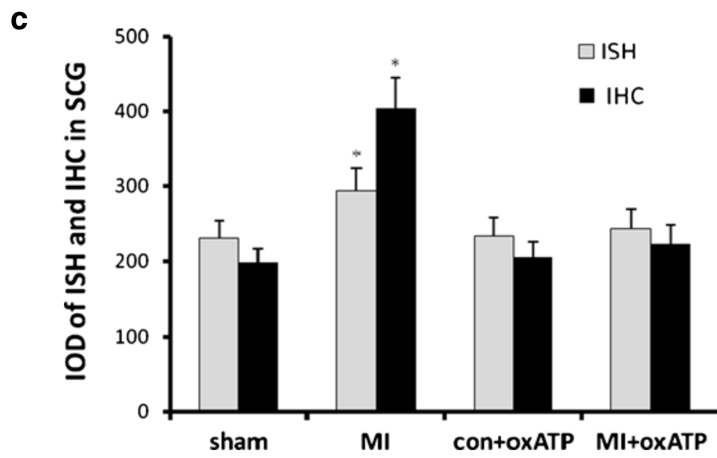
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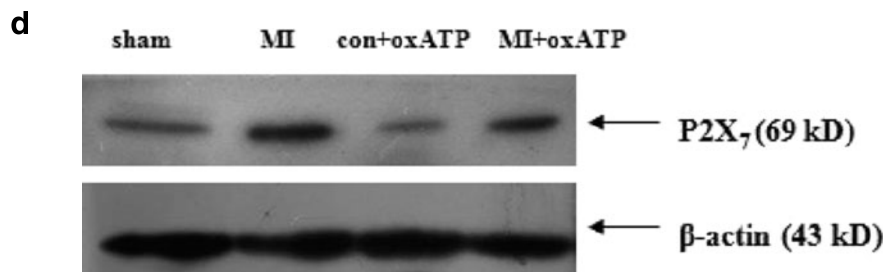
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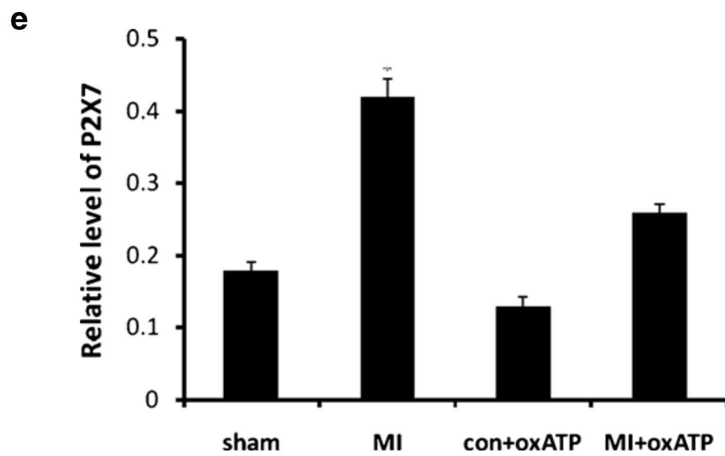
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MI+oxATP

◀ **Fig. 2** Effects of oxATP on the increased $P2X_7$ expression induced by ischemic injury. The expressions of $P2X_7$ mRNA and protein were measured by in situ hybridization (**a**), immunohistochemistry (**b**), and Western blotting (**d**). The bar graphs (**c**, **e**) showed the statistical results for expression of $P2X_7$ mRNA or protein. The relative expression in Western blotting was expressed by the IOD ratio of $P2X_7$ protein to β-actin. The results showed that the expression levels of $P2X_7$ mRNA or protein in MI group ($n=8$) were significantly higher than those in sham group ($n=8$), con+oxATP group ($n=8$), and MI+oxATP group ($n=8$; $p<0.05$). No difference was found among sham group, con+oxATP group, and MI+oxATP group ($p>0.05$). *Arrows* indicate the immunostaining neurons. *Scale bars*, 20 μm. Results are mean ± SE. *$p<0.05$ vs sham group, con+oxATP group, and MI+oxATP group

and 15 min later, the 320-μL siRNA solution was injected into the sublingual vein. The siRNA target sequence 5′-GUGCAGUGAAUGAGUACUATT-3′ (Invitrogen) was selected for the $P2X_7$ receptor. Rats were assigned in a random blind fashion to one of four groups, as follows: sham operation group (sham group), myocardial ischemic group (MI group), $P2X_7$ siRNA vector-treated myocardial ischemic rats group (MI+$P2X_7$ siRNA group), and MI rats treated with scramble siRNA vector group (MI+scramble siRNA group).

Data analysis

Statistical analyses of the data were performed using SPSS 11.5. All results were expressed as mean ± SE. Statistical significance was determined by one-way analysis of variance followed by the Fisher post hoc test for multiple comparisons. $p<0.05$ was considered as significant difference.

Results

Effects of oxATP or knockdown $P2X_7$ on blood pressure, heart rate, and electrocardiogram

After 20 days of myocardial ischemia, systolic blood pressure and heart rate in the MI group (MI; $n=8$) were increased in comparison with those in sham group and control rats treated with oxATP (con+oxATP; $n=8$, respectively; $p<0.05$). After the treatment with $P2X_7$ antagonist oxATP in the MI rats (MI+oxATP) ($n=8$), systolic blood pressure and heart rate were lower than those in MI rats ($p<0.05$; A of Table 1). Heart rate in MI+oxATP group was still higher than con+oxATP group and sham group ($n=8$; $p<0.05$). There was no significant difference between con+oxATP group and sham group ($p>0.05$; A of Table 1). After ligating the left anterior descending coronary artery, ST segment in the ECG was highly upward. After 20 days of myocardial ischemia, the abnormal Q wave appeared obviously in MI rats compared with that in sham or oxATP-treated control rats. After MI rats treated with oxATP, abnormal Q wave induced by myocardial ischemia was improved in comparison with that in MI group (Fig. 1a).

In order to validate $P2X_7$ inhibition in SCG after the myocardial ischemic injury, siRNA $P2X_7$ receptor in vivo was used. After the treatment with siRNA $P2X_7$ in myocardial ischemic rats (MI+siRNA $P2X_7$), systolic blood pressure and heart rate were lower than those in MI rats and MI+scramble siRNA group ($n=8$, respectively; $p<0.05$). There was no significant difference between sham group and MI+$P2X_7$ siRNA group ($n=8$, respectively; $p>0.05$; B of Table 1). After MI rats treated with siRNA $P2X_7$, abnormal Q wave induced by myocardial ischemia was improved in comparison with that in MI group and MI+scramble siRNA group (Fig. 1b).

Effects of oxATP on serum TNF-α and IL-6

The serum levels of TNF-α and IL-6 were measured by ELISA kit. Both TNF-α and IL-6 were higher in MI group than in sham group, con+oxATP group, or MI+oxATP group ($n=6$; $p<0.01$). There were no significant differences among sham group, con+oxATP group, and MI+oxATP group ($n=6$; $p>0.05$; Fig. 1c).

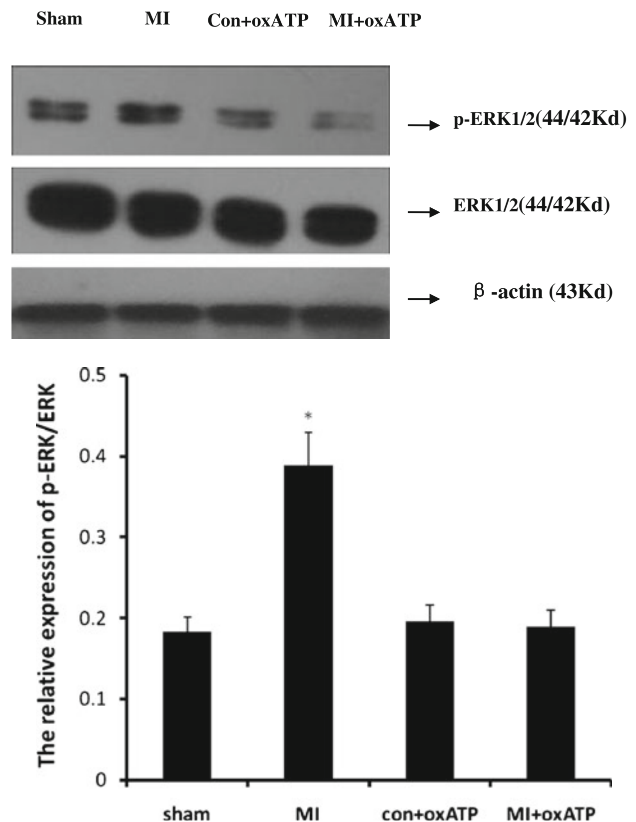
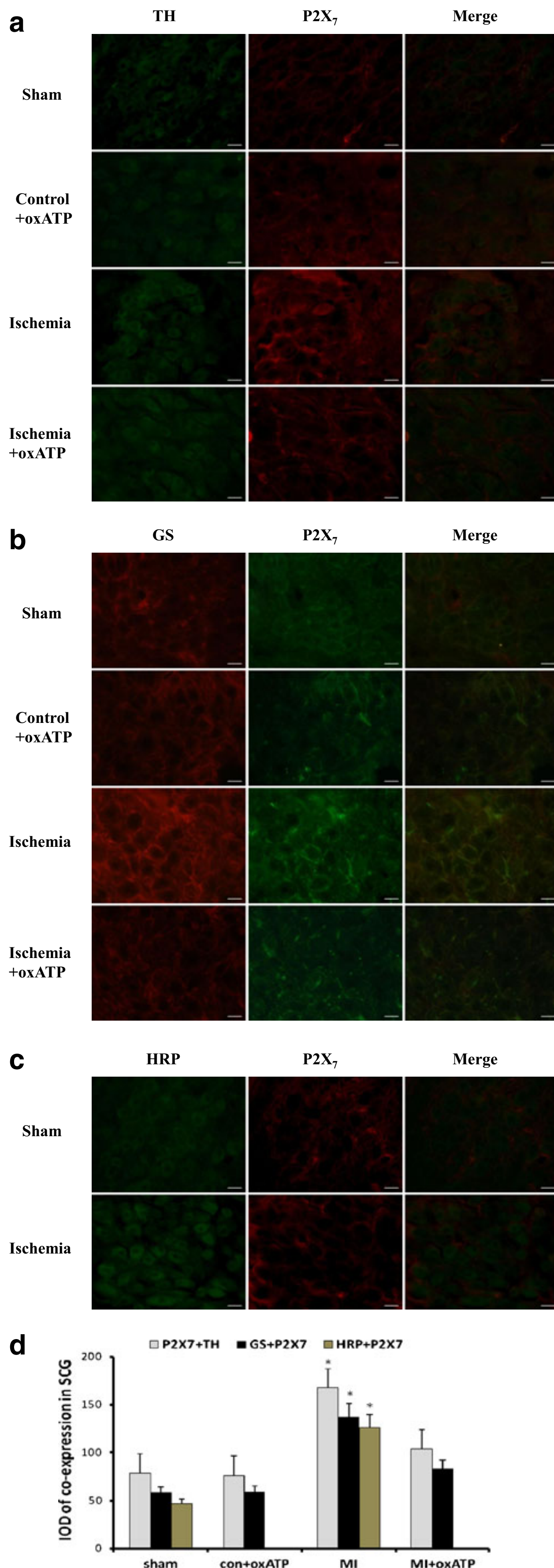


Fig. 3 The expression of ERK1/2 and p-ERK1/2 protein in SCG. The IOD ratio of p-ERK1/2 to total ERK1/2 in SCG in MI group ($n=8$) was higher than that in sham group ($n=8$), con+oxATP group ($n=8$), and MI+oxATP group ($n=8$; $p<0.05$). No difference was found among sham group, con+oxATP group, and MI+oxATP group ($p>0.05$). There was no difference in ERK1/2 protein among the four groups ($p>0.05$). Results are mean ± SE. *$p<0.05$ vs sham group, con+oxATP group, and MI+oxATP group



◀ **Fig. 4** The double-label immunofluorescence of $P2X_7$ and TH, GS or HRP in SCG. **a** There was the coexpression of $P2X_7$ and TH in SCG. *Red* signal represents $P2X_7$ staining with TRITC, and *green* signal indicates TH staining with FITC. *Merge* represents $P2X_7$ and TH double staining image. *Scale bar*, 20 μm. **b** GS is the typical feature of satellite glial cells (SGCs) in sympathetic ganglia. $P2X_7$ receptor was co-expressed with GS, so it was mainly expressed in SGCs. *Red* signal represents $P2X_7$ staining with TRITC, and *green* signal indicates GS staining with FITC. *Merge* represents $P2X_7$ and GS double staining image. *Scale bar*, 20 μm. **c** There was double-label immunofluorescence of $P2X_7$ and HRP from the cardiac afferent endings to the SCG, as assessed by the retrograde tracing neuronal labeling using HRP as a retrograde tracing marker. *Red* signal represents $P2X_7$ staining with TRITC, and *green* signal indicates HRP staining with FITC. *Merge* represents $P2X_7$ and HRP double staining image. *Scale bar*, 20 μm. **d** The bar graphs showed the statistical results for co-expression of $P2X_7$ and TH, GS or HRP in SCG (n=6 in all groups)

Effects of oxATP or knockdown $P2X_7$ on serum myocardial enzymes

MI injury induced elevations in serum cardiac enzymes. CK-MB, CK, LDH, and cTn-I in the blood serum were markers for the ischemic injury of myocardial tissue. After 20 days of MI, serum concentrations of CK-MB, CK, LDH, and cTn-I in the MI rats were significantly increased compared with those in the sham or con+oxATP rats ($p<0.05$). $P2X_7$ receptor antagonist oxATP significantly attenuated the elevated serum concentrations of these enzymes due to myocardial ischemia, indicative of reduced myocardial injury. Serum concentrations of CK-MB, CK, LDH, and cTn-I in MI+oxATP group were higher than those in con+oxATP group or sham group ($p<0.05$; A of Table 2). After MI rats treated with siRNA $P2X_7$, serum concentrations of CK-MB, CK, LDH, and cTn-I were decreased in comparison with that in MI group and MI+scramble siRNA group ($p<0.05$; B of Table 2).

Effects of oxATP on the expressions of $P2X_7$ mRNA and protein in SCG

The expressions of $P2X_7$ mRNA and protein in SCG were assessed by ISH (Fig. 2a), immunohistochemistry (Fig. 2b), and Western blotting (Fig. 2d). Higher expression of $P2X_7$ mRNA and protein occurred in MI group in comparison with sham group, con+oxATP group, and MI+oxATP group (n=8; $p<0.05$; Fig. 2a–e). The bar graphs (Fig. 2c, e) showed the result of ISH, IHC, and Western blotting. The relative expression was the IOD ratio of $P2X_7$ to β-actin. The results showed that the expression of $P2X_7$ receptor in MI group was significantly higher than that in sham group, con+oxATP, and MI+oxATP ($p<0.05$). No significant difference was found among sham group, con+oxATP group, and MI+oxATP group (n=8; $p>0.05$; Fig. 2).

Effects of oxATP on the levels of ERK1/2 and p-ERK1/2 in SCG

Phosphorylation and activation of ERK1/2 is involved in the activation of inflammatory mediators. The expressions of ERK1/2 and p-ERK1/2 in SCG were analyzed by Western blotting. The IOD ratio of ERK1/2 to β-actin was not significantly different among four groups ($p>0.05$). But the IOD ratio of p-ERK1/2 to total ERK1/2 in MI group was higher than that in sham group ($n=8$). In addition, oxATP attenuated nearly 50 % of the upregulated expression due to MI (Fig. 3).

Double-label immunofluorescence of $P2X_7$ and TH, GS, or HRP in SCG

The double-label immunofluorescence of $P2X_7$ and TH in SCG was detected with fluorescence immunohistochemistry in four groups after 20 days of myocardial ischemia (Fig. 4a). $P2X_7$ and TH were co-expressed in SCG. The double-label immunofluorescence of $P2X_7$ and TH in MI group exhibited higher density than that in sham group, con+oxATP group, and MI+oxATP group ($n=6$, respectively; $p<0.05$; Fig. 4a, d). There was no significant difference among con+oxATP group, sham group, and MI+oxATP group ($n=6$, respectively; $p>0.05$; Fig. 4a, d).

The main type of glial cells in most sympathetic ganglia is the satellite glial cells (SGCs). SGCs usually form envelopes around individual ganglionic neurons. The presence of glutamine synthetase (GS) is the typical feature of glial cells. The coexpression of GS and $P2X_7$ receptor in SCG was measured by double-label immunofluorescence (Fig. 4b). After 20 days of myocardial ischemia, the staining of GS and $P2X_7$ by double-label immunofluorescence in MI group was more intense than that in con+ oxATP group, sham group, and MI+oxATP group ($n=6$, respectively; $p<0.05$; Fig. 4b, d). There was no significant difference among sham group, con+oxATP group, and MI+oxATP group ($n=6$, respectively; $p>0.05$; Fig. 4b, d). Horseradish peroxidase (HRP) was used as a retrograde tracing marker to observe the retrograde neuronal labeling from the cardiac afferent endings to SCG. Two groups of rats, sham control and myocardial ischemia, were studied in these experiments. In MI group, HRP was injected into cardiac apex and conus arteriosus at 13 days after MI operation. The double immunofluorescence labelling of HRP and $P2X_7$ in the SCG of sham group was observed 7 days later, which displayed barely staining. By contrast, the staining of $P2X_7$ and HRP by double immunofluorescence labelling in MI group was more intense ($n=6$, respectively; $p<0.05$; Fig. 4c, d), suggesting the neuronal labeling of $P2X_7$ receptor from the cardiac afferent endings to SCG.

Effects of knockdown $P2X_7$ on the expression or co-expression of $P2X_7$ or/and GS in SCG

In order to validate $P2X_7$ inhibition interrupting the formation of sensory–sympathetic coupling in SCG after the myocardial ischemic injury, we adopted a second approach based on the expression of siRNA $P2X_7$ receptor. The expressions of $P2X_7$ or GS were assessed by immunohistochemistry (Fig. 5a, b). After siRNA $P2X_7$ in myocardial ischemic rats, the expression levels of GS or $P2X_7$ were significantly lower than those in MI group ($n=6$, respectively; $p<0.05$). There was no significant difference among sham group, MI+$P2X_7$ siRNA group, and MI+scramble siRNA group ($n=6$, respectively; $p>0.05$; Fig. 5c).

The coexpression of GS and $P2X_7$ receptor in SCG was measured by double-label immunofluorescence (Fig. 5d). After siRNA $P2X_7$ in myocardial ischemic rats, the coexpression staining of GS and $P2X_7$ was significantly lower than that in MI group ($n=6$, respectively; $p<0.05$). There was no significant difference among sham group, con+scramble siRNA group, and MI+$P2X_7$ siRNA group ($n=6$, respectively; $p>0.05$; Fig. 5e). The results further revealed that $P2X_7$ receptor was involved in myocardial ischemic injurious response.

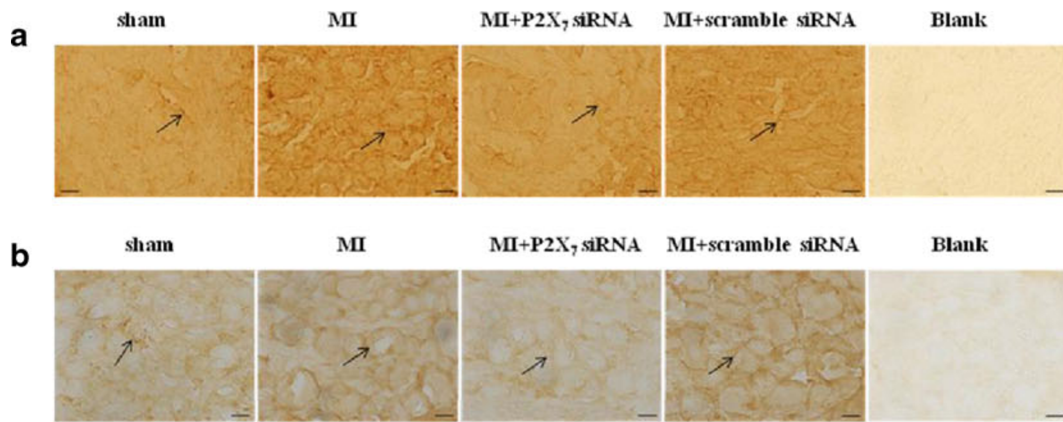
Double immunofluorescence labelling of $P2X_3$ and NeuN or HRP in SCG

NeuN is the marker of neurons. The presence of NeuN immunoreactivity is the typical feature of sympathetic neurons. After 20 days of myocardial ischemia, the staining of NeuN and $P2X_3$ by double-label immunofluorescence in SCG of MI group was more intense than that in sham group, con+oxATP group, and MI+oxATP group($n=6$, respectively; $p<0.05$; Fig. 6a, c). There was no significant difference among the latter three groups ($n=6$, respectively; $p>0.05$; Fig. 6a, c). The results indicated that $P2X_3$ receptor was mainly expressed in SCG neurons.

The retrograde neuronal labeling from the cardiac afferent endings to SCG was also performed after injection of HRP as above. A little staining of $P2X_3$ and HRP was observed in the SCG of sham group by double-label immunofluorescence. The staining of $P2X_3$ and HRP in MI group was more intense than that in sham group ($n=6$, respectively; $p<0.05$; Fig. 6b, c), indicating the presence of $P2X_3$ from the cardiac afferent endings to SCG.

Double-label immunofluorescence of HRP and $P2X_7$, $P2X_3$, CGRP, or SP in DRG

HRP was used to observe the retrograde neuronal labeling from the superior cervical ganglia (SCG) to the cervical dorsal root ganglia (DRG). In MI group, HRP was injected


a
sham
MI
MI+P2X$_7$ siRNA
MI+scramble siRNA
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b
sham
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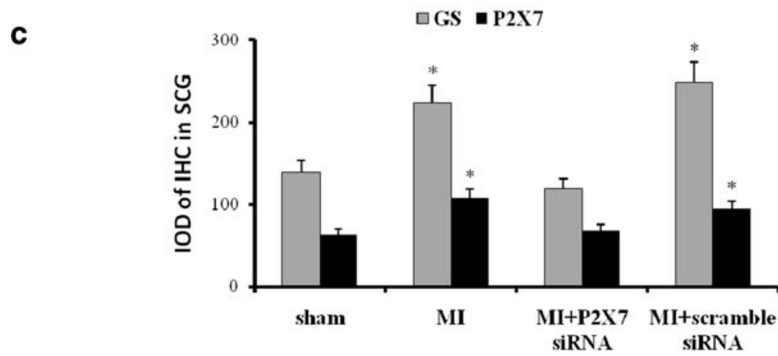
c
GS
P2X7
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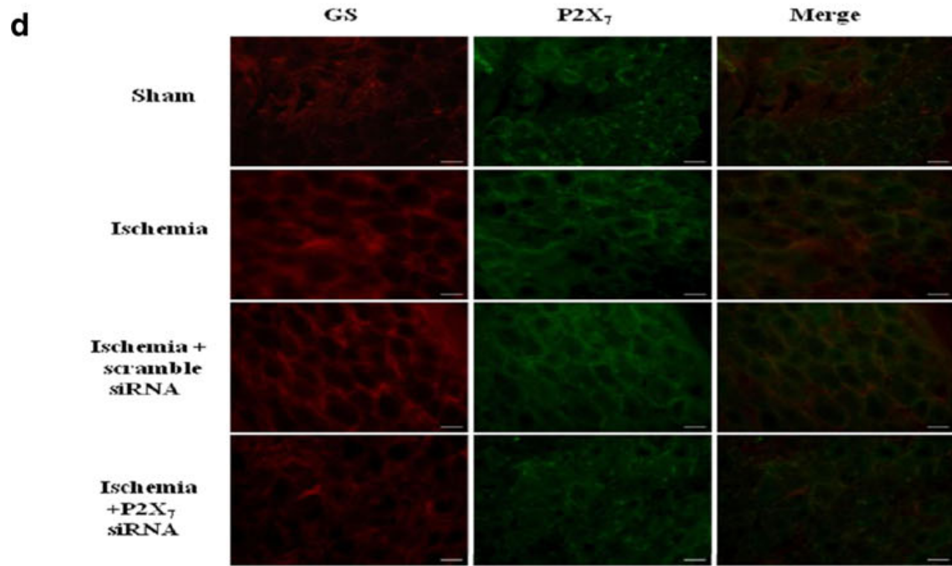
d
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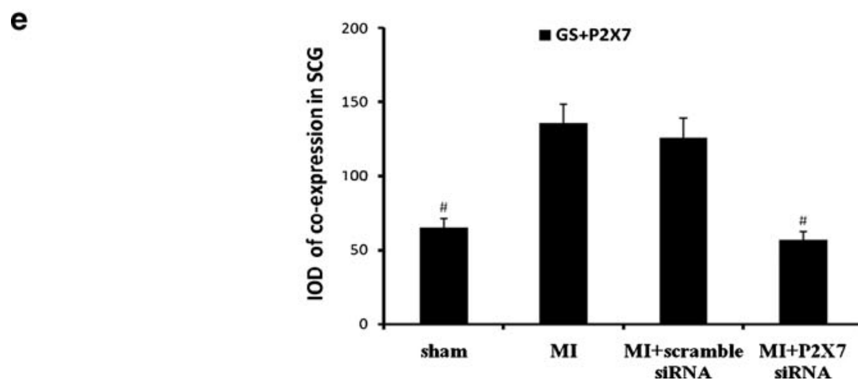
e
GS+P2X7
IOD of co-expression in SCG
200
150
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MI+P2X7 siRNA

◀ **Fig. 5** Effects of siRNA $P2X_7$ on the expression or co-expression of $P2X_7$ or/and GS in SCG. The expressions of $P2X_7$ (**a**) or GS (**b**) were assessed by immunohistochemistry. After siRNA $P2X_7$ in myocardial ischemic rats, the expression levels of GS or $P2X_7$ were significantly lower than those in MI group and MI+scramble siRNA group (n=6, respectively; p<0.05). There was no significant difference among sham group and MI+$P2X_7$ siRNA group (n=6, respectively; p>0.05). *Arrows* indicate the immunostaining neurons. *Scale bars*, 20 μm. The bar graphs (**c**) showed the statistical results for expression of GS or $P2X_7$ immunoactivity. Results are mean ± SE. *p<0.05 vs sham group, con+oxATP group, and MI+oxATP group. The co-expression of GS and $P2X_7$ receptor in SCG was measured by double-label immunofluorescence after siRNA $P2X_7$ receptor (**d**). After siRNA $P2X_7$ in myocardial ischemic rats, the co-expression staining of GS and $P2X_7$ was significantly lower than that in MI group and MI+scramble siRNA group (n=6, respectively; p<0.05). There was no significant difference among sham group and MI+$P2X_7$ siRNA group (n=6, respectively; p>0.05). The results further revealed that $P2X_7$ receptor was involved in myocardial ischemic injury. *Red* signal represents GS staining with TRITC, and *green* signal indicates $P2X_7$ staining with FITC. *Merge* represents GS and $P2X_7$ double staining image. *Scale bar*, 20 μm. The bar graphs showed the statistical results for co-expression of GS and $P2X_7$ in SCG (**e**)

into SCG at 13 days after MI operation. HRP was injected into SCG for 7 days in sham group.

The double-label immunofluorescence of HRP and $P2X_7$ in the DRG was observed. The staining of HRP and $P2X_7$ in the DRG of sham group was lower than that in MI group (n=6, respectively; p<0.05; Fig. 7a, e). In addition, double-label immunofluorescence of HRP and $P2X_3$ in the DRG of sham group was also lower than that in MI group (n=6, respectively; p<0.05; Fig. 7b, e). Likewise, the staining of double-label immunofluorescence of HRP and CGRP (Fig. 7c, e) or HRP and SP (Fig. 7d, e) in the DRG of sham group was lower than that in MI group (n=6, respectively; p<0.05). These double-label immunofluorescence results indicated that there was the retrograde neuronal labeling from DRG to SCG.

Effects of oxATP on the expression of GS, CGRP, or SP immunoreactivity in SCG

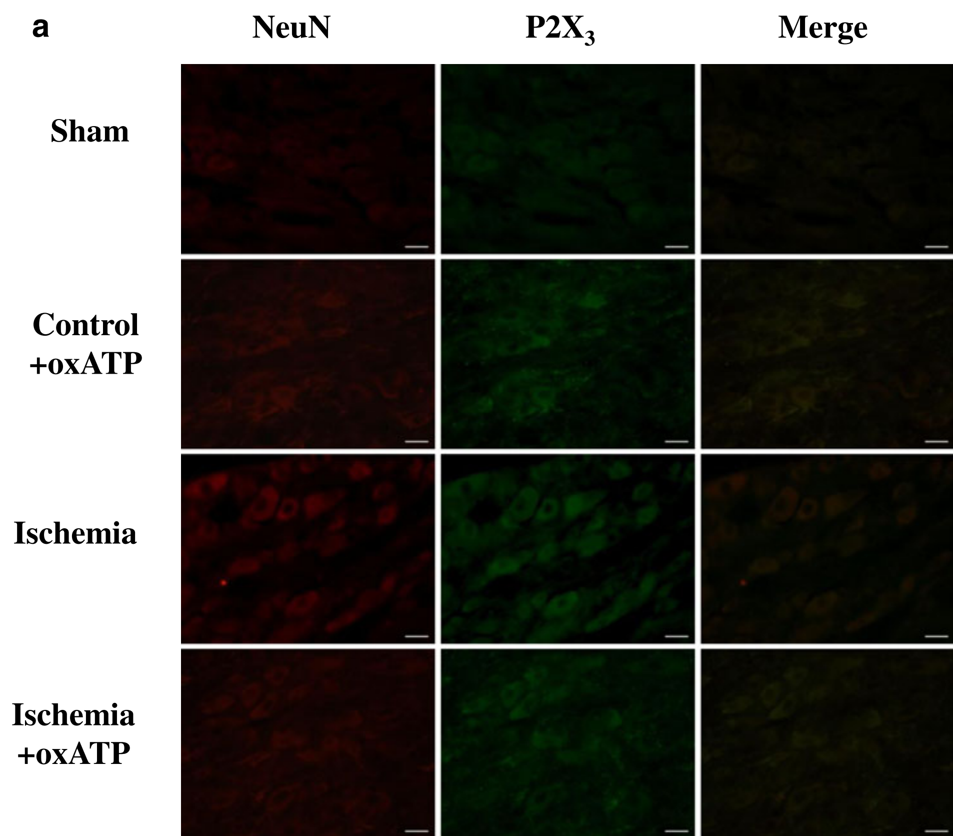
Glutamine synthetase (GS) is the marker of glial cells. Its expression in SCG was examined by immunohistochemistry. The integrated optical density of GS immunoreactivity in sham group, con+oxATP group, MI group and MI+oxATP group was observed (n=8, respectively) (Fig 8a). The levels of GS immunoreactivity in MI group were higher than those in sham group and con+oxATP group (n=8) (p<0.05) (Fig. 8a, d). oxATP treatment could completely reverse the up-regulation of GS due to MI. There was no significant difference among sham group, con+oxATP group and MI+oxATP group (n=8) (p>0.05) (Fig. 8a, d).

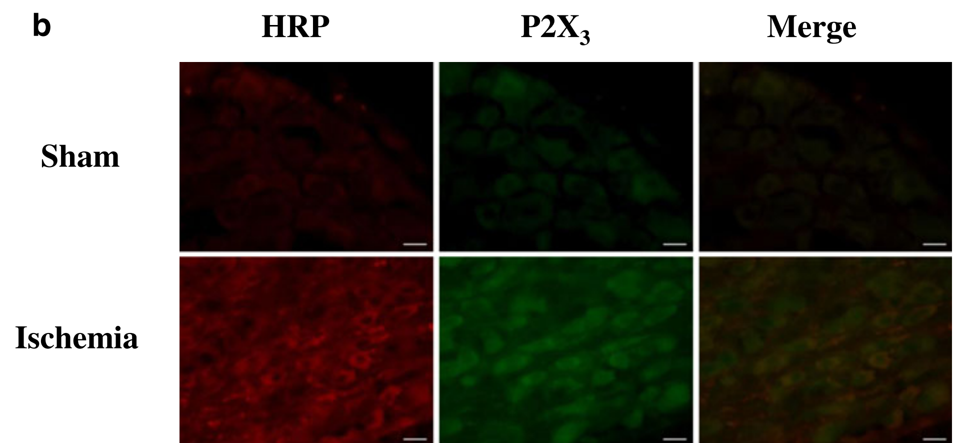
Calcitonin gene-related peptide (CGRP) and substance P (SP) are the neurochemical markers of primary sensory afferent fibers. The integrated optical density CGRP immunoreactive in sham group, oxATP control group, MI group, and MI+oxATP group were observed by immunohistochemistry (n=8, respectively) (Fig 8b, c). MI group exhibited higher CGRP or SP immunoreactivity than sham group and con+oxATP group (n=8; p<0.05; Fig. 8b–d). oxATP treatment could abolish MI-induced upregulation of CGRP and SP. No significant difference was observed among sham group, con+oxATP group, and MI+oxATP group (n=8; p>0.05; Fig. 8b–d).

Discussion

Efferent nerves of cervical sympathetic ganglia control the function of heart and blood vessels [34]. The sympathetic ganglia do not simply relay the pre-ganglionic signals but play an integrative role [6, 30, 58, 59]. Acute myocardial ischemia activates cardiac sympathetic afferent nerves, which are often associated with an increase in blood pressure and sympathetic nerve activity [4, 26–29, 50, 51, 58, 59]. The results in our laboratory showed that the systolic blood pressure and heart rate in the MI rats were higher than those in control rats. This means that the activation of sympathetic afferent fibers after myocardial ischemia gives rise to the sympathoexcitatory reflex. After treated with the $P2X_7$ receptor antagonist oxATP, the systolic blood pressure and heart rate were decreased, suggesting the possible involvement of activation of $P2X_7$ receptor in sympathoexcitatory action after MI injury. $P2X_7$ receptor is expressed in SCG [20, 41]. In some tissue $P2X_7$ antibody gives positive reaction in $P2X_7$ KO mice probably interacting with splice variants of the $P2X_7$ receptors [8]. Our results showed that MI rats displayed higher levels of $P2X_7$ immunoreactivity, mRNA, and protein in SCG. After treated with oxATP, the upregulated expression of $P2X_7$ receptor in MI rats was significantly decreased. This further indicated that ATP and $P2X_7$ receptor in SCG participated in the sympathoexcitatory transmission after MI injury.

Acute MI injury elevated the levels of serum CK, CK-MB, LDH, and cTn-I activities [24, 27, 28, 36, 50, 51]. Serum cTn-I seems more sensitive and more specific than CK, CK-MB, and LDH in minor myocardial injury [2, 5, 16]. In this study, $P2X_7$ receptor antagonist oxATP significantly inhibited the elevations in serum CK, CK-MB, LDH, and cTn-I after acute MI injury. Our ECG records showed that after ligating the left anterior descending coronary artery, ST segment was highly upward, which indicated the appearance of acute MI injury. After 20 days of ligating, abnormal Q wave appeared obviously in MI rats. When MI rats were treated with oxATP, such abnormal Q wave was improved. $P2X_7$ antagonist oxATP may inhibit the nociceptive transmission of sympathoexcitatory action in SCG after acute MI injury to produce the cardioprotective action. Inflammatory mediators could augment the nociceptive responses to ATP [7, 9, 11]. The activation of $P2X_7$ receptor





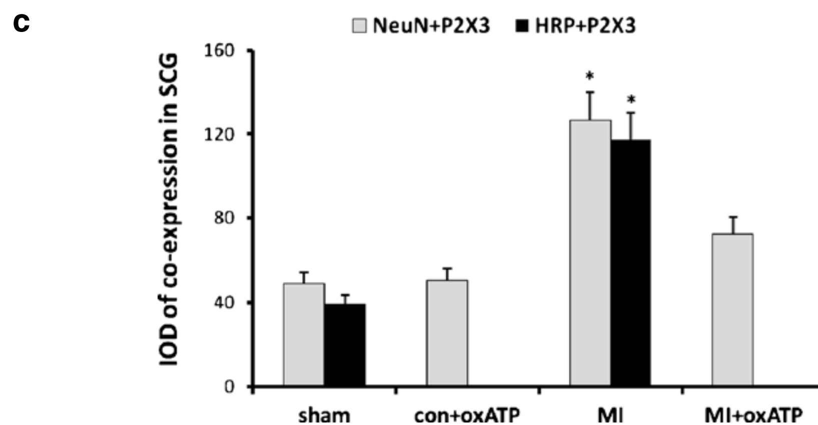


Fig. 6 The double-label immunofluorescence of $P2X_3$ and NeuN or HRP in SCG. **a** NeuN is the marker of neurons. The image shows that $P2X_3$ receptor was mainly expressed in SCG neurons. *Red* signal represents $P2X_3$ staining with TRITC, and *green* signal indicates NeuN staining with FITC. *Merge* represents $P2X_3$ and NeuN double staining image. *Scale bar*, 20 μm. **b** There was double immunofluorescence of $P2X_3$ and HRP from the cardiac afferent endings to SCG, as tested by the retrograde neuronal labeling. *Red* signal represents $P2X_3$ staining with TRITC, and *green* signal indicates HRP staining with FITC. *Merge* represents $P2X_3$ and HRP double staining image. *Scale bar*, 20 μm. **c** The bar graphs showed the statistical results for co-expression of $P2X_3$ and NeuN or HRP in SCG (n=6 in all groups)

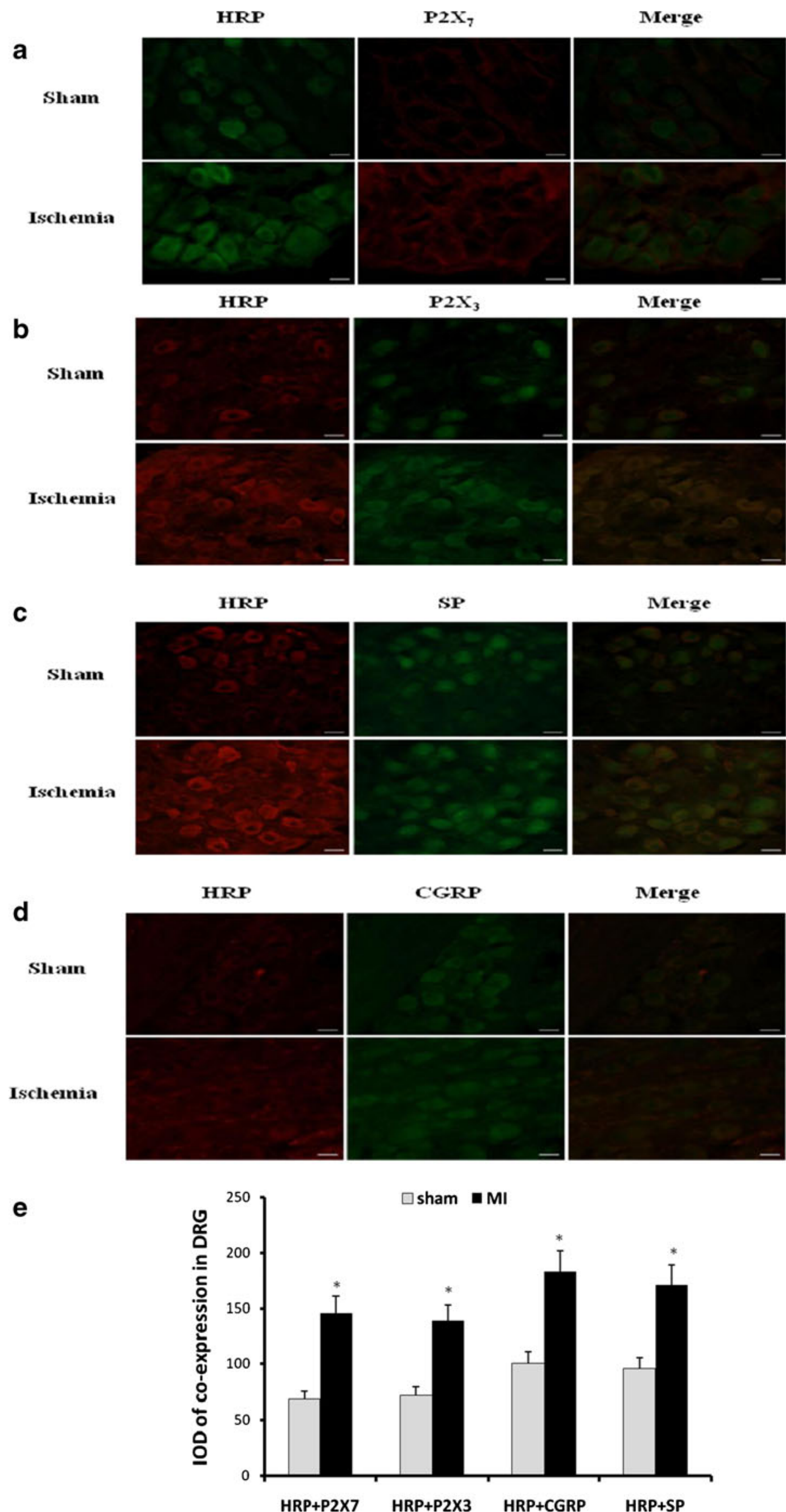


Fig. 7 The double-label immunofluorescence of $P2X_7$, $P2X_3$, CGRP, or SP with HRP in DRG. HRP was used as retrograde tracing marker. **a** There was double immunofluorescence of $P2X_7$ and HRP from the cervical DRG to SCG, tested by the retrograde tracing neuronal labeling. *Red* signal represents $P2X_7$ staining with TRITC, and *green* signal indicates HRP staining with FITC. *Merge* represents $P2X_7$ and HRP double staining image. *Scale bar*, 20 μm. **b** Double immunofluorescence of $P2X_3$ and HRP from the cervical DRG to SCG was detected by the retrograde tracing neuronal labeling. *Red* signal represents $P2X_3$ staining with TRITC, and *green* signal indicates HRP staining with FITC. *Merge* represents $P2X_3$ and HRP double staining image. *Scale bar*, 20 μm. **c** There was double-label immunofluorescence of CGRP and HRP coexpression from the cervical DRG to SCG, tested by the retrograde tracing neuronal labeling. *Red* signal represents CGRP staining with TRITC, and *green* signal indicates HRP staining with FITC. *Merge* represents CGRP and HRP double staining image. *Scale bar*, 20 μm. **d** SP and HRP coexpression from the cervical DRG to SCG was detected by the retrograde tracing neuronal labeling. *Red* signal represents SP staining with TRITC, and *green* signal indicates HRP staining with FITC. *Merge* represents SP and HRP double staining image. *Scale bar*, 20 μm. **e** The bar graphs showed the statistical results for co-expression of $P2X_7$, $P2X_3$, CGRP, or SP with HRP in DRG (n=6 in all groups)

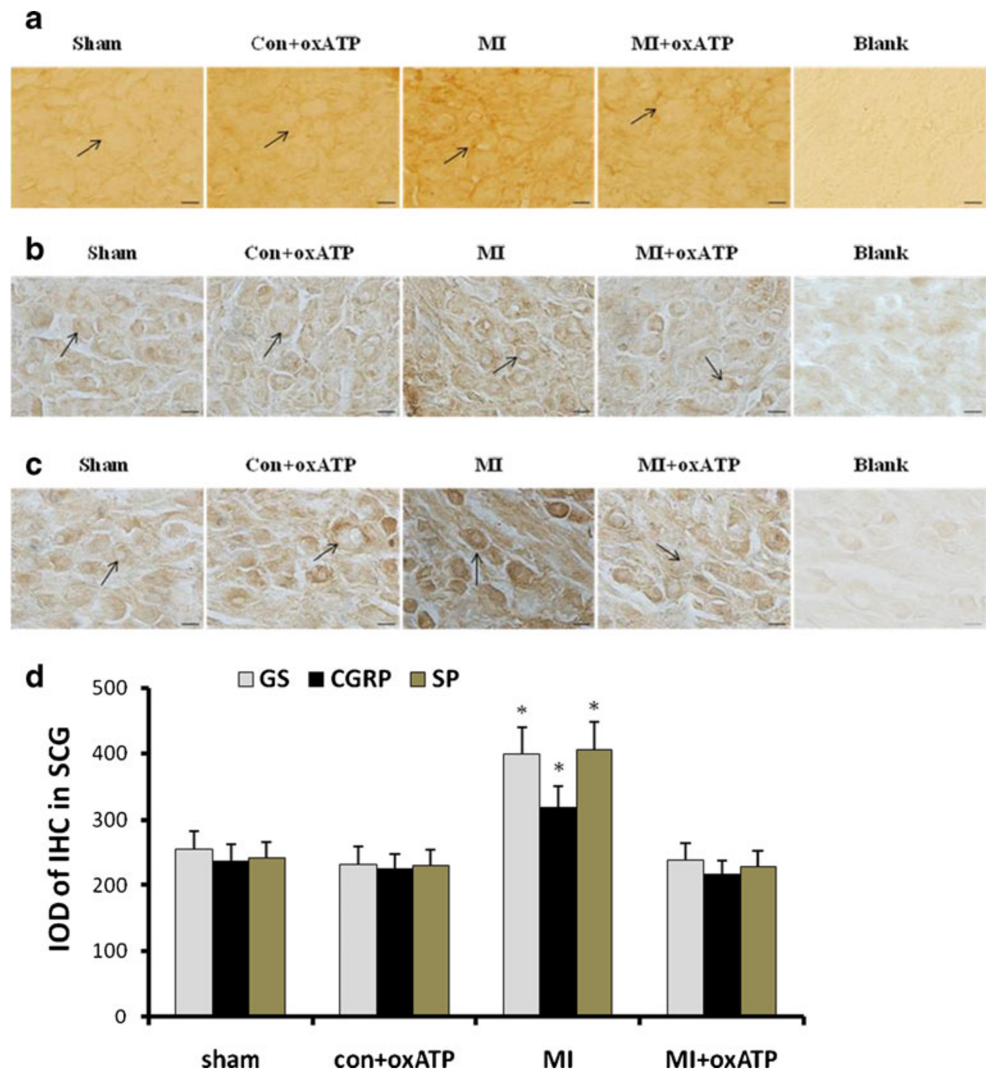


Fig. 8 Effects of oxATP on the immunoreactivity of GS, CGRP, or SP in SCG. The results show that the intensity of immunostaining for GS (**a**), CGRP (**b**), and SP (**c**) in SCG was higher in MI group (n=8) than that in sham group (n=8), con+oxATP group (n=8), and MI+oxATP group (n=8; p<0.05). No difference was found for these proteins among sham group, con+oxATP group, and MI+oxATP group (p>0.05). *Arrows* indicate the immunostained neurons. *Scale bars*, 20 μm. The bar graphs (**d**) show the statistical analysis of immunoreactivity for GS, CGRP, or SP

enhanced release of inflammatory mediators and cytokines [11, 12, 18]. TNF-α and the pro-inflammatory cytokine IL-6 are implicated in the pathogenesis of heart ischemic injury [17, 32]. Both IL-6 and TNF-α were significantly increased in the rats of our experiments after MI injury. $P2X_7$ antagonist oxATP could mitigate the enhanced IL-6 and TNF-α due to MI injury. Combined with the results in ECG, it appeared that $P2X_7$ antagonist oxATP could alleviate MI injury via modulating the inflammatory response.

Tyrosine hydroxylase (TH) is a noradrenergic marker [7, 27, 34, 58]. Our study showed that there was the coexpression of $P2X_7$ and TH in SCG, indicating that $P2X_7$ receptor was present in the sympathetic nerves. The coexpression values of $P2X_7$ receptor and TH in the SCG of MI rats were higher than those in sham rats. The upregulated $P2X_7$ receptor may participate in the signal transmission of sympathetic nerve activity after MI injury and enhance the sympathoexcitatory action. $P2X_7$ antagonist oxATP significantly decreased the enhanced $P2X_7$ and TH in the SCG of MI rats. Thus, oxATP could inhibit the activation of $P2X_7$ receptor after the MI injury to decrease the transmission of sympathoexcitatory action.

The mitogen-activated protein kinase (MAPK) ERK1 and ERK2 are the critical mediators of signaling pathways linking to the activation of membrane receptors [14, 52]. Our result showed that p-ERK1/2 in SCG of MI rats was significantly higher than that in control rats. ATP leaking out from the damaged cells can facilitate P2X receptors to cause the phosphorylation of ERK [35, 37]. Therefore, the activation of $P2X_7$ receptor in SCG after myocardial ischemia might be involved in the enhancement of intracellular ERK signaling. Treatment of MI rats with oxATP inhibited the increase of p-ERK1/2 in SCG. Thus, these results suggested that the phosphorylation of ERK 1/2 after the activation of $P2X_7$ receptor may participate in the underlying pathological mechanism for the sympathoexcitatory action during MI injury.

SGCs in sympathetic ganglia form envelopes around individual neurons, which create a distinct functional unit consisting of a neuron and its attending SGCs [21, 46].

$P2X_3$ and $P2X_7$ receptors are closely associated with the transmission of nociceptive signals in DRGs [9–11]. $P2X_3$ receptor is expressed mainly in small- and medium-sized DRG neurons [9–11]. $P2X_7$ receptor is expressed in SGCs [9–11]. Endogenously released ATP activates ischemia-sensitive cardiac afferents through the activation of $P2X_3$ receptor located on the cardiac sensory neurites [19, 27, 28, 30, 38, 49–51, 58, 59]. Glutamine synthetase (GS) as the glial marker is also found in the SGCs of the cervical sympathetic ganglia [21]. NeuN is a marker of neuron. The results of double-label immunofluorescence in our study showed that $P2X_7$ receptor is expressed both neurons and SGCs in SCG. However, $P2X_7$ receptor was mainly co-expressed with GS, whereas $P2X_3$ receptor was mainly co-expressed with NeuN in SCG. There is evidence for ATP release from neurons in sympathetic ganglia [48]. SGCs in SCG may be a target for ATP. Purinergic signaling is a major mode of neuron-glial communication [11, 45, 53, 60]. GS expression in SCG after MI injury was increased. This indicated that there was a communication between neurons and glial cells via $P2X_7$ receptor or $P2X_3$ receptor in SCG. GS expression in SCG was decreased when MI rats were treated with oxATP. The communication between neurons and glial cells in the SCG may be involved in the nociceptive transmission of MI injury. $P2X_7$ receptor antagonist oxATP could inhibit the communication. After $P2X_7$ siRNA in myocardial ischemic rats, the expressions of $P2X_7$ or GS assessed by immunohistochemistry and the co-expression staining of GS and $P2X_7$ tested by double-label immunofluorescence were lower than those in MI group. The results further indicated that $P2X_7$ receptor participated in the nociceptive response of MI injury.

HRP was used as a tracing marker to observe the retrograde neuronal labeling [31]. Evidence in our study for the afferent component from the primary sensory ganglia (cervical DRG) to SCG has been provided by combining retrograde tracing with labeling immunofluorescence. Results showed that the retrograde tracer HRP injected into SCG was found to label the cervical DRG. Double-label immunofluorescence results indicated that there was the coexpression of retrograde tracer HRP and $P2X_7$ receptor in the cervical DRG. After myocardial ischemia, the coexpression values of HRP and $P2X_7$ receptor in the cervical DRG were higher than those in control. This indicated that there was a communication between the afferent nerves of primary sensory ganglia and the efferent nerves of SCG. ATP can activate the cardiac sympathetic afferent nerves during myocardial ischemia [19, 27, 28, 30, 38, 49–51, 58, 59]. Signaling mediated by $P2X_7$ receptor in SCG and the cervical DRG may be involved in the communication which can be facilitated after MI injury.

Substance P (SP) and calcitonin gene-related peptide (CGRP) are the markers of primary sensory nerves [23]. The sympathetic–sensory coupling in dorsal root ganglia occurred following peripheral nerve injury [39, 55]. Our results showed that there was the expression of CGRP or SP immunoreactivity in SCG, which might be the primary sensory nerve terminals. Retrograde tracing test showed that there was coexpression of HRP and CGRP or SP when the retrograde tracer HRP injected into SCG to label the cervical DRG. The coexpression values of HRP and CGRP or SP in the cervical DRG were increased after MI injury. The results further indicated that there was a communication between the afferent nerves of DRG and the efferent nerves of SCG. The levels of CGRP and SP immunoreactivity in SCG were enhanced after the myocardial ischemia. $P2X_7$ receptor antagonist oxATP decreased such upregulation of CGRP and SP in SCG. DRG afferent terminals may be involved in the interaction with the sympathetic postganglionic efferent neurons. Therefore, our data suggested that there was a sensory–sympathetic coupling between the cervical DRG sensory afferent nerves and the sympathetic postganglionic efferent neurons.

Inflammatory mediators and cytokines (such as ATP, TNF-α, and IL-6) may facilitate the formation of sensory–sympathetic coupling. After ischemic injury, inflammatory mediators and cytokines upregulated the expression of SP, CGRP, and $P2X_3$ in the sensory afferent fibers surrounded the SCG neurons. ATP activated $P2X_7$ receptor on the surface membrane of SGCs, which resulted in activation of the SGCs. Activation of SGCs may be the sprouting factor of primary sensory nerves. After activation of SGCs, the plasma membrane in contact with the sympathetic postganglionic neurons revealed numerous ruffles, which promoted the interaction between neurons and SGCs [21]. Inflammatory factors and cytokines also sensitized sympathetic postganglionic neurons. Sensory–sympathetic coupling increased the activity of sympathetic postganglionic neurons thus affecting the efferent outputs into the cardiovascular system. DRG sensory sprouting into SCG neurons may increase the abnormal neuronal discharges and aggravate the sympathoexcitatory action. $P2X_7$ antagonist oxATP inhibited the activation of $P2X_7$ receptor on SGCs, which, in turn, decreased primary sensory sprouting. By interrupting the activation of SGCs, $P2X_7$ antagonist inhibited the nociceptive transmission of sympathoexcitatory action after acute MI injury to bring about the cardioprotective action.

It was reported that angina pain symptoms in 50–60 % of patients were completely relieved after sympathectomy [33, 42]. Our previous studies showed that after the treatment with A-317491 in the MI rats, the upregulated systolic blood pressure and heart rate were decreased [27, 28, 38, 50, 51, 58, 59]. The arrhythmia after the myocardial ischemia could be improved by A-317491 [27, 28, 38, 50, 51, 58, 59]. A-

317491 might inhibit the nociceptive transmission of sensory–sympathetic coupling. In the current study, $P2X_7$ receptor antagonist oxATP decreased the up-expression of $P2X_3$, CGRP, and SP in SCG after the myocardial ischemia, which inhibited the nociceptive transmission of sensory–sympathetic coupling. After $P2X_7$ siRNA in myocardial ischemic rats, the co-expression staining of GS and $P2X_7$ was decreased in comparison with that in MI group. Therefore, the activation of $P2X_7$ receptor in SCG was associated with the increased sympathoexcitatory action via sensory–sympathetic coupling induced by MI injury.

Conclusions

In summary, there was a sensory–sympathetic coupling between the rat cervical DRG afferent nerves and the SCG neurons after the MI injury. ATP activated $P2X_7$ receptor, which resulted in activation of the SGCs. The activation of SGCs participated in the increased sympathoexcitatory action after MI injury. $P2X_7$ antagonist oxATP could inhibit the activation of SGCs and interrupt the nociceptive transmission of sensory–sympathetic coupling between the cervical DRG nerves and the SCG neurons after the MI injury.

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References

1. Abbracchio MP, Burnstock G, Verkhratsky A, Zimmermann H (2009) Purinergic signalling in the nervous system: an overview. Trends Neurosci 32:19–29
2. Adams JE III, Bodor GS, Davila-Roman VG, Delmez JA, Apple FS, Ladenson JH, Jaffe AS (1993) Cardiac troponin I. A marker with high specificity for cardiac injury. Circulation 88:101–106
3. Arbeloa J, Pérez-Samartín A, Gottlieb M, Matute C (2012) P2X7 receptor blockade prevents ATP excitotoxicity in neurons and reduces brain damage after ischemia. Neurobiol Dis 45:954–961
4. Armour JA (1999) Myocardial ischaemia and the cardiac nervous system. Cardiovasc Res 41:41–54
5. Bertinchant JP, Larue C, Pernel I, Ledermann B, Fabbro-Peray P, Beck L, Calzolari C, Trinquier S, Nigond J, Pau B (1996) Release kinetics of serum cardiac troponin I in ischemic myocardial injury. Clin Biochem 29(6):587–594
6. Boehm S, Kubista H (2002) Fine tuning of sympathetic transmitter release via ionotropic and metabotropic presynaptic receptors. Pharmacol Rev 54:43–99
7. Bours MJ, Swennen EL, Di Virgilio F, Cronstein BN, Dagnelie PC (2006) Adenosine 5′-triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation. Pharmacol Ther 112:358–404
8. Brass D, Grably MR, Bronstein-Sitton N, Gohar O, Meir A (2012) Using antibodies against P2Y and P2X receptors in purinergic signaling research. Purinergic Signal 8(Suppl 1):S61–S79
9. Burnstock G (2006) Purinergic P2 receptors as targets for novel analgesics. Pharmacol Ther 110:433–454
10. Burnstock G (2007) Physiology and pathophysiology of purinergic neurotransmission. Physiol Rev 87:659–797
11. Burnstock G, Krügel U, Abbracchio MP, Illes P (2011) Purinergic signalling: from normal behaviour to pathological brain function. Prog Neurobiol 95:229–274
12. Colomar A, Marty V, Medina C, Combe C, Parnet P, Amedee T (2003) Maturation and release of interleukin-1beta by lipopolysaccharide-primed mouse Schwann cells require the stimulation of P2X7 receptors. J Biol Chem 278:30732–30740
13. Cotrina ML, Nedergaard M (2009) Physiological and pathological functions of P2X7 receptor in the spinal cord. Purinergic Signal 5:223–232
14. Dai Y, Fukuoka T, Wang H, Yamanaka H, Obata K, Tokunaga A, Noguchi K (2004) Contribution of sensitized P2X receptors in inflamed tissue to the mechanical hypersensitivity revealed by phosphorylated ERK in DRG neurons. Pain 108(3):258–266
15. Erlinge D, Burnstock G (2008) P2 receptors in cardiovascular regulation and disease. Purinergic Signal 4:1–20
16. Falahati A, Sharkey SW, Christensen D, McCoy M, Miller EA, Murakami MA, Apple FS (1999) Implementation of serum cardiac troponin I as marker for detection of acute myocardial infarction. Am Heart J 137:332–337
17. Feldman AM, Combes A, Wagner D, Kadakomi T, Kubota T, Li Y, McTiernan C (2000) The role of tumor necrosis factor in the pathophysiology of heart failure. J Am Coll Cardiol 35:537–544
18. Ferrari D, Pizzirani C, Adinolfi E, Lemoli RM, Curti A, Idzko M, Panther E, Virgilio FD (2006) The $P2X_7$ receptor: a key player in IL-1 processing and release. J mmunol 176:3877–3883
19. Fu LW, Longhurst JC (2010) A new function for ATP. Am J Physiol Heart Circ Physiol 299:H1762–H1771
20. Gourine AV, Wood JD, Burnstock G (2009) Purinergic signalling in autonomic control. Trends Neurosci 32:241–248
21. Hanani M (2010) Satellite glial cells in sympathetic and parasympathetic ganglia: in search of function. Brain Res Rev 64:304–327
22. Higuchi T, Stephan G, Nekolla RS, Poethko T, Yu M, Wester HJ, Casebier DS, Robinson SP, Botnar RM, Schwaiger M (2008) A new 18F-labeled myocardial PET tracer: myocardial uptake after permanent and transient coronary occlusion in rats. Nucl Med 49:1715–1722
23. Hukkanen M, Platts LAM, Corbett SA, Santavirta S, Polak JM, Konttinen YT (2002) Reciprocal age-related changes in GAP-43/B-50, substance P and calcitonin gene-related peptide (CGRP) expression in rat primary sensory neurones and their terminals in the dorsal horn of the spinal cord and subintima of the knee synovium. Neurosci Res 42:251–260
24. Ioannidis JP, Karvouni E, Katritsis DG (2003) Mortality risk conferred by small elevations of creatine kinase-MB isoenzyme after percutaneous coronary intervention. J Am Coll Cardiol 42:1406–1411
25. Jardine DL, Charles CJ, Ashton RK, Bennett SI, Whitehead M, Frampton CM, Nicholls MG (2004) Increased cardiac sympathetic nerve activity following acute myocardial in a sheep model. J Physiol 565:325–333
26. Li DP, Pan HL (2000) Responses of neurons in rostral ventrolateral medulla to activation of cardiac receptors in rats. Am J Physiol Heart Circ Physiol 279:H2549–H2557
27. Li GL, Liu SM, Zhang J, Xu CS, Lin JR, Li X, Liang SD (2010) Increased sympathoexcitatory reflex induced by myocardial ischemic nociceptive signaling via $P2X_{2/3}$ receptor in rat superior cervical ganglia. Neurochem Int 56(8):984–390

28. Li GL, Liu SM, Yang Y, Xie JY, Liu J, Kong FJ, Tu GH, Wu RP, Li GD, Liang SD (2011) Effects of oxymatrine on sympathoexcitatory reflex induced by myocardial ischemic signaling mediated by $P2X_3$ receptors in rat SCG and DRG. Brain Res Bull 84:419–424
29. Li W, Knowlton D, Van Winkle DM, Habecker BA (2004) Infarction alters both the distribution and noradrenergic properties of cardiac sympathetic neurons. Am J Physiol Heart Circ Physiol 286:H2229–H2236
30. Liang SD, Xu CS, Li GL, Gao Y (2010) P2X receptors and modulation of pain transmission: focus on effects of drugs and compounds used in traditional Chinese medicine. Neurochem Int 57:705–712
31. Lieu TM, Kollarik M, Myers AC, Undem BJ (2011) Neurotrophin and GDNF family ligand receptor expression in vagal sensory nerve subtypes innervating the adult guinea pig respiratory tract. Am J Physiol Lung Cell Mol Physiol 300:L790–L798
32. Matsmori A (2003) Roles of cytokines in the pathogenesis of heart failure. Nippon Rinsho 61:745–750
33. Meller ST, Gebhart GF (1992) A critical review of the afferent pathways and the potential chemical mediators involved in cardiac pain. Neuroscience 48:501–24
34. Pather N, Partab P, Singh B, Satyapal KS (2003) The sympathetic contribution to the cardiac plexus. Surg Radiol Anat 25(3–4):210–215
35. Ponnusamy M, Liu N, Gong R, Yan H, Zhuang S (2011) ERK pathway mediates P2X7 expression and cell death in renal interstitial fibroblasts exposed to necrotic renal epithelial cells. Am J Physiol Renal Physiol 301:F650–F659
36. Ricciardi MJ, Davidson CJ, Gubernikoff G, Beohar N, Eckman LJ, Parker MA, Bonow RO (2003) Troponin I elevation and cardiac events after percutaneous coronary intervention. Am Heart J 145:2–8
37. Seino D, Tokunaga A, Tachibana T, Yoshiya S, Dai Y, Obata K, Yamanaka H, Kobayashi K, Noguchi K (2006) The role of ERK signaling and the P2X receptor on mechanical pain evoked by movement of inflamed knee joint. Pain 123:93–203
38. Shao LJ, Liang SD, Li GL, Xu CS, Zhang CP (2007) Exploration of $P2X_3$ in the rat stellate ganglia after myocardial ischemia. Acta Histochem 109:330–337
39. Shinder V, Govrin-Lippmann R, Cohen S, Belenky M, Ilin P, Fried K, Wilkinson HA, Devor M (1999) Structural basis of sympathetic–sensory coupling in rat and human dorsal root ganglia following peripheral nerve injury. J Neurocytol 28:743–761
40. Skaper SD, Debetto P, Giusti P (2010) The $P2X_7$ purinergic receptor: from physiology to neurological disorders. FASEB J 24:337–345
41. Sperlagh B, Vizi ES, Wirkner K, Illes P (2006) $P2X_7$ receptors in the nervous system. Prog Neurobiol 78:327–346
42. Stritesky M, Dobias M, Demes R, Semrad M, Poliachova E, Cermak T, Charvat J, Malek I (2006) Endoscopic thoracic sympathectomy—its effect in the treatment of refractory angina pectoris. Interact Cardiovasc Thorac Surg 5:464–468
43. Swissa M, Zhou SM, Gonzalez-Gomez I, Chang CM, Lai AC, Cates AW, Fishbein MC, Karagueuzian HS, Chen PS, Chen LS (2004) Long-term subthreshold electrical stimulation of the left stellate ganglion and a canine model of sudden cardiac death. J Am Coll Cardiol 43:858–864
44. Thames MD, Kinugawa T, Dibner-Dunlap ME (1993) Reflex sympathoexcitation by cardiac afferents during myocardial ischemia: role of adenosine. Circulation 87:1698–704
45. Verderio C, Matteoli M (2011) ATP in neuron–glia bidirectional signaling. Brain Res Rev 66:106–114
46. Verkhratsky A, Parpura V, Rodríguez JJ (2011) Where the thoughts dwell: the physiology of neuronal–glial “diffuse neural net”. Brain Res Rev 66:133–151
47. Vassort G (2001) Adenosine 5′-triphosphate: a P2-purinergic agonist in the myocardium. Physiol Rev 81:767–806
48. Vizi ES, Liang SD, Sperlágh B, Kittel A, Jurányi Z (1997) Studies on the release and extracellular metabolism of endogenous ATP in rat superior cervical ganglion: support for neurotransmitter role of ATP. Neuroscience 79:893–903
49. Wan F, Li GL, Liu SM, Zhu GC, Xu SH, Lin JR, Zhang J, Li X, Liang SD (2010) P2X2/3 receptor activity of rat nodose ganglion neurons contributing to myocardial ischemic nociceptive signaling. Auton Neurosci: Basic Clin 158:58–64
50. Wang YX, Li GL, Liang SD, Wan F, Xu CS, Gao Y, Liu SM, Lin JR (2009) Expression of $P2X_2$ and $P2X_3$ receptors in rat nodose neurons after myocardial ischemia injury. Auton Neurosci 145:71–75
51. Wang YX, Li GL, Liang SD, Zhang AX, Xu CS, Gao Y, Zhang CP, Wan F (2008) Role of $P2X_3$ receptor in myocardial ischemia injury and nociceptive sensory transmission. Auton Neurosci 139:30–37
52. Watters JJ, Sommer JA, Pfeiffer ZA, Prabhu U, Guerra AN, Bertics PJ (2002) A differential role for the mitogen-activated protein kinases in lipopolysaccharide signaling: the MEK/ERK pathway is not essential for nitric oxide and interleukin 1beta production. J Biol Chem 277:9077–9087
53. Weick M, Cherkas PS, Härtig W, Pannicke T, Uckermann O, Bringmann A, Tal M, Reichenbach A, Hanani M (2003) P2 receptors in satellite glial cells in trigeminal ganglia of mice. Neuroscience 120:969–977
54. Xiang Z, Bo X, Burnstock G (1998) Localization of ATP-gated P2X receptor immunoreactivity in rat sensory and sympathetic ganglia. Neurosci Lett 256:105–108
55. Xie W, Strong JA, Zhang JM (2010) Increased excitability and spontaneous activity of rat sensory neurons following in vitro stimulation of sympathetic fiber sprouts in the isolated dorsal root ganglion. Pain 151:447–459
56. Yan Z, Li S, Liang Z, Tomic M, Stojilkovic SS (2012) The P2X7 receptor channel pore dilates under physiological ion conditions. J Gen Physiol 132:563–573
57. Yao H, Zhang Y, He F, Wang C, Xiao Z, Zou J, Wang F, Liu Z (2012) Short hairpin RNA targeting 2B gene of coxsackievirus B3 exhibits potential antiviral effects both in vitro and in vivo. BMC Infect Dis 12:177
58. Zhang CP, Liang SD, Li GL, Xu CS, Gao Y, Wang YX, Zhang AX, Wan F (2007) The involvement of $P2X_3$ receptors of rat sympathetic ganglia in cardiac nociceptive transmission. J Physiol Biochem 63(3):249–258
59. Zhang CP, Li GL, Liang SD, Xu CS, Zhu GC, Wang YX, Zhang AX, Wan F (2008) Myocardial ischemic nociceptive signaling mediated by $P2X_3$ receptor in rat stellate ganglion neurons. Brain Res Bull 75:77–82
60. Zhang X, Chen Y, Wang C, Huang LY (2007) Neuronal somatic ATP release triggers neuron-satellite glial cell communication in dorsal root ganglia. Proc Natl Acad Sci USA 104:9864–9869