



FUNDC1: a key mediator of adenosine A2BR activation-induced inhibition of cardiac mitophagy under ischemia/reperfusion conditions

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Background: Mitophagy is an essential factor in mitochondrial quality control and myocardial ischaemia/reperfusion (I/R) injury protection. Because adenosine A2B receptor (A2BR) activation exerts a major role in reducing myocardial I/R injury, the effects of adenosine A2BR activation on cardiac mitophagy under reperfusion conditions were investigated.

Methods: 110 adult Wistar rats (7–10 w), weighing 250–350 grams, were cultured in specific-pathogen-free (SPF) conditions before experiments. All hearts were removed and reperfused by Langendorff device. Six hearts with coronary flow (CF) values >28 or <10 mL/min were excluded. Others were arbitrarily divided into the following groups: sham operation group, I/R group, BAY60-6583 (BAY) (1–1,000 nM) + I/R group, PP2 + BAY + I/R group. After ischemia in rats, reperfusion was performed. H9c2 cells were placed in an imitated ischemic environment followed by Tyrode's solution to stimulate hypoxia/reoxygenation (H/R) injury. The mitochondrial fluorescence indicator MitoTracker Green and lysosomal fluorescence indicator LysoTracker Red were used to examine mitochondria and lysosomes, respectively. Colocalization of mitochondrial and autophagy marker proteins was determined by immunofluorescence. Autophagic flow currents were tested by Ad-mCherry-GFP-LC3B. Protein-protein interactions were predicted using a database and analyzed by co-immunoprecipitation. Autophagy marker protein, mitophagy marker protein, and mitophagy protein FUNDC1 were detected by immunoblotting.

Results: Compared with those in the I/R group, myocardial autophagy and mitophagy were suppressed by the selective adenosine A2BR agonist BAY, and this effect was inhibited by the selective Src tyrosine kinase inhibitor PP2, indicating that adenosine A2BR activation could inhibit myocardial autophagy and mitophagy by activating Src tyrosine kinase. In support, in H9c2 cells, the selective Src tyrosine kinase inhibitor PP2 inhibited the effect of BAY on TOM20 with LC3 or mitochondria with lysosomes colocalization and autophagy flow. Here, we showed that mitochondrial FUNDC1 co-precipitated with Src tyrosine kinase after BAY was added. Consistently, the immunofluorescence and western blotting results demonstrated that compared to that in the H/R group, the expression of mitochondrial FUNDC1 was reduced by BAY, but this effect was reversed by PP2.

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Conclusions: Adenosine A2BR activation may inhibit myocardial mitophagy by downregulating expression of the mitochondrial FUNDC1 by activating Src tyrosine kinase under I/R conditions and could increase the interaction between Src tyrosine kinase and FUNDC1.

Keywords: Adenosine A2B receptor; Src tyrosine kinase; FUNDC1

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Introduction

Background

Myocardial ischaemia/reperfusion (I/R) injury, which is caused by the massive production of reactive oxygen species (ROS), inflammatory cell infiltration, calcium overload and other factors, is a type of serious injury that occurs when the ischaemic myocardium is reperfused during the treatment of ischaemic heart disease (1). Improving the outcomes of ischaemia heart disease requires as little I/R injury as possible. Langendorff technology, which is relatively mature, and it is convenient to obtain the information about myocardial and coronary vessel status of a single heart, has been used to set myocardial I/R model for many years (2,3). Because mitochondria are the main source of adenosine triphosphate (ATP) and can trigger apoptosis or necrosis by generating ROS, the stability of mitochondrial morphology and function controls the life and death of the cell (4). ROS can open the mitochondrial permeability

transition pore (mPTP) through various mechanisms, thereby causing the mitochondrial membrane to expand and rupture. Cytochrome C and other pro-apoptotic factors in mitochondria will enter the body, causing the death of cardiomyocytes (5). Therefore, it is important to inhibit the damage to the reperfused myocardium through preventing and reducing mitochondrial dysfunction.

Rationale and knowledge gap

The dysfunctional relationship between mitochondrial fission, mitochondrial fusion and mitophagy has been proven to be a key factor leading to myocardial I/R injury (6). Mitophagy, a kind of selective autophagy, exerts a key role in the quality control of mitochondria. Mitophagy not only selectively removes damaged mitochondria but also promotes the synthesis of new mitochondria, thereby ensuring mitochondrial quality (7). In 1987, adenosine was first reported to pronouncedly decrease infarct size and improve regional ventricular function in canine hearts under I/R conditions, and adenosine and its analogues have been considered to be the most promising drugs for treating myocardial I/R injury (8). Adenosine and its analogues induce a cardioprotective effect by activating different adenosine receptors (9). Studies on the effects of adenosine receptor activation on autophagy and mitophagy are insufficient, and the results are contradictory and complex (Table 1).

Among the 4 well-known adenosine receptors, the adenosine 2B receptor (A2BR) subtype was proven to mediate the cardioprotective effects induced by both ischaemic preconditioning and postconditioning (19). Mitochondrial oxidative stress injury was alleviated by inhibiting mitochondrial complex I activity by activating Src tyrosine kinase via the activation of adenosine A2A receptor (A2AR) or A2BR (20). Meanwhile, Src tyrosine kinase regulates the activity of a variety of mitophagy-

Highlight box

Key findings

- FUNDC1 is a key mediator of adenosine A2BR activation-induced inhibition of cardiac mitophagy under ischemia/reperfusion conditions.

What is known and what is new?

- Mitophagy is an essential factor in mitochondrial quality control against myocardial I/R injury.
- A2BR activation may decrease the binding of FUNDC1 to mitochondrial outer membrane by increasing FUNDC1 tyrosine phosphorylation via increasing Src tyrosine kinase activity.

What is the implication, and what should change now?

- It is helpful to fully understand the influence of adenosine receptor activation on mitochondrial structure and function which may promote the development of new methods against myocardial I/R injury.

Table 1 Previous studies of the effect of AR activation on autophagy and mitophagy

AR subtypes	Model	The effect of AR activation on autophagy and mitophagy
AR (10)	Primary bovine PAECs	AR↑ Mitophagy↑
A1R (11)	HL-1/isolated adult rat cardiomyocytes simulated I/R	A1R↑ Autophagy↑
A1R (12)	Starvation model of human islets, human pancreatic neuroendocrine cell line BON-1 and in hepatocytes	Low A1R↑ Autophagy↑ Low A1R↑ Autophagy↓
A2R (13)	Isolated Wistar rat heart I/R model	A2R↑ Autophagy↓
A2AR (14)	Chondrocytes	A2AR↑ Autophagy↑
A2AR (15)	Neutrophils	A2AR↑ Autophagy↓
A2BR (16)	Mice AMI <i>in vivo</i>	A2BR↓ Autophagy↓
A3R (17)	Lung cancer cell lines (A549 and H1299)	A3R↑ Autophagy↑
A3A3R (18)	A375 melanoma cells	A3R↑ Autophagy↑

AR, adenosine receptor; PAEC, pulmonary artery endothelial cell; I/R, ischaemia/reperfusion; AMI, acute myocardial infarction.

associated proteins (21). Although adenosine A2 receptor could decrease myocardial autophagy after reperfusion, the mechanism by which adenosine A2BR activates autophagy or mitophagy under I/R conditions is still unclear.

Objective

Our aim was to explore the effects of adenosine A2BR activation on cardiac autophagy and mitophagy under reperfusion conditions and the potential mechanism, to demonstrate that adenosine A2BR activation may inhibit myocardial autophagy and mitochondrial phagocytosis induced by I/R, and that Src tyrosine kinase may mediate adenosine A2BR activation to inhibit myocardial autophagy and mitosis induced by I/R by regulating FUNDC1. We present this article in accordance with the ARRIVE reporting checklist (available at <https://cdt.amegroups.com/>

[article/view/10.21037/cdt-22-468/rc](https://doi.org/10.21037/cdt-22-468/rc)).

Methods

Chemicals

BAY60-6583 (BAY, cat. No. 4472) and PP2 (cat. No. P0042) were purchased from Tocris Bioscience (Ellisville, MO, USA). Antibodies against LC3 (cat. No. 51520, RRID: AB_881429) and TOM20 (cat. No. 56783, RRID: AB_945896) were obtained from Abcam (San Francisco, CA, USA). The COX IV (cat. No. 4850S, RRID: AB_2085424) antibody was obtained from Cell Signaling Technology (Beverly, MA, USA). Antibodies against Src (cat. No. 2109, AB_2106059) and FUNDC1 (cat. No. 251933) were purchased from ZEN BIO (Chengdu, China). β -Tubulin (cat. No. TDY043CTDY) was obtained from TDR Biotech (Qingdao, China). The fluorescent polymer microspheres

were purchased from Duke Scientific Corp. (Fremont, CA, USA), and 2,3,5-triphenyltetrazolium chloride (TTC) was purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). MitoTracker Green (cat. No. C1048), LysoTracker Red (cat. No. C1046) and mCherry-GFP-LC3B (cat. No. C3011) were obtained from Beyotime Biotechnology (Shanghai, China).

Experimental animals

Adult male Wistar (7–10 w) weighing 250–350 g was purchased from the Laboratory Animal Research Center of North China University of Science and Technology. They were put into a cage (4 rats/cage) under 22 ± 2 °C and $55 \pm 5\%$ humidity with a 12 h/12 h light/dark cycle. Supply standard laboratory food and drinking water. All of the experimental protocols were approved by the Ethics Committee of North China University of Science and Technology (application approval No. 2017023) and conducted under NIH Guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised in 1996).

Regional I/R model of isolated rat hearts

The rat heart was operated according to the above method (22). Male Wistar rats (250–350 g) were anesthetized using sodium thiobarbital (100 mg/kg ip). Take out the heart quickly and wash it with cold Krebs-Henseleit buffer (pH 7.4), which contains 118.5 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.8 mM $CaCl_2$, 24.8 mM $NaHCO_3$, 1.2 mM KH_2PO_4 , and 10 mM glucose. Then, after the aortic intubation, the heart was fixed on the Langendorff device, perfused with Krebs-Henseleit buffer, and inflated with 95% O_2 /5% CO_2 . The whole process took only 30 seconds. A latex balloon attached to a pressure sensor is inserted through the left atrium into the left ventricle. Using the physiological signal collection and processing system (Chengdu Instrument Manufactory, China) to continuously record various cardiac dynamic parameters including heart rate (HR) and left ventricular systolic pressure (LVSP). All hearts can be held steady for at least 20 min. After the blood pressure is stabilized, coronary flow (CF) is measured. Heart with a CF value of >28 or <10 mL/min will be excluded. Initially, left ventricular end-diastolic pressure (LVEDP) was recorded between 5 and 10 mmHg. A 5-0 suture is wound around the left coronary artery, with one end of the suture threaded through a small

piece of soft vinyl tube to form a sleeve. The sleeve is pulled and fixed to the catheter with a small hemostat, causing ischemia (20). In the I/R group, the isolated myocardium was subjected to ischemia for 30 min, and then reperfused for 1 h. In the sham operation group, a silk thread was implanted, but there was no suture. BAY (1–1,000 nM) was injected for 65 min within 5 min of reperfusion. The Src tyrosine inhibitor PP2 (1 μ M) was added for 70 min before reperfusion for 10 min.

Measurement of infarct size

The area at risk (AAR) and infarct area were assessed through fluorescent polymer microspheres and TTC staining as described previously (23). In order to achieve randomness, the reagents were prepared by another person and named by number every day. The person who did the animal experiments did not know what reagents were given. The number of groups has been added to each presentation of figures. In addition, 4 hearts were excluded from the study because of CF <10 mL/min, while 2 hearts were excluded from the study because of CF values >28 or 28 mL/min. Briefly, the coronary artery was reoccluded, and fluorescent polymer microspheres were infused. Then, the hearts were weighed, stored at -20 °C for 2 h, frozen and cut into 1 mm slices. Immerse the section in 10 mL of 10% formalin to increase its contrast, and squeeze it into the middle of a glass plate of only 1 mm. The myocardial ischemia and myocardial ischemia were qualitatively analyzed by using transparent acetate section. Background subtraction, brightness and contrast enhancement are performed on each image to improve sharpness and sharpness. The area was converted to volume by multiplying the section thickness, and the infarct size was expressed as a percentage (%) of AAR.

Western blotting

Apical heart tissues were prepared as protein lysates. The protein content on each swimming lane of Western Blot is 40 μ g/mL, and the polyvinylidene fluoride (PVDF) membrane of 0.45 μ m is used for all blocking membranes. All membranes were incubated overnight with primary antibodies (LC3, COX IV, TOM20, or FUNDC1) at 4 °C, and each band was tested for the second antibody in an hour at room temperature. All thin films were visualized using enhanced chemiluminescence (ECL) method. The membrane was prepared again with anti-Tubulin antibody,

and the equal load of the sample was determined. Use ImageJ to record the pictures in the computer and quantify them. All our Western Blot results are repeated 6 times.

Cell culture

H9c2 myocytes were obtained from the American Standard Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured at 37 °C in 5% CO₂/95% O₂ air in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) (Gibco) and 100 U penicillin/streptomycin (24).

Hypoxia/reoxygenation (H/R) model in H9c2 cells

H9c2 cells were placed in a glucose-free Tyrode solution in a simulated ischemic environment (10 mM 2-deoxy-D-glucose and 10 mM sodium dithionite) for 30 min, followed by treatment with normal Tyrode reperfusion for 20 min (22). BAY treatment was performed within 5 min before reoxygenation. The selected Src tyrosine inhibitor PP2 (1 μM) was added 10 min before reoxygenation.

Immunofluorescence analysis

Cells were plated in a specific culture dish (50,000 cells/dish) for 36 h. After being treated according to the experimental protocol in 2.7, the cells were fixed in 4% formaldehyde for 1 h at room temperature. The cells were cleaned with phosphate buffered saline (PBS) three times. After the first cleaning, the cells were infiltrated with 0.1% TritonX-100 at room temperature for 15 min. The second time, the cells were closed with 5% BSA at room temperature for 1 h. Then the cells were rinsed with PBS 5 times, FITC or Cy3 secondary antibody was added, and incubated at 37 °C for 1 h. The confocal microscope was used for laser scanning. FITC fluorescence was excited by 488 nm helium-neon laser and imaged by 520 nm long path filter. The fluorescence of Cy3 was excited by a 543 nm helium-neon laser, and Cy3 was imaged by a 570 nm long path filter.

Colocalization of lysosomes and mitochondria in H9c2 cells

Cells were plated in specific culture dishes (50,000 cells/dish) for 36 h. The cells were washed 3 times using PBS and then treated with DMEM containing LysoTracker Red (1:20,000)

and MitoTracker Green (1:10,000) for 30 min. After being incubated with fluorescence probes, the cells were treated as follows. After being washed with PBS, culture dishes were placed on the objective table and imaged with a laser scanning confocal microscope. The green fluorescence of MitoTracker is excited by a 488 nm He-Ne laser and processed by a 516 nm long path filter. LysoTracker Red light was studied with 543 nm He-Ne laser, and the image was processed with 590 nm long path filter.

Autophagy flow assay

Cells were seeded in particular culture dishes (50,000 cells/dish) for 24 h. After culturing to a certain density, Ad-mCherry-GFP-LC3B was included, reaching an ultimate concentration of 40 vp/mL. After 24 h of excitation, mCherry and GFP fluorescent spots were analyzed by confocal microscopy. A 488 nm He-Ne laser was used to excite GFP fluorescence, and a 516 nm long optical path filter was used for image processing. Under the He-Ne laser at 543 nm, mCherry fluorescence is excited, and then the image is processed with a 590 nm optical path filter (25).

Protein-protein interaction (PPI) network construction

We used online databases (STRING, <https://string-db.org/>; INBIO, <https://inbio-discover.com/>) to construct the PPI network.

Mitochondrial isolation

According to the instructions of the manufacturer, mitochondria and cytoplasm were separated by standard homogenization, protease digestion, and differential centrifugation and so on, using tissue mitochondrial separation reagent (Beyotime Institute of Biotechnology, Shanghai, China). Simply put, the weighed heart sample was placed into a frozen PBS (v/v, 1/10) and manually cut using small scissors. The sliced tissue was separated from PBS at a speed of 600 rpm, and then cultured on ice using trypsin digest (v/v, 1/8) for 20 min. The digestion solution of trypsin was discarded and homogenized with a mixture of protease inhibitors in the mitochondrial separation buffer (solution A). Centrifuge the supernatant at 3,000 ×g for 10 min. The isolated mitochondrial particles were then suspended to the storage buffer (40 μL/100 mg tissue). Each process is carried out at 4 °C.

Table 2 Coronary flow and cardiodynamic data

Groups	CF		HR		LVSP		LVEDP	
	Baseline	End	Baseline	End	Baseline	End	Baseline	End
Sham	15.50±1.08	13.13±0.75	205.00±2.00	200.00±10.00	109.85±4.56	93.53±4.30	6.54±1.77	8.72±0.33
I/R	15.38±0.75*	11.13±1.11*	201.00±5.00	188.00±6.00	109.23±7.16*	84.05±4.21*	5.82±0.78*	18.86±0.58*

Values are means ± SEM of 4 experimental observations. *, P<0.05 vs. sham. CF, coronary flow; HR, heart rate; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; I/R, ischaemia/reperfusion; SEM, standard error of the mean.

Co-immunoprecipitation (CoIP)

Src tyrosine kinase antibody was cross-linked to magnetic beads according to Pierce Crosslink Magnetic IP/CoIP Kit (88805; Thermo Scientific, Rockford, IL, USA). One milligram mitochondrial protein was cross-linked with Src antibody with 1 mg Src antibody and incubated overnight at 4 °C. The protein-Src antibody-bead complexes were washed with IP-lysis/wash buffer twice and then ultrapure water before eluting the bound antigen. The eluate was then immunoblotted.

Statistical analysis

Statistical analysis was conducted using the SigmaPlot 12.5 version software (Systat Software, Inc., San Jose, CA, U.S.A.). The data are expressed as the mean ± SEM and were obtained from 3 to 6 separate experiments. A comparison of two groups was assessed by Student's *t*-test. One-way ANOVA followed by Tukey's test was recruited to determine differences between more than two groups. A value of P<0.05 was considered statistically significant. A protocol was prepared before the study without registration.

Results

Adenosine A2BR activation inhibits I/R-induced myocardial autophagy and mitophagy

As shown in *Table 2*, no obvious differences were found in baseline cardiodynamic variables between the sham and I/R groups, CF and LVSP were decreased, while LVEDP was increased in the I/R group (*Table 2*). Moreover, infarct size was pronouncedly boosted in the I/R group (*Figure 1A-1C*). These results showed that our regional I/R model of isolated rat hearts was successfully established.

To determine the effects of adenosine A2BR activation on myocardial autophagy and mitophagy under I/R conditions, we measured the levels of autophagy and mitophagy

marker protein expression. Western blotting showed that the ratio of LC3 II/β-Tubulin of I/R group (−0.39, 95% CI: −0.53 to −0.26; P=0.026), which is a major indicator of autophagy, was significantly increased, while the changes in mitophagy marker proteins (COX IV and TOM20) were not significant compared to those in the sham group (*Figure 2A-2D*). However, the selective adenosine A2BR agonist BAY reduced the ratio of LC3 II/β-Tubulin and increased COX IV and TOM20 expression in a dose-dependent manner (0.43, 95% CI: 0.15 to 0.71; P=0.011; −0.53, 95% CI: −0.74 to −0.31; P=0.009; 0.34, 95% CI: −0.05 to 0.73; P=0.032) (*Figure 2A-2D*). Moreover, confocal microscopy showed that the colocalization of TOM20 and LC3 was inhibited by BAY under H/R conditions (0.34, 95% CI: −0.05 to 0.73; 982.00, 95% CI: −156.05 to 2,120.05; P=0.004; 25.66, 95% CI: 17.80 to 35.52; P=0.004) (*Figure 3A,3B*). In addition, we found that after BAY was given under H/R conditions, it also inhibited co-localization of mitochondria and lysosomes (−46.79, 95% CI: −93.89 to 18.32; P=0.032) (*Figure 3C,3D*). The results showed that after BAY treatment, the number of yellow fluorescent dots and red fluorescent dots increased significantly, indicating that the number of autophagosomes and autophagolysosomes increased (25.50, 95% CI: 19.64 to 30.36; P<0.001; 25.50, 95% CI: 14.75 to 36.25; P<0.001) (*Figure 4A,4B*).

Src tyrosine kinase participates in the effects of adenosine A2BR activation against myocardial autophagy and mitophagy under I/R conditions

As shown in *Figure 3A*, the selective Src tyrosine kinase inhibitor PP2 inhibited the effect of BAY on TOM20 and LC3 colocalization. Moreover, Western blotting showed that the effects of A2BR activation on autophagy and mitophagy marker proteins, including LC3-II/β-Tubulin, COX IV and TOM20, were also inhibited by PP2 (0.04, 95% CI: −0.08 to 0.16; P=0.976) (*Figure 5A-5D*). In addition, comparing to the BAY group, the number of

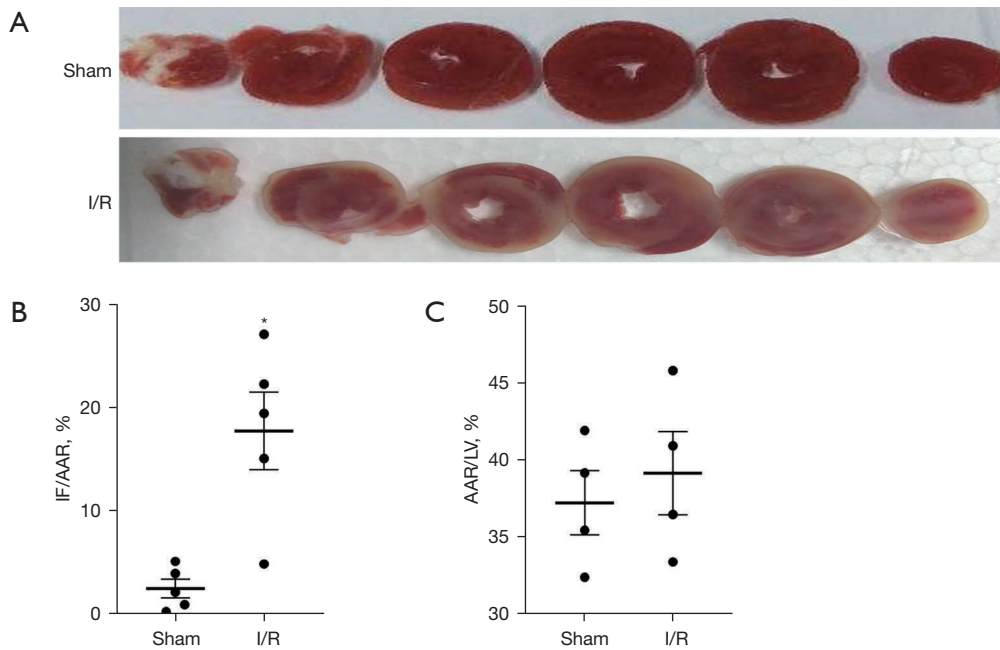


Figure 1 Infarct size in isolated rat hearts. Each bar is the mean ± SEM of 4 experiments. *, $P < 0.05$ vs. sham. I/R, ischaemia/reperfusion; IF, infarction area; AAR, area at risk; LV, left ventricular; SEM, standard error of the mean.

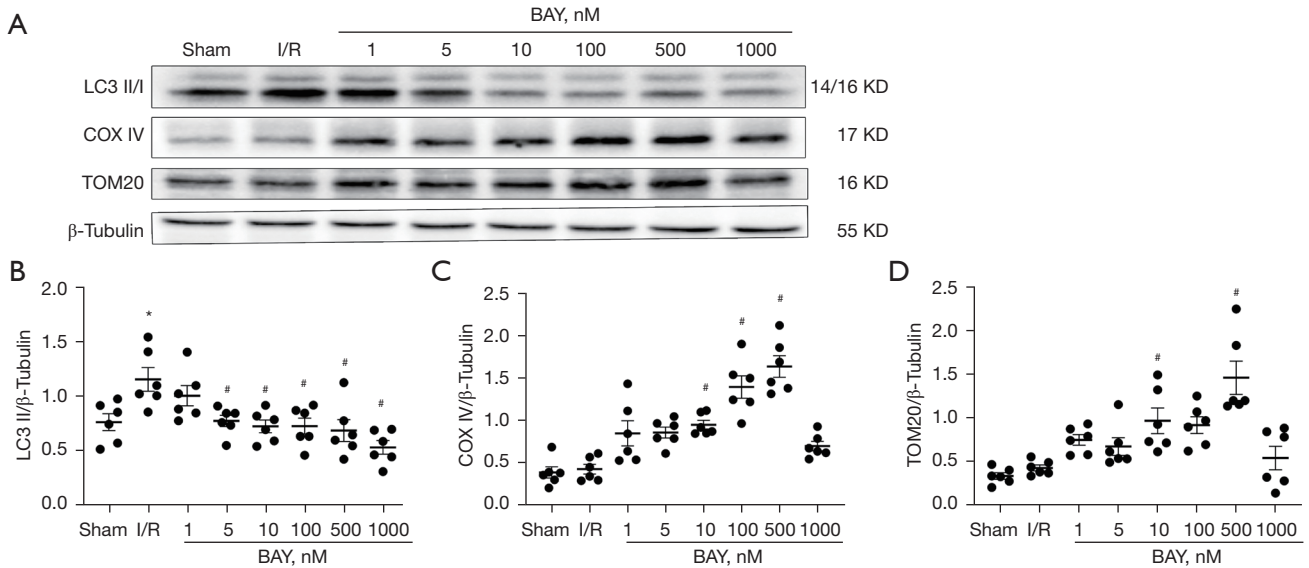


Figure 2 The effects of adenosine A2BR activation on myocardial autophagy and mitophagy under I/R conditions. Western blotting results of LC3, COX IV and TOM20 protein expression. Each bar is the mean ± SEM of 6 experimental. *, $P < 0.05$ vs. Control; #, $P < 0.05$ vs. I/R. SEM, standard error of the mean; I/R, ischaemia/reperfusion.

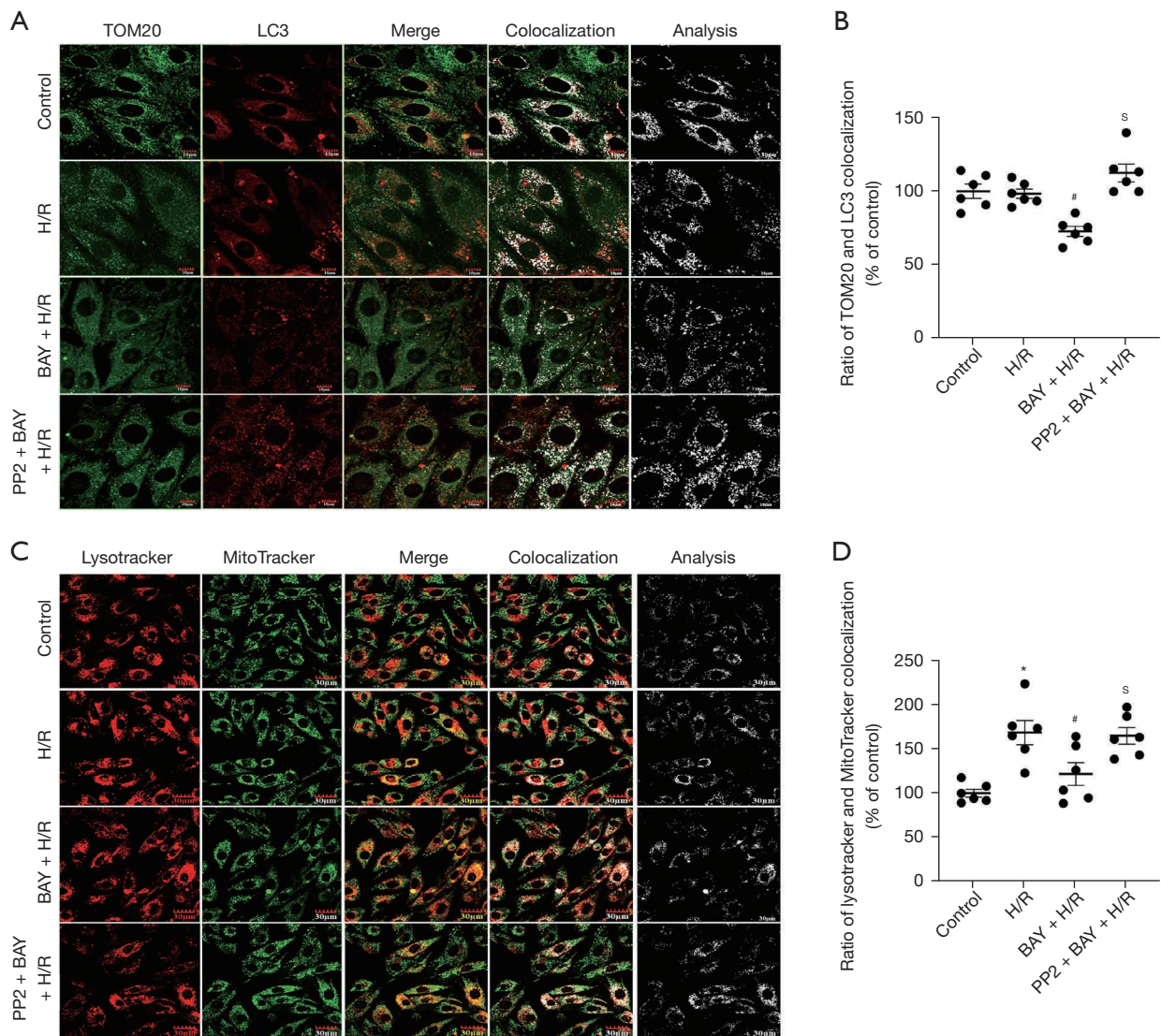


Figure 3 Confocal images of Cy3-labeled LC3 (red) and FITC-labeled TOM20 (green). MitoTracker Green and LysoTracker Red were utilized for mitochondria (green) and lysosomes (red) staining (A,C). Summarized data for colocalization (B,D). Each bar is the mean \pm SEM of 6 experiments. *, $P < 0.05$ vs. Control; #, $P < 0.05$ vs. H/R; ^s, $P < 0.05$ vs. BAY + H/R. SEM, standard error of the mean; H/R, hypoxia/reoxygenation.

yellow fluorescent dots and red fluorescent dots reduced by PP2 treatment (Figure 4A,4B). These data demonstrated that adenosine A2BR activation could inhibit myocardial autophagy and mitophagy by activating Src tyrosine kinase under I/R conditions.

Adenosine A2BR activation inhibits myocardial autophagy and mitophagy under I/R conditions by regulating FUNDC1

The immunofluorescence results demonstrated that

compared to that in the H/R group, the expression of mitochondrial FUNDC1 was reduced by BAY, but this effect was reversed by PP2 (Figure 6A,6B). To identify mitophagy-associated proteins that may play an important role in adenosine A2BR-induced mitophagy inhibition in I/R conditions, we used online databases (STRING, <https://string-db.org/>; INBIO, <https://inbio-discover.com/>) to analyse the interaction between Src tyrosine kinase and different mitophagy receptors and found that there was a strong correlation between Src tyrosine kinase

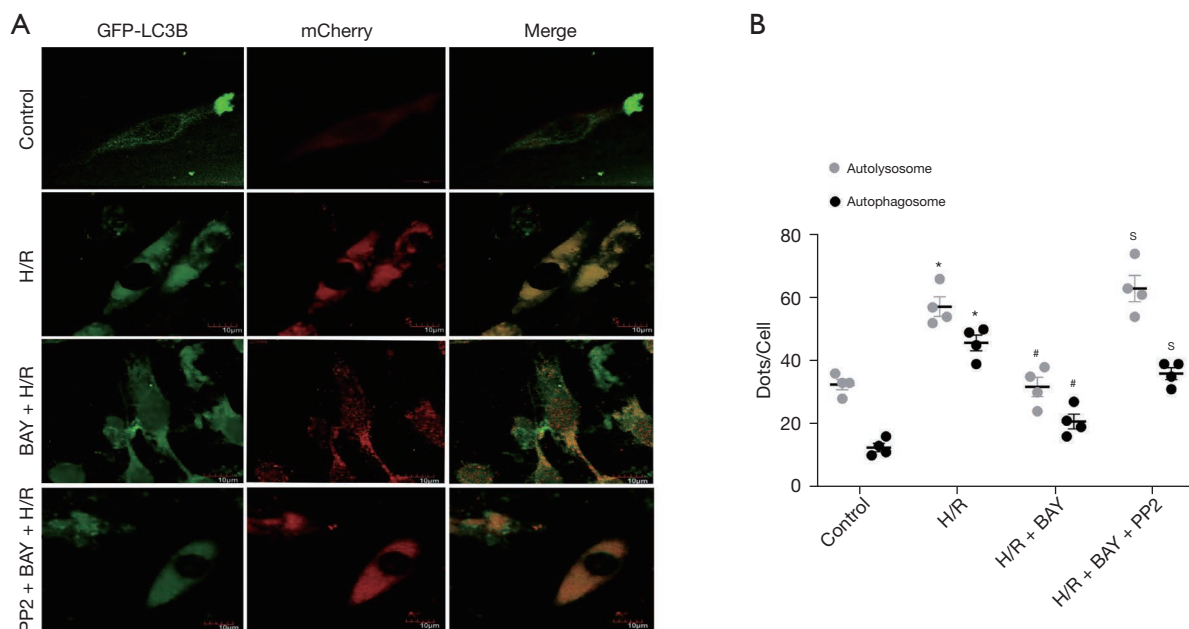


Figure 4 The yellow and red puncta represent autophagosome and autolysosomes, respectively. Summarized data for colocalization (A,B). Each bar is the mean \pm SEM of 4 experiments. *, $P < 0.05$ vs. Control; #, $P < 0.05$ vs. H/R; ^s, $P < 0.05$ vs. BAY + H/R. SEM, standard error of the mean; H/R, hypoxia/reoxygenation.

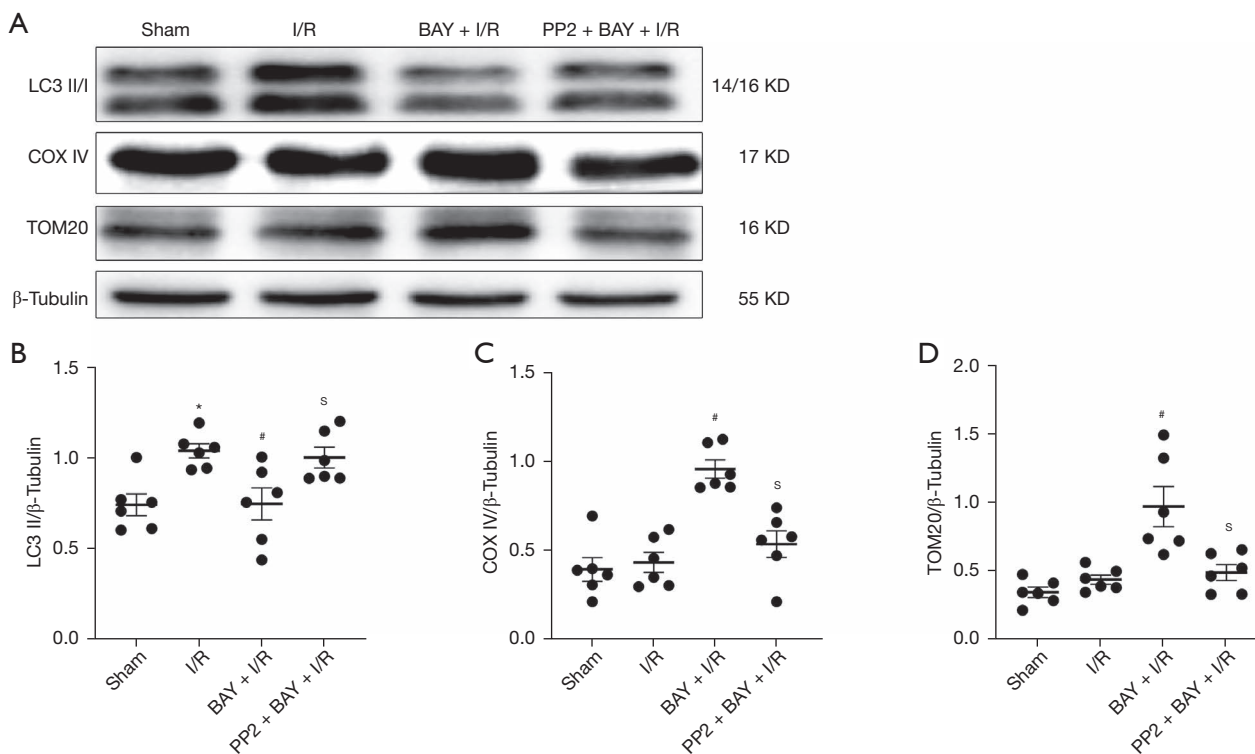


Figure 5 LC3, COX IV and TOM20 protein expression (A). Summarized data for LC3 II, COX IV and TOM20 expressions (B-D). Each bar is the mean \pm SEM of 6 experiments. *, $P < 0.05$ vs. Control; #, $P < 0.05$ vs. I/R; ^s, $P < 0.05$ vs. BAY + I/R. SEM, standard error of the mean; I/R, ischaemia/reperfusion.

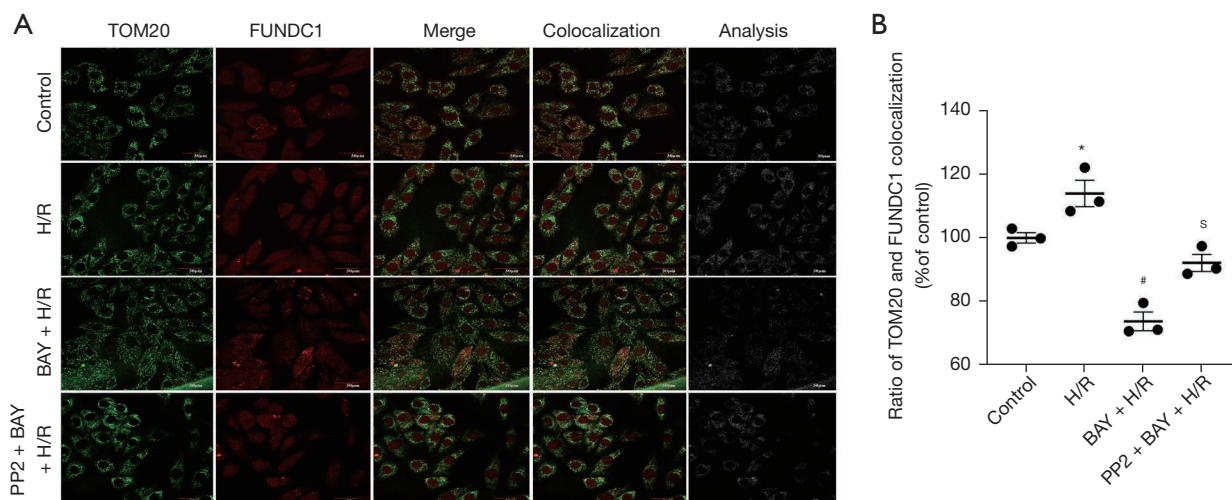


Figure 6 Confocal images of Cy3-labeled FUNDC1 (red) and FITC-labeled TOM20 (green) (A). Ad-mCherry-GFP-LC3B was used to assess autophagy flow (B). Each bar is the mean \pm SEM of 3 experiments. *, $P < 0.05$ vs. Control; #, $P < 0.05$ vs. H/R; ^s, $P < 0.05$ vs. BAY + H/R. SEM, standard error of the mean; H/R, hypoxia/reoxygenation.

and FUNDC1 (Figure 7A,7B). To further confirm the relationship between Src tyrosine kinase and FUNDC1, CoIP was performed. The results showed that mitochondrial FUNDC1 co-precipitated with Src tyrosine kinase after BAY was added (Figure 7C). Western blotting demonstrated that mitochondrial FUNDC1 expression was significantly decreased by BAY, and this effect was inhibited by PP2 (-0.07 , 95% CI: -0.37 to -0.22 ; $P=1.000$) (Figure 7D). These results suggested that adenosine A2BR activation may inhibit myocardial autophagy and mitophagy by regulating mitochondrial FUNDC1 by activating Src tyrosine kinase.

Discussion

Key findings

In the present study, adenosine A2BR activation may inhibit myocardial mitophagy by downregulating expression of the mitochondrial FUNDC1 by activating Src tyrosine kinase under reperfusion conditions and could increase the interaction between Src tyrosine kinase and FUNDC1.

Explanations of findings

The mitochondrion, which is called the “power factory” of the cell, is an important and unique organelle in eukaryotes. The main function of mitochondria is to synthesize ATP through the tricarboxylic acid cycle and oxidative

phosphorylation. Therefore, mitochondria are the centre of energy conversion and output via the metabolic process in most cells. In addition, mitochondria also exert a critical role in the generation of ROS, fatty acid metabolism, calcium balance, and cell signal transduction (26). Mitochondrial dysfunction involves many cell activities, such as apoptosis, aging and disease. Mitochondrial damage is a key factor in cardiac ischemia reperfusion injury (27). It is widely accepted that autophagy is a double-edged sword in myocardial I/R injury (28). Increasing mitophagy can alleviate cardiac function injury and ventricular remodelling after coronary ligation, while decreasing mitophagy can exacerbate injury (29).

Adenosine A2BR is a kind of G-protein coupled receptor (GPCR) that is 332 amino acids in length with a molecular weight of approximately 36 kDa (30). In this study, because 10 nM BAY could induce stable and significant inhibitory effects on myocardial autophagy and mitophagy, this do was used in further experiments. Until recently, adenosine A2BR remained poorly characterized due to its low affinity for endogenous ligands. Several studies demonstrated that A2BR activation may reduce inflammation and promote tissue restitution by regulating immune cells alternative macrophage activation (31-33). According to previous studies, adenosine stimulates IL-10 through A2BR. In addition, adenosine receptor activation plays a role by interfering with the translation inhibition of IL-10 3'-UTR (34). In the study of sepsis, there are

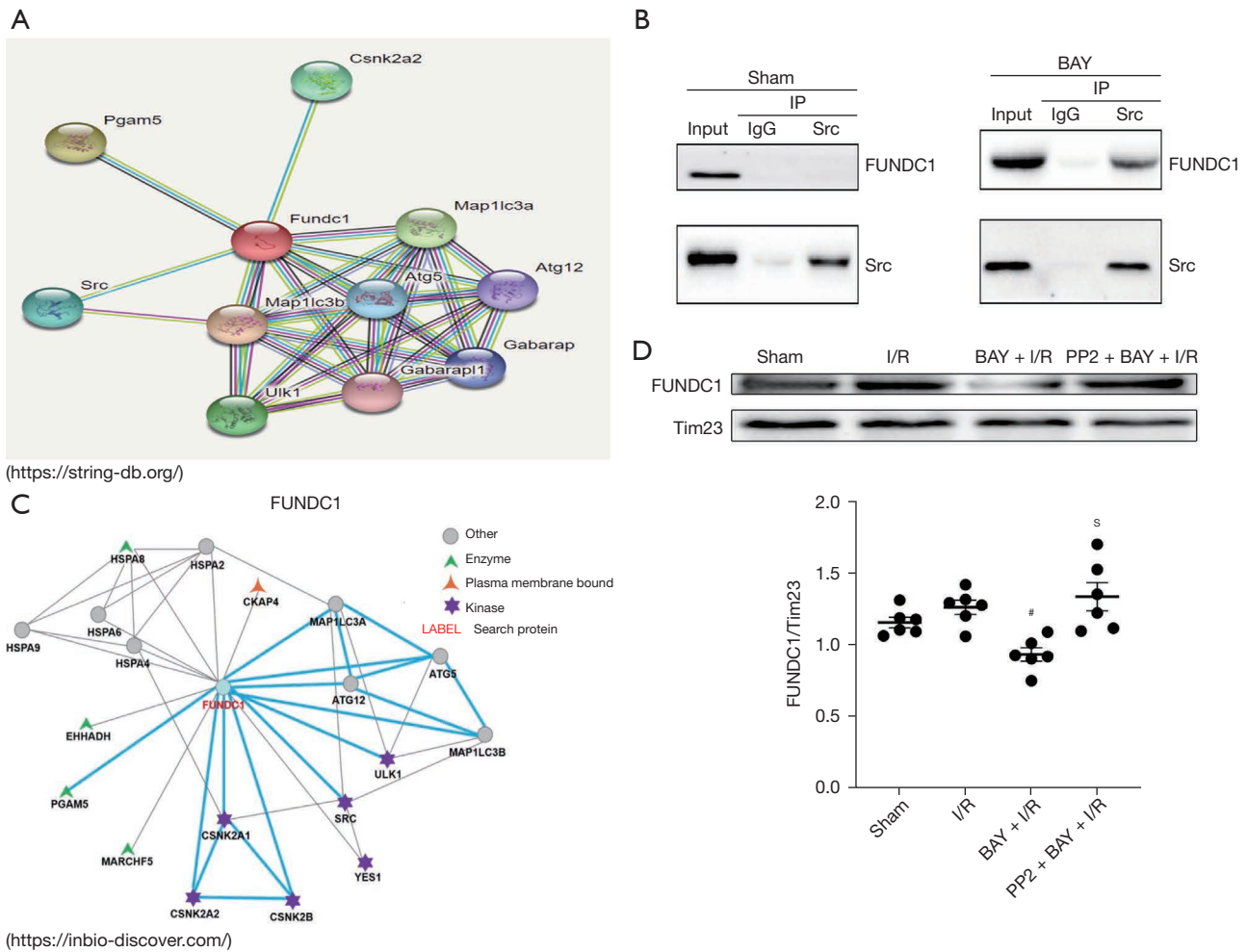


Figure 7 The relationship between Src tyrosine kinase and FUNDC1 in adenosine A2BR activation provoked myocardial autophagy and mitophagy inhibition under I/R conditions. The protein-protein interaction was analyzed by STRING (A) and INBIO (B). Mitochondrial FUNDC1 and Src tyrosine kinase were immunoprecipitated with an anti-Src antibody. Normal IgG was used as a control (C). Western blotting images of mitochondrial FUNDC1 expression. Summarized data for FUNDC1 expression (D). Each bar is the mean ± SEM of 6 experiments. #, P<0.05 vs. I/R; §, P<0.05 vs. BAY + I/R. IP, immunoprecipitation; SEM, standard error of the mean; I/R, ischaemia/reperfusion.

data proving that A2BR can prevent sepsis-induced death by inhibiting excessive inflammation (35). Not only that, but also study have pointed out that adenosine activation can enhance IL-10-mediated STAT3 signal transduction and M2c macrophage activation (36). A previous study showed that A2BR activation may induce strong protection against I/R injury by reducing oxidative stress by targeting mitochondria (20,37). Therefore, adenosine A2BR is an essential target in myocardial I/R injury (38). According to our research, Adenosine A2BR activation market is affected by myco-cardial autophagy and mitophagy under I/R conditions The mCherry-GFP-LC3B expression vector, A

tandem fluorescent LC3, was used to further monitor the autophagy flow. Our study demonstrated that adenosine A2BR activation could reduce myocardial I/R injury by inhibiting mitochondrial complex I by activating Src tyrosine kinase (20). However, we showed that adenosine A2BR activation markedly inhibited myocardial autophagy and mitophagy, which may attenuate the protective effect of adenosine A2BR in I/R conditions.

Comparison with similar researches

Mitophagy-associated proteins are key factors in

initiating mitophagy and determining selectivity, and Src tyrosine kinase regulates the activity of a variety of mitophagy-associated proteins by promoting tyrosine phosphorylation (21). This selectivity is achieved by identifying the LC3-interacting region (LIR). We demonstrated that adenosine A2BR activation could inhibit myocardial autophagy and mitophagy by activating Src tyrosine kinase under I/R conditions. In our study, the adenosine A2B receptor is activated through the selective adenosine A2B receptor agonist BAY60-6583, while in the literature, the adenosine A2B receptor is activated and suppressed through adenovirus injection, and the different activation methods may affect the final experimental results. Moreover, The animal models and animal species used in the research in the literature are different from our research (16). We use adult male Wistar rats to simulate the ischemia reperfusion model. In the article, C57 mice are used to establish the myocardial infarction model. Different models and animal strains have a significant impact on the experimental results. In the future, we can further use transgenic animals for verification.

Strengths and limitations

To date, FUNDC1, which is composed of 155 amino acids, is involved in mitophagy induced by hypoxia. FUNDC1 is a transmembrane protein that is located in the outer mitochondrial membrane and contains a typical sequence that interacts with the LC3 protein family. When tyrosine 18 and serine 13 of FUNDC1 are phosphorylated by Src tyrosine kinase or the protein kinase CK2, their activity is inhibited. When these sites are dephosphorylated, FUNDC1 is activated and binds to the LC3 protein family (39). Besides these phosphorylation sites, the effects of other FUNDC1 tyrosine sites are still not clear. In this study, only FUNDC1 has been discussed, but the Src tyrosine kinase regulates the content of FUNDC1 and thus affects autophagy. How to regulate FUNDC1 on the mitochondrial membrane and whether FUNDC1 has the binding site of the Src tyrosine kinase still need further study. In the current study, adenosine A2BR activation may inhibit myocardial autophagy and mitophagy by downregulating mitochondrial FUNDC1 by activating Src in I/R conditions. In this study, we found that adenosine A2BR may be activated by activating Src tyrosine kinase and decreasing FUNDC1 expression level, thus suppressing the autophagy of fungal mitochondria in I/R model. It betters the study on occurrence and regulation mechanism of

mitochondrial autophagy, which is helpful to future targeted treatment of adenosine on mitochondrial damage through biochemical and pharmacological methods.

Implications and actions needed

We propose that the tyrosine phosphorylation of FUNDC1 may alter its spatial conformation and thus affect its binding to mitochondria. Moreover, the interaction between Src tyrosine kinase and FUNDC1 could be strengthened by adenosine A2BR activation. All the data show that A2BR activation may decrease the binding of FUNDC1 to mitochondrial outer membrane by increasing FUNDC1 tyrosine phosphorylation via increasing Src tyrosine kinase activity.

Conclusions

Adenosine A2BR activation may inhibit myocardial mitophagy by downregulating the mitochondrial FUNDC1 by activating Src tyrosine kinase under reperfusion conditions and could increase the interaction between Src tyrosine kinase and FUNDC1.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at <https://cdt.amegroups.com/article/view/10.21037/cdt-22-468/rc>

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[com/article/view/10.21037/cdt-22-468/coif](https://doi.org/10.21037/cdt-22-468/coif)). All authors report that this work was supported by grant 81700324 from the National Natural Science Foundation of China, grant 226Z7711G from the Hebei Provincial Special Fund for Central Guiding Local Science and Technology Development Plan and grant C20220354 from Hebei Provincial Financial Assistance for the Introduction of Overseas Researchers, and grant X2019173 from the NCST University Student Innovation Project. The authors have no other conflicts of interest to declare.

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