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Hydrogen sulfide protects against myocardial ischemia and reperfusion injury by activating AMP-activated protein kinase to restore autophagic flux

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ABSTRACT

How hydrogen sulfide (H_2S) protects against myocardial ischemia–reperfusion (I/R) injury is poorly understood. By using a slow-releasing H_2S donor, we investigated if H_2S protected against myocardial I/R injury by activating AMPK and restoring I/R-impaired autophagic flux. Male rats received anterior descending coronary artery occlusion followed by reperfusion. The H_2S donor ADT and/or the AMPK inhibitor, compound C (CC), were administered after occlusion. Infarction was analyzed histologically. AMPK activation was assessed in the ischemic heart by analyzing phosphorylation of AMPK and S6 ribosomal protein. Autophagy was assessed by analyzing the following markers: microtubule-associated protein 1 light chain 3 (LC3) I and II, lysosome associated membrane protein-2 (LAMP-2), P62 and beclin-1. We further investigated if blocking autophagic flux with chloroquine abolished ADT cardioprotection in vivo. Myocardial I/R reduced serum H_2S levels, which was elevated by ADT. ADT enhanced AMPK activation and reduced infarction following I/R, and both effects were abolished by AMPK inhibition. Myocardial I/R induced autophagosome accumulation, as evidenced by the increased ratios of LC3-II/LC3-I, upregulation of beclin-1 and P62 and reduction in LAMP-2. ADT blunted these autophagic changes induced by I/R, indicating that ADT restored I/R-impaired autophagic flux. The AMPK inhibitor CC blocked ADT effects on restoring I/R-impaired autophagy flux. Moreover, chloroquine pretreatment abolished cardioprotection of ADT and increased autophagosome accumulation in the ADT-treated heart following I/R. In conclusion, AMPK activation and subsequent restoration of I/R-impaired autophagic flux are unrecognized mechanisms underlying cardioprotective effects conferred by H_2S donors.

1. Introduction

Swift coronary reperfusion is a common strategy to treat myocardial ischemia. However, reperfusion itself leads to damage, known as ischemia–reperfusion (I/R) injury [1]. Hence, to explore effective treatments to mitigate myocardial I/R injury is needed [2]. Hydrogen sulfide (H_2S), a well-known poisonous gas, is increasingly recognized as an important gasotransmitter [3]. Although increasing evidence suggested that H_2S donors are potentially novel drug therapies for myocardial ischemia [4-5], we know little about the mechanisms underlying H_2S protection following

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myocardial I/R. We recently reported that ADT [5-(4-methoxyphenyl)-3H-1,2-dithiole-3-thione], a clinical drug approved in China and Europe, protected against cerebral ischemia as a slow-releasing organic H_2S donor [6]. We also reported that ADT-based H_2S donors activated AMPK more robustly than the well-established AMPK activator metformin [7]. However, it remains unknown if H_2S donors act through AMPK activation to protect against myocardial I/R injury.

Activated AMPK inhibits mTOR and thereby induces autophagy. However, whether autophagy plays a beneficial or deleterious role following myocardial I/R is elusive. By degrading cytosolic lipids and proteins into free fatty acids and amino acids that can be reused, myocardial ischemia-induced autophagy may play a pro-survival role. On the other hand, autophagy also promotes cell death and thus exacerbates infarct damage following myocardial I/R [8-9]. Ischemia enhances AMPK activation in the mouse heart, which in turn induces protective autophagy. In addition, reperfusion further enhances autophagosome formation. However, AMPK activation is not enhanced by reperfusion. Instead, myocardial I/R-enhanced autophagosome formation is dependent on beclin-1 upregulation [10]. More strikingly, I/R-induced beclin-1-dependent autophagosome formation is detrimental, since myocardial I/R only promotes autophagosome formation but not autolysosome clearance, thus leading to impairment of autophagic flux [11]. To elucidate the mechanism underlying H_2S cardioprotection, we investigated if enhancement of AMPK activation by a H_2S donor following myocardial I/R restored I/R-impaired autophagic flux and if this is the mechanism by which H_2S protects against myocardial I/R injury.

2. Materials and methods

2.1. Rat model of myocardial ischemia–reperfusion injury

The animal protocols were approved by the Committee for Experimental Animals of Soochow University and in accordance with the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication vol. 25 no. 28, revised 1996). Adult male Sprague-Dawley rats weighing 270–320 g were purchased from JOINN Laboratories (Suzhou, China). Before surgery, rats were housed in quiet rooms and had free access to food and water for one week. Rat model of myocardial ischemia–reperfusion injury was built as our previous reports [8,12], and myocardial I/R injury were induced in rats via 30 min of left descending coronary artery occlusion, followed by 4 h of reperfusion.

2.2. Experimental groups and drug administration

As Supplementary Fig. 1 and Supplementary Table 1 illustrated, rats were randomly divided to nine groups. ADT, control vehicle for ADT, the autophagic flux blocker chloroquine (CQ) and normal saline (NS) were administered via intraperitoneal injection while the AMPK inhibitor compound c and DMSO administered via right jugular vein injection. CQ and NS were given 1 h before surgical operation. All the rest of drugs were administered immediately at the beginning of reperfusion. The dose and administration route for ADT were chosen based on our previous publication, which showed that intraperitoneally administered ADT at 50 mg/kg conferred robust protection in a well-established mouse model of cerebral ischemia [6]. In addition, it has been reported that compound c at 250 μg/kg abolishes cardioprotection conferred by AMPK activators [13] and that CQ at 10 mg/kg blocks the autophagy flux following myocardial I/R [11,14].

2.3. Hemodynamics

A catheter full of normal saline containing 10 IU/mL heparin was inserted into the right carotid artery. The catheter was connected to the Medlab-U/4C501H system (Nanjing Meiyi Technology, China) to continuously monitor heart rate (HR), mean arterial blood pressure (MAP) and rate pressure product (RPP; systolic blood pressure × heart rate).

2.4. Determination of myocardial infarct sizes

At 4 h after reperfusion, the coronary artery was immediately re-occluded. Evans Blue solution (5%, Sigma–Aldrich, St. Louis, MO, USA) was administered via right jugular vein to stain normal region blue and show the area at risk (AAR) of left ventricle (LV). Then, the LV was cut into 6 transverse slices of 2-mm thickness. Slices were incubated in phosphate buffer (PBS, pH 7.4) containing 1% TTC (Sigma–Aldrich, St. Louis, MO, USA) at 37 °C for 15 min. Then, the slices were fixed in 10% formaldehyde solution (pH 7.4) for 24 h. The infarct tissue was separated from the AAR under a dissecting microscope. Infarct sizes were expressed as the percent of the weight of the tissue collected from infarct areas to the weight of the tissue from AAR [8,12].

2.5. Histopathology

At 4 h after reperfusion, the hearts were harvested, rinsed with ice-cold PBS and fixed in 10% formaldehyde solution for 24 h. After dehydration and embedding in paraffin wax, the heart was sectioned into 4-μm thick slices. The slices were then stained with hematoxylin-eosin (HE) as previously described [1].

2.6. Determination of H_2S concentrations in the blood

At 45, 120 and 240 min after reperfusion, the blood was collected and centrifuged for 3 min at 3000 rpm. The serum (100 μL) was collected and mixed with 1% zinc acetate (150 μL) and 10% trichloroacetic acid (300 μL), followed by the addition of 30 mM $FeCl_3$ (133 μL) in 1.2 M HCl and 20 mM N,N-dimethylphenylendiamine sulfate (100 μL) in 7.2 M HCl. After incubation for 20 min, absorbance was measured at 670 nm with infinite M200 PRO (TECAN). The concentrations of H_2S were calculated from a standard curve of NaHS (6.25–200 μM) [6,16].

2.7. Measurement of reactive oxygen species (ROS) levels

The oxidant-sensitive fluorogenic probe dihydroethidium (DHE, Sigma–Aldrich, St. Louis, MO, USA) was used to measure ROS generation as previously described [15]. Briefly, at 4 h after reperfusion, the hearts were collected and sectioned into 10-μm thick slices. The slices were incubated with 10 μM DHE in PBS (pH 7.4) in the dark for 30 min at 37 °C. Fluorescent images were obtained at the excitation wavelength of 555 nm using a laser scanning confocal microscope (Carl ZEISS LSM700, Prenzlauer, Berlin, Germany).

2.8. Western blot analysis

Proteins were separated by SDS-PAGE gels and transferred to PVDF membranes. After blocking with non-fat milk, the membranes were incubated with primary antibodies against following proteins overnight at 4 °C: AMPK, phosphorylated AMPK (p-AMPK), S6 ribosomal protein, phosphorylated S6 ribosomal protein, beclin-1 (1:1000; Cell Signaling Technology, Danvers, MA, USA), microtubule-associated protein 1 light chain 3 (LC3, 1:1000;

Abcam, Cambridgeshire, UK), lysosome associated membrane protein-2 (LAMP-2, 1:200; Santa Cruz Biotechnology, California, USA) and P62 (1:1000; Sigma–Aldrich, St. Louis, MO, USA). After rinsing, membranes were probed with appropriate horseradish peroxidase-conjugated secondary antibodies (1:1000; Cell Signaling Technology, Danvers, MA, USA). β-actin was assayed as loading controls.

2.9. Statistical analysis

All data were expressed as means ± SEM. One-way analysis of variance (ANOVA), followed by Tukey–Kramer post-hoc tests, were used for the comparisons of data using GraphPad Prism (San Diego, CA, USA).

3. Results

3.1. Effects of ADT on mortality and systemic hemodynamics

As shown in Supplementary Table 1, there were no differences in mortality among groups ($P > 0.05$). The basal heart rates (HRs), mean arterial blood pressure (MAP) and the rate-pressure product (RPP) were comparable among all groups. During the occlusion and reperfusion, myocardial I/R significantly decreased MAP and RPP ($P < 0.05$, I/R + vehicle *vs.* Sham). Compared to I/R rats receiving vehicle (I/R + vehicle), there was a significant recovery in MAP and RPP in I/R rats treated with ADT (I/R + ADT) after 2 h of reperfusion ($P < 0.05$). The recovery effect was abrogated by the AMPK inhibitor compound c (CC) ($P < 0.05$, I/R + ADT + CC *vs.* I/R + ADT + DMSO or I/R + ADT). During occlusion or reperfusion, HR was not different among all groups.

3.2. ADT decreased myocardial infarction and elevated serum H_2S levels

There were no differences in the areas at risk (AAR) among the groups receiving I/R (Fig. 1B, $P > 0.05$). Myocardial I/R resulted in infarction (I/R + vehicle), which was significant reduced by ADT (Fig. 1C, $P < 0.05$, I/R + ADT *vs.* I/R + vehicle). ADT protection was abrogated by the AMPK inhibitor CC (Fig. 1C, I/R + ADT + CC *vs.* I/R + ADT + DMSO or I/R + ADT).

We also confirmed the protective effects of ADT with HE staining. Compared to sham-operated rats, the loss of the myoplasm, reduction in striation and rupture of myocardial cells were evident

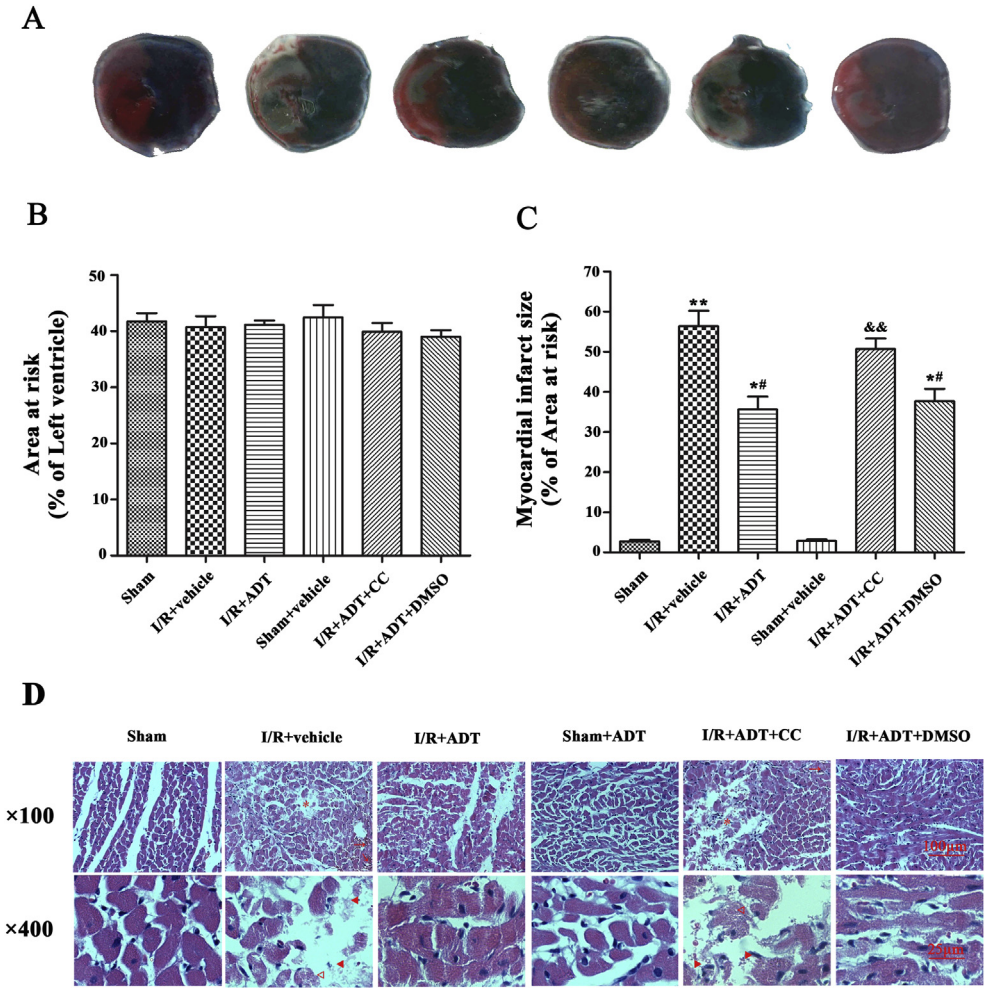

Fig. 1. The hydrogen sulfide donor ADT reduced myocardial I/R injury and the protective effects were abolished by the AMPK inhibitor CC. (A) Representative slices of the left ventricle (LV) stained by Evans blue and TTC. (B) The weight of the area at risk (AAR), presented as the percentage of the weight of AAR/the weight of LV, was not different among the experimental groups. (C) Infarct volumes (presented as % of AAR) at 4 h reperfusion. $n = 7$ for Sham group or Sham + ADT group and $n = 10$ for other groups, $^{**}P < 0.01$ and $^{*}P < 0.05$ *vs.* Sham, $^{\#}P < 0.05$ *vs.* I/R + vehicle, $^{\&\&}P < 0.01$ *vs.* I/R + ADT or I/R + ADT + DMSO. (D) HE staining further confirmed ADT protective effects following myocardial I/R. The images were shown at 100× or 400× magnifications (scale bars: 25 μm or 100 μm). Representative images from three independent experiments, ↑ denoted erythrocyte extravasation, * denoted striation reduction, △ and ▲ denoted myocyte loss and rupture.

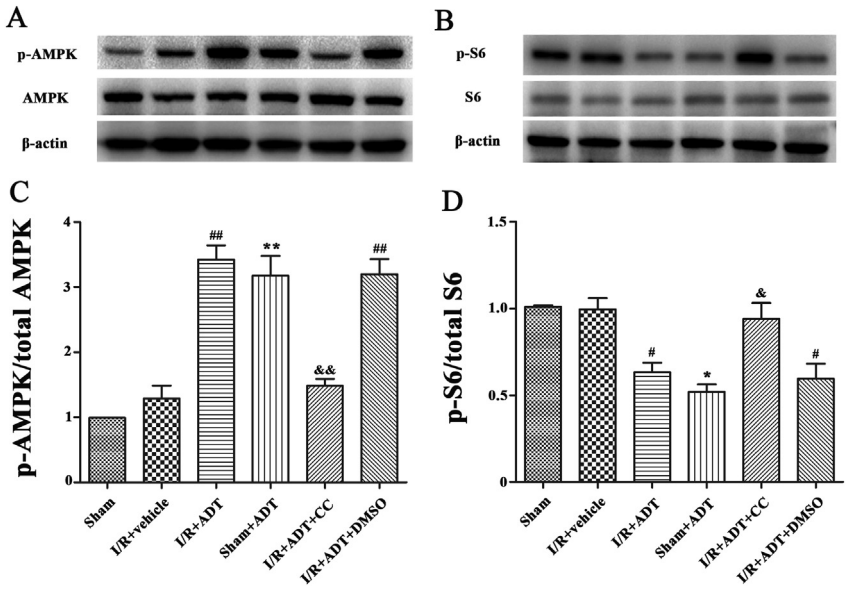

Fig. 2. The hydrogen sulfide donor ADT enhanced AMPK activation (phosphorylation) following myocardial I/R. (A, C) Representative immunoblots and densitometric analysis of p-AMPK and total AMPK in the ischemic heart at 4 h reperfusion. (B, D) Representative immunoblots and densitometric analysis of p-S6 and total S6 in the ischemic heart at 4 h reperfusion. $n = 5$ per group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ *vs.* I/R + vehicle, $^{*}P < 0.05$ and $^{**}P < 0.01$ *vs.* Sham, $^{\&}P < 0.05$ and $^{\&\&}P < 0.01$ *vs.* I/R + ADT or I/R + ADT + DMSO.

and the outlines of the myocardial fibers were hardly recognized in some areas in the heart of rats receiving myocardial I/R (Fig. 1D, I/R + vehicle). These pathologic changes were ameliorated in I/R rats treated with ADT (I/R + ADT). ADT-improved histological outcomes were largely abolished by the AMPK inhibitor compound c (I/R + ADT + CC).

To investigate if ADT released H_2S, we measured serum H_2S concentrations. Compared to sham, myocardial I/R significantly reduced serum H_2S concentrations after 45 min of reperfusion (Supplementary Fig. 2, I/R + vehicle *vs.* Sham). ADT, administered immediately after reperfusion, persistently elevated serum H_2S levels from 45 min till 4 h after injection ($P < 0.05$, I/R + ADT and I/R + ADT + CC *vs.* I/R + vehicle or Sham).

3.3. ADT enhanced AMPK activation and autophagic flux following myocardial I/R

As shown in Fig. 2, myocardial I/R did not enhance AMPK activation (phosphorylation) in the ischemic heart (I/R + Vehicle) 4 h after reperfusion. ADT significantly increased the ratio of p-AMPK/AMPK in the ischemic heart ($P < 0.01$, I/R + ADT *vs.* I/R + vehicle), which was blunted by the AMPK inhibitor compound c ($P < 0.01$, I/R + ADT + CC *vs.* I/R + ADT or I/R + ADT + DMSO). Phosphorylation of S6 protein has been reported to be inhibited by activated AMPK. Consistently, the ratio of p-S6/S6 was also reduced by ADT ($P < 0.05$, I/R + ADT *vs.* I/R + vehicle), which was blunted by CC ($P < 0.05$, I/R + ADT + CC *vs.* I/R + ADT + DMSO). Moreover, ADT increased AMPK phosporylation and decreased p-S6/S6 in the heart at baseline (Sham + ADT *vs.* Sham).

Increased ratios of LC3-II/I are the markers for autophagosome formation. LAMP-2, a critical determinant of autophagosome-lysosome fusion, is essential for autophagosome clearance and autophagic flux [17]. Beclin-1 up-regulation and LAMP-2 down-regulation are the mechanisms underlying impaired autophagic flux following myocardial I/R [11,14]. Moreover, P62, an adaptor protein that links aggregated proteins in autophagosomes and is degraded in autolysosomes, has been used to indicate autophagic flux [10,17]. Compared to sham, myocardial I/R increased LC3-II/I ratios, up-regulated beclin-1 and P62 protein levels but reduced LAMP-2 protein levels in the ischemic heart at 4 h after reperfusion (Fig. 3). These results suggested that myocardial I/R led to the impairment of autophagic flux by inducing autophagosome formation but impairing autophagosome clearance. ADT blunted I/R-induced increase in LC3-II/I ratios, beclin-1 and P62 levels and decrease in LAMP-2 protein levels ($P < 0.05$, I/R + ADT *vs.* I/R + vehicle), indicating that ADT restored I/R-impaired autophagy flux. The effects of ADT on LC3-II/I ratios, beclin-1, P62 and LAMP-2 was blunted by CC. At basal conditions, ADT had no effects on beclin-1, P62 and LAMP-2 protein levels ($P > 0.05$), but enhanced LC3-II/I ratios ($P < 0.05$, Sham + ADT *vs.* Sham).

ROS likely accounts for beclin-1 upregulation and LAMP-2 down-regulation induced by I/R [11,14]. We used DHE to measure ROS generation as previously described [15]. ADT remarkably lowered the DHE fluorescent intensity 4 h after reperfusion in the rats receiving I/R (Supplementary Fig. 3, $P < 0.05$, I/R + ADT *vs.* I/R + vehicle). The inhibitory effects of ADT on ROS generation were blunted by co-administration of the AMPK inhibitor CC ($P < 0.05$, I/R + ADT + CC *vs.* I/R + ADT + DMSO).

3.4. The autophagic flux blocker CQ abolished ADT cardioprotection following myocardial I/R

To further investigate if ADT cardioprotection was dependent on improved autophagic flux, we pretreated the rats with CQ to block autophagic flux as previously reported [11]. As shown in Supplementary Table 1, the recovery effect of ADT on MAP and RPP was abrogated by CQ ($P < 0.05$, I/R + ADT + CQ *vs.* I/R + ADT + NS). Compared to normal saline (NS), CQ significantly increased the infarction in ADT-treated rats (Supplementary Fig. 4, $P < 0.05$, I/R + ADT + CQ *vs.* I/R + ADT or I/R + ADT + NS). CQ alone had no effects on infarction ($P > 0.05$, I/R + vehicle *vs.* I/R + CQ). Moreover, ADT effects on restoring I/R-impaired autophagic flux, as indicated by the attenuation of I/R-induced increase in LC3-II/I ratios and P62 protein levels and reduction in LAMP-2 protein levels, were reversed by CQ (Fig. 4, $P < 0.05$, I/R + ADT + CQ *vs.* I/R + ADT + NS or I/R + ADT). CQ alone had no effect on these markers compared to vehicle (Fig. 4, $P > 0.05$, I/R + vehicle *vs.* I/R + CQ).

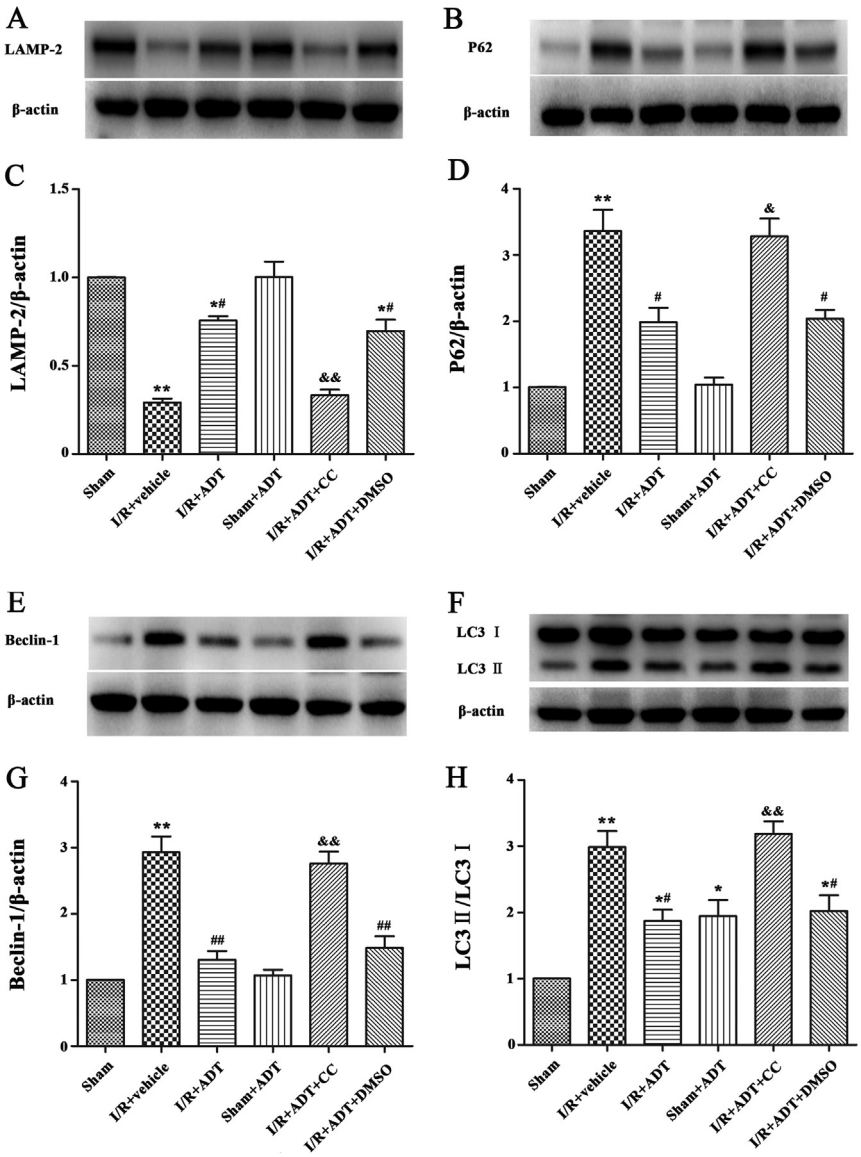

Fig. 3. The H_2S donor ADT enhanced autophagic flux following myocardial I/R at 4 h reperfusion. (A, C) Representative immunoblots and densitometric analysis of LAMP-2 in the ischemic heart. (B, D) Representative immunoblots and densitometric analysis of P62 in the ischemic heart. (E, G) Representative immunoblots and densitometric analysis of beclin-1 in the ischemic heart. (F, H) Representative immunoblots and densitometric analysis of LC3 I and LC3 II in the ischemic heart. $^{*}P < 0.05$ and $^{**}P < 0.01$ *vs.* Sham, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ *vs.* I/R + vehicle, $^{\&}P < 0.05$ and $^{\&\&}P < 0.01$ *vs.* I/R + ADT or I/R + ADT + DMSO, $n = 5$ per group.

4. Discussion

Our study presented three major findings. First, ADT administered after ischemia elevated serum levels of H_2S and protected against myocardial I/R injury. Second, cardioproection conferred by the H_2S donor ADT was dependent on AMPK activation. Third, ADT-induced post-reperfusion AMPK activation restored myocardial I/R-impaired autophagic flux, which is likely the mechanism underlying cardioprotection conferred by H_2S.

H_2S exerts a wide influence on the cardiovascular system. Overexpressing cystathionine-γ-lyase, the major H_2S synthase, in the cardiovascular system, ameliorates, while deletion of cystathionine-γ-lyase exacerbates myocardial ischemia injury following ischemia–reperfusion [18]. Consistently, a cystathionine-γ-lyase inhibitor increases whereas the H_2S donor NaHS reduces the myocardial infarction in vivo and in vitro [4,5]. Gaseous H_2S also protects against myocardial ischemia injury [19,20]. However, the effects of H_2S are concentration-dependent. Excessive H_2S release from inorganic donors such as NaHS and Na_2S may lead to serious toxic effects. Thus, we focused on slow-releasing organic H_2S donors since they have greater clinical translatability.

ADT is clinically used as a hepatoprotective and anti-xerostomia drug [21]. ADT, as a slow-releasing H_2S donor, protected neurons from glutamate oxidative toxicity and protected the brain from cerebral ischemia [6,23]. In vivo, ADT is rapidly metabolized into ADT-OH, which is the most widely used moiety for synthesis of slow releasing H_2S donors [22]. In this study, myocardial I/R reduced endogenous H_2S levels, implying that reduction in endogenous production of H_2S may contribute to pathogenesis following myocardial I/R. ADT, administered after ischemia, persistently increased serum H_2S levels starting at 45 min till 4 h after injection and remarkably reduced myocardial infarction. These results suggest that slow-releasing H_2S donors have great translational significance in terms of treating myocardial ischemia.

Recently we showed that H_2S donors activated AMPK in several cell types [7,23]. In this study, ADT at the protective dose up-

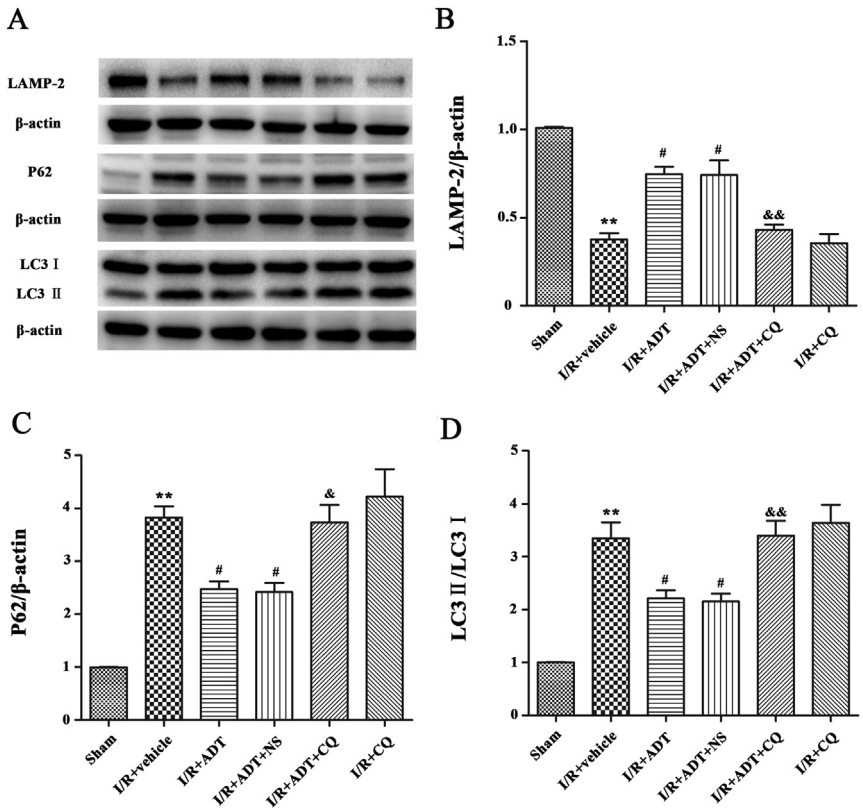

Fig. 4. The autophagic flux blocker CQ abolished ADT effects on restoring autophagic flux in the ischemic heart following myocardial I/R. (A) Representative immunoblots of LAMP-2, P62, LC3 I and LC3 II in the ischemic heart at 4 h reperfusion. (B–D) Densitometric analysis of LAMP-2, p62 and the ratios of LC3 II/I. $^{**}P < 0.01$ *vs.* Sham, $^{\#}P < 0.05$ *vs.* I/R + vehicle, $^{\&}P < 0.05$ and $^{\&\&}P < 0.01$ *vs.* I/R + ADT or I/R + ADT + NS, $n = 5$ per group.

regulated AMPK activation following myocardial I/R. Moreover, the AMPK inhibitor CC down-regulated AMPK activation and abolished the cardioprotection of ADT, suggesting that activating AMPK is the mechanism underlying cardioprotection of H_2S donors.

AMPK activation is well known to induce autophagy. Macroautophagy consists of two stages [24]. First, the cargos are segregated and wrapped into the double membrane-bound autophagosomes, then, the autophagosomes are fused with lysosomes and degraded. This complete autophagic flux is essential for keeping intracellular homeostasis and cell survival. The role of myocardial I/R-induced autophagy is elusive. Myocardial ischemia alone induces AMPK activation-dependent autophagy, which is protective during the ischemic phase. However, reperfusion-enhanced autophagosome formation exacerbates infarct damage during the reperfusion phase [10]. Moreover, AMPK activation is not enhanced by reperfusion. Indeed, myocardial I/R-induced autophagosome formation is mediated by beclin-1 upregulation and LAMP2 reduction. It is suggested that myocardial I/R stimulate autophagosome formation but not lysosome-autophagosome formation, thus impairing autophagosome clearance and exacerbating myocardial ischemia injury following reperfusion [11,14].

Since AMPK activation-induced autophagy during the ischemic phase is protective and since AMPK activation is not enhanced following myocardial reperfusion, we hypothesize that enhancing AMPK activation following reperfusion can restore I/R-impaired autophagic flux and this is the mechanism underlying cardioprotection conferred by H_2S following myocardial I/R. In support of the hypothesis, we observed that ADT enhanced AMPK activation and improved myocardial I/R-impaired autophagic flux in the ischemic heart. Moreover, the promoting effects of ADT on autophagosome clearance were blunted by the AMPK inhibitor CC and by the autophagic flux blocker CQ. More relevantly, both CC and CQ significantly attenuated ADT protection against myocardial I/R injury. These results suggested that restoration of I/R-impaired autophagic flux by H_2S is likely to be AMPK activation-dependent, which was critical to H_2S cardioprotection against myocardial I/R.

In conclusion, the slow-releasing H_2S donor ADT, administered after ischemia, protected against myocardial I/R injury by activating AMPK. Moreover, we provided evidence suggesting that AMPK activation and consequent restoration of I/R-impaired autophagic flux are mechanisms underlying cardioprotective effects of H_2S donors.

Conflict of interest

All authors declare that they have not conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.017.

Transparency document

Transparency document associated with this article can be found in the online at http://dx.doi.org/10.1016/j.bbrc.2015.02.017.

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