Basic Research in Cardiology (2019) 114:39
https://doi.org/10.1007/s00395-019-0747-9

ORIGINAL CONTRIBUTION

Cardioprotection of post-ischemic moderate ROS against ischemia/reperfusion via STAT3-induced the inhibition of MCU opening

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Received: 31 March 2019 / Accepted: 19 August 2019 / Published online: 28 August 2019
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Abstract

Enhanced reactive oxygen species (ROS) at the beginning of reperfusion activated signal transducer and activator of transcription 3 (STAT3) in intermittent hypobaric hypoxia (IHH)-afforded cardioprotection against ischemia/reperfusion (I/R). However, its mechanism remains largely unknown. This study aimed to investigate the role and the downstream of STAT3 in exogenous enhanced post-ischemic ROS-induced cardioprotection using the model of moderate hydrogen peroxide postconditioning (H_2O_2PoC) mimicking endogenous ROS in IHH. Moderate H_2O_2PoC not only improved the post-ischemic myocardial contractile recovery and reduced the infarct size in isolated rat I/R hearts, but also alleviated mitochondrial calcium overload and ameliorated Ca^{2+} transients, cell contraction, and mitochondrial membrane potential in rat I/R cardiomyocytes. However, the cardioprotective effects of moderate H_2O_2PoC were abrogated by Janus kinase 2 (JAK2)/STAT3 inhibitor AG490 in rat hearts as well as adenovirus-delivered short hairpin RNA specific for STAT3 and the opener of mitochondrial calcium uniporter (MCU) spermine in rat cardiomyocytes. Notably, the moderate H_2O_2PoC-afforded cardioprotection abrogated by spermine could be rescued by STAT3 over-expression with adenovirus in rat I/R cardiomyocytes. Besides, moderate H_2O_2PoC enhanced mitochondrial STAT3 expression during I/R. A co-localization/interaction of STAT3 or phospho-STAT3^{ser727} and MCU was observed in rat cardiomyocytes with moderate H_2O_2PoC at 5 and 30 min of reperfusion but not in rat I/R cardiomyocytes. Further, STAT3 interacted with the N-terminal domain (NTD) of MCU in rat cardiomyocytes with moderate H_2O_2PoC. These findings indicated that post-ischemic moderate ROS activate STAT3 against cardiac I/R by inhibiting MCU opening via its interaction with the NTD of MCU to alleviate mitochondrial calcium overload.

Keywords Post-ischemic moderate ROS · Hydrogen peroxide postconditioning · Mitochondrial Ca^{2+} concentration · Cardiac contraction · Signal transducer and activator of transcription 3 · Mitochondrial calcium uniporter

Introduction

Myocardial ischemia/reperfusion (I/R) injury is the common clinical manifestation of the pathological scenario of ischemic heart disease. Myocardial contractile dysfunction due to I/R is partly incited by calcium overload [9]. Calcium overload in myocytes, including mitochondrial $[Ca^{2+}]_m$ and cytosolic free calcium concentration $[Ca^{2+}]_c$ overload, is especially considered as one of the primary elements resulting in cardiac I/R injury [44]. $[Ca^{2+}]_m$ overload has been proved to be the cause of $[Ca^{2+}]_c$ overload and contractile dysfunction [10, 15]. Thus, the reduction of $[Ca^{2+}]_m$ overload is an essential way to achieve cardioprotection. During myocardial I/R, mitochondrial calcium uniporter (MCU) selectively transport significant amounts of calcium from the cytosol into mitochondria [14]. The observations show that MCU serves as an important ion

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00395-019-0747-9) contains supplementary material, which is available to authorized users.

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channel in the regulation of $[Ca^{2+}]_m$ overload, such as treatment with MCU inhibitor Ru_{360} decreasing $[Ca^{2+}]_m$ overload in cardiac I/R [13], small interfering RNA targeting MCU reducing $[Ca^{2+}]_m$ overload in cardiomyocytes [40] and conditional cardiac-specific $MCU^{-/-}$ mice subjected to I/R resulting in a significant reduction of $[Ca^{2+}]_m$ overload [27]. However, the regulatory mechanism of MCU during cardiac I/R injury and cardioprotection remains unveiled.

Reactive oxygen species (ROS) production during early reperfusion should reach a threshold to trigger the ischemic preconditioning (IPC)-, ischemic postconditioning (IPoC)-, remote ischemic preconditioning (RIPC)- and intermittent hypobaric hypoxia (IHH) adaptation-induced cardioprotection [20, 42, 52, 53]. Meanwhile, ROS generated inadequately or excessively during early reperfusion are main factors of I/R injury [4, 53]. The stable form of ROS is hydrogen peroxide (H_2O_2) [4]. It has been found that moderate concentrations of H_2O_2 postconditioning (H_2O_2PoC), a model of enhanced ROS at early reperfusion in an exogenous manner, significantly promote post-ischemic left ventricular (LV) contractile recovery [53]. Besides, moderate H_2O_2 preconditioning alleviates calcium paradox in hearts and cardiomyocytes [12, 37]. However, whether moderate H_2O_2PoC protects the hearts and cardiomyocytes from calcium overload and contractile dysfunction due to I/R remains elusive. Although the inhibition of MCU seems to play a crucial role in IPC and IPoC [57, 60], whether moderate H_2O_2PoC can reduce calcium overload and improve cardiac contraction via inhibiting MCU is unknown.

Signal transducer and the activator of transcription 3 (STAT3) is a significant protein contributing to the signal transduction of cardioprotection [5, 18, 55]. Not only STAT3 acts as a transcription factor in the nucleus with its phosphorylation at tyrosine 705 and serine 727 for regulating gene transcription [3, 39, 41], but also STAT3 phosphorylated at tyrosine 705 (pY-STAT3) and serine727 (pS-STAT3) is required in the mitochondria for its fast cardioprotective effect in pig and mouse [6, 22, 23, 25]. Endogenous enhanced ROS in IHH can activate STAT3 during early reperfusion via improving postischemic sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA2) activity through increasing SR B cell lymphoma-2 (Bcl-2), which interacts with SERCA2 and maintains mitochondrial function [55]. However, whether STAT3 preserves mitochondrial function in moderate H_2O_2PoC is still unknown. Tumor necrosis factor-alpha (TNF-α) preconditioning only activates STAT3 during reperfusion [29] or inhibits the opening of MCU [16] to achieve cardioprotection in isolated rat I/R hearts, nevertheless, whether activated STAT3 in moderate H_2O_2PoC can alleviate myocyte calcium overload and improve cardiac contractile via inhibiting MCU against cardiac I/R insult is unclear.

To address the aforementioned issues, a model of moderate H_2O_2PoC was designed to examine (1) the effects of post-ischemic moderate ROS on $[Ca^{2+}]_c$ in myoctes, $[Ca^{2+}]_m$ in myocytes, mitochondrial membrane potential, Ca^{2+} transients, and myocytes contractile during I/R; (2) the role of STAT3 in post-ischemic moderate ROS-induced cardioprotection; (3) the role of MCU in post-ischemic moderate ROS-induced cardioprotection; (4) the correlation of STAT3 with MCU and its role in post-ischemic moderate ROS-induced cardioprotection; and (5) the molecular mechanism of action of STAT3 on MCU in post-ischemic moderate ROS-induced cardioprotection.

Methods

Animals

In this study, adult male Sprague–Dawley (SD) rats (Shanghai Slac Laboratory Animal Co. Ltd.) were used according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011) and animal procedures approved by the Institutional Review Board of Shanghai University of Medicine & Health Sciences (Shanghai, China). The weight of rats was from 280 to 320 g.

Langendorff-perfused rat I/R hearts

Sodium pentobarbital (50 mg/kg i.p.) was used to anesthetize the rats. The hearts were rapidly isolated from the rats and mounted on the Langendorff apparatus with the Krebs–Henseleit solution at 37 °C as previously described [12, 32]. The pressure transducer (Gould P23Db, AD Instrument, New South Wales, Australia) was coupled to a fluid-filled balloon and we inserted it into the LV cavity. After LV end-diastolic pressure (LVEDP) was maintained at 5–10 mmHg by inflating the balloon, the isolated heart was put onto the Langendorff system with global non-flow I/R (30-min/45-min). The PowerLab system (AD Instrument, New South Wales, Australia) was used to evaluate LV developed pressure (LVDP), LVEDP, maximum rates of pressure development or decay over time ($\pm dP/dt$ max) and heart rate (HR). H_2O_2 (20 μmol/L, Merck, Darmstadt, Germany) [53] and AG490 (20 μmol/L, Merck, Darmstadt, Germany), a Janus kinase 2 (JAK2)/STAT3 inhibitor, were used at the beginning of reperfusion for 5 min (Online Fig. 1a).

Infarct size estimation

The isolated rat hearts were subjected to global non-flow I/R (30-min/2-h), following which they were frozen the hearts and cut LV into 2-mm-thick slices. Subsequently, 1% (w/v)

triphenyltetrazolium chloride (phosphate buffer, pH 7.4) was used to stain the slices and used 10% formaldehyde to fix them [8, 31, 52, 61]. Image-Pro Plus software (Media Cybernetics, Maryland, USA) was used to calculate the infarct size and the infarct area was expressed as a percentage of the LV area at risk.

Construction of recombinant adenoviruses

Recombinant adenoviruses expressing rat STAT3 (AdSTAT3), short hairpin RNA of STAT3 (AdSTAT-3shRNA), rat Flag-MCU (AdFlag-MCU WT), rat Flag-MCU with N-terminal domain (NTD) deletion (AdFlag-MCUΔ_{75-165}), vector (AdVector) and short hairpin RNA of scramble (AdScramble) were prepared as described previously [32] using the pHBAd vector system (Hanbio, Shanghai, China). The detailed method is available in Supplementary materials online.

Isolation, culture, and adenoviral infection of myocytes

A standard method was used to isolate and culture LV myocytes as previously described [11, 32]. Then, the LV myocytes were infected with recombinant adenoviruses. The detailed method is available in Supplementary materials online.

Simulated I/R in isolated cardiomyocytes

A simulated I/R (20-min/30-min) model in isolated cardiomyocytes was set up as previously described [11, 15]. First, the myocytes were perfused in the modified Krebs–Henseleit solution (35 °C, pH 7.4) and then in ischemic solution, containing (mmol/L): NaCl, 123.0; KCl, 8.0; $NaHCO_3$, 6.0; NaH_2PO_4, 0.9; $MgSO_4$, 0.5; Na-lactate, 20.0; and $CaCl_2$, 1.8, gassed with 95% N_2/5% CO_2, (pH 6.8) for 20 min. Finally, they were reperfused in the modified Krebs–Henseleit solution again for 30 min. Different concentrations of H_2O_2 (0.1, 0.3, 1, 3, 10 and 30 µmol/L), N-(2-mercaptopropionyl) glycine (2-MPG, 300 µmol/L, Merck), a ROS scavenger, AG490 (300 nmol/L), and spermine (2 µmol/L, Merck), a MCU agonist [36, 57, 58], were administered at the beginning of reperfusion for 5 min. LV myocytes were harvested from the same heart for protein extraction at preischemia (Pre), reperfusion 5 min (R5) and reperfusion 30 min (R30).

Measurement of $[Ca^{2+}]_c$, $[Ca^{2+}]_m$, Ca^{2+} transients and cell shortening

An IonOptix system (Milton, Massachusetts, USA) was used to measure Ca^{2+} transients and cell shortening simultaneously as previously described [11, 15, 32]. The calcium indicator Indo-1 AM (5 µmol/L, Thermo Fisher, Massachusetts, USA) was used to measure $[Ca^{2+}]_c$ and Rhod 2-AM (5 µmol/L, Thermo Fisher, Massachusetts, USA) was used to measure $[Ca^{2+}]_m$ as previously described [12]. The detailed method is available in Supplementary materials online.

ROS detection

ROS production in isolated cardiomyocytes during simulated I/R was detected using 5 (and 6)-carboxy-2,7-dichlorodihydrofluerescein diacetate (DCF; Thermo Fisher, Massachusetts, USA) as previously described [52]. Cardiomyocytes from control or moderate H_2O_2PoC groups were loaded with DCF (20 µmol/L) for 10 min before plating on the dish. The acetate groups of the probe can be removed by intracellular esterases, which allow the retention of the probe by the cells. Because DCF is nonfluorescent until it is oxidized by ROS within the cell, the intracellular generation of ROS was reflected by monitoring the increase in DCF fluorescence. Fluorescence was detected using an inverted microscope (Nikon, Tokyo, Japan) and recorded (excitation/emission: 495/525 nm) every 5 min during I/R with a fixed field of view including 30–50 rod-shaped cardiomyocytes. The excitation light source was set at low power to avoid inducing oxidation and opened only when it was needed for imaging. Fluorescence intensity was analyzed with Image-Pro Plus software (Media Cybernetics).

ATP quantification by luminescence

The ATP level within the mitochondria was quantified using the ATPlite luminescence ATP detection assay system (Perkin Elmer, Massachusetts, USA) as per the kit instructions.

Mitochondrial function

The electron transport chain supercomplex activities of mitochondrial function were estimated spectrophotometrically using specific donor–acceptors chemistry following the kit (GENMED, Shanghai, China) instructions. For complex I, rotenone-sensitive NADH-oxidoreductase was used; succinate decylubiquinone dichlorophenolindophenol (DCPIP) reductase was used to assess complex II. Ubiquinol reductase was used to assess the activities of complex III and cytochrome c was used to assess complex IV. All the mitochondrial activities were normalized with protein.

Mitochondrial membrane potential ($\Delta\Psi m$) assay

$\Delta\Psi m$ was determined by a 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylimidacarbocyanine iodide (JC-1) assay kit (Beyotime, Nantong, China) using a Gemini™ EM fluorescence microplate reader (Molecular Devices, California, USA) as

previously described [55]. The detailed method is available in Supplementary materials online.

Western blot analysis

The standard western blot analysis for mitochondrial protein or immunoprecipitates from LV myocytes was performed as previously described [11, 12, 32] with antibodies against STAT3 (1:2000, Cell Signaling Technology, Massachusetts, USA), MCU (1:2000, Cell Signaling Technology, Massachusetts, USA), Flag (1: 5000, Proteintech, Illinois, USA), phospho-STAT3^{ser727} (pS-STAT3) (1:2000, Cell Signaling Technology, Massachusetts, USA), cytochrome c oxidase IV (COXIV) (1:2000, Santa Cruz, California, USA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:8000, Cell Signaling Technology, Massachusetts, USA). The detailed method is available in Supplementary materials online.

Co-immunoprecipitation assay

The co-immunoprecipitation experiments were performed as described previously [32]. The LV myocytes lysates were immunoprecipitated with anti-STAT3 (Cell Signaling Technology), anti-pS-STAT3 (Cell Signaling Technology) anti-MCU (Cell Signaling Technology) and anti-Flag (Proteintech) as well as with negative control antibodies, normal rabbit (Proteintech) or mouse IgG antibodies (Santa Cruz). The detailed method is available in Supplementary materials online.

Immunofluorescence analysis

The immunofluorescence experiments were performed as described previously [32]. First, the myocytes were fixed with paraformaldehyde and then co-immunostained with antibodies against STAT3 (Cell Signaling Technology), MCU (Abcam, Cambridge, UK). Finally, laser-scanning confocal microscopy (Leica, Heidelberg, Germany) was used to observe them. The detailed method is available in Supplementary materials online.

Statistical analysis

All values were expressed as mean ± standard error of the mean (SEM) and analyze the differences among groups by one-way repeated or muti-way analysis of variance (ANOVA) followed by the Tukey–Kramer multiple-comparison post hoc tests using SPSS v 22.0 (SPSS). $P < 0.05$ was considered statistically significant.

Results

Moderate H_2O_2PoC promoted the post-ischemic myocardial contractile function recovery and decreased infarct size via activated STAT3

To determine the role of STAT3 in the cardioprotection afforded by moderate H_2O_2PoC, LV contractile function and infarct size were detected in the I/R injury model of Langendorff-perfused rat hearts. LVDP, LVEDP, $+dP/dt_{max}$ and $-dP/dt_{max}$ were remarkably suppressed after global non-flow I/R (30-min/45-min, Fig. 1a), while the suppression was markedly attenuated by moderate H_2O_2PoC (20 μmol/L, Fig. 1a) [53]. These protections were abrogated by AG490 (20 μmol/L) administered during reperfusion for the first 5 min (Fig. 1a, Online Fig. 1a), although the HR of the different groups remained similar after global non-flow I/R (30-min/45-min, Online Fig. 1b). Consistently, moderate H_2O_2PoC significantly attenuated myocardial infarct size after global non-flow I/R (30-min/2-h) and such a protection was attenuated in the AG490 group (28.8 ± 1.2% in H_2O_2PoC vs. 58.5 ± 2.9% in H_2O_2PoC with AG490, $P < 0.001$), but no effect was observed in the control I/R heart (Fig. 1b). These results suggested a key role of the activated STAT3 during early reperfusion in cardioprotection afforded by moderate H_2O_2PoC.

Moderate H_2O_2PoC achieved cardioprotection in cardiomyocytes and simulated enhanced ROS production during early reperfusion

To determine the different roles of H_2O_2 during reperfusion in I/R injury and cardioprotection in cardiomyocytes, the concentration-dependent effects of H_2O_2PoC on post-ischemic $[Ca^{2+}]_c$, Ca^{2+} transients and cell shortening were examined in isolated cardiomyocytes from rat hearts. Moderate H_2O_2PoC (1 μmol/L and 3 μmol/L) significantly reduced $[Ca^{2+}]_c$ overload induced by I/R in cardiomyocytes, whereas low-dose H_2O_2PoC (0.1 μmol/L and 0.3 μmol/L) and high-dose H_2O_2PoC (10 μmol/L and 30 μmol/L) were the same with control I/R, without statistical significance (Fig. 2a). Moreover, moderate H_2O_2PoC (3 μmol/L) obviously reversed the I/R-altered parameters, including amplitude, maximum upstroke velocity (upstroke V_{max}), and half-decay time (decay rate τ) or maximum return velocity (return V_{max}) of Ca^{2+} transients (Online Fig. 2a) and cell shortening (Online Fig. 2b). However, H_2O_2PoC (1 μmol/L) improved the decay rate of Ca^{2+} transients (Online Fig. 2a) and return V_{max} of cell shortening (Online Fig. 2b). Thus, H_2O_2PoC (3 μmol/L) was chosen as the moderate H_2O_2PoC.

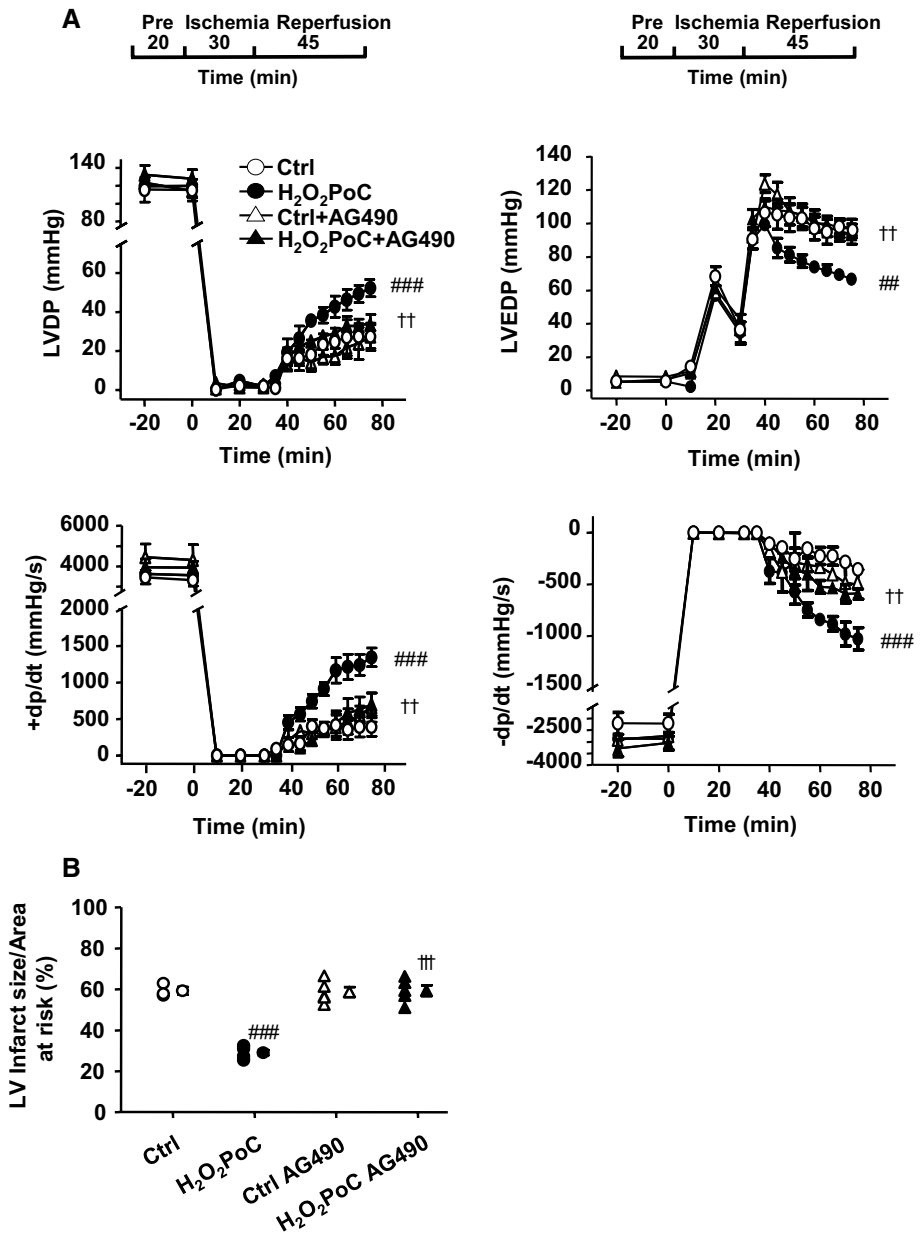

Fig. 1 Effects of H_2O_2PoC (20 µmol/L) during global no-flow I/R (30-min/45-min) on post-ischemic myocardial contractile function recovery and infarct size with or without the suppression of STAT3 activation by AG490 (20 µmol/L). **a** Summarized effects of H_2O_2PoC on post-ischemic left ventricular (LV) developed pressure (LVDP) (left upper), LV end-diastolic pressure (LVEDP) (right upper), maximum left ventricular *dP*/*dt* (+ *dp*/*dt*) (left bottom) and minimum left ventricular *dP*/*dt* (− *dp*/*dt*) (right bottom) recovery with or without AG490. **b** Effects of H_2O_2PoC on myocardial infarct size with or without AG490 expressed as a percentage of the LV area at risk. $n = 3$–6 each. Pre, preischemia; Ctrl, I/R control; H_2O_2PoC, hydrogen peroxide postconditioning; AG490, inhibitor of JAK2/STAT3. Statistical significance was achieved by muti-way ANOVA followed by Tukey–Kramer multiple comparison: $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. corresponding I/R control; $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ vs. corresponding H_2O_2PoC values

To determine whether H_2O_2PoC (3 µmol/L) could mimic the endogenously enhanced ROS production during early reperfusion seen in IHH [52], the ROS production was monitored in isolated cardiomyocytes subjected to simulated I/R using DCF fluorescence. The DCF fluorescence intensity was significantly enhanced during early reperfusion in the simulated I/R cardiomyocytes isolated from control rat hearts and was further enhanced by H_2O_2PoC. Such enhancement was totally suppressed by 2-MPG (Online Fig. 3a), a similar pattern seen in the IHH-enhanced ROS production during the early reperfusion [52]. Further, the mitochondrial function was detected. Moderate H_2O_2PoC significantly improved post-ischemic mitochondrial function, including the content of ATP (Online Fig. 3b) and the activities of complex I (Online Fig. 3c), complex II (Online Fig. 3d) and complex IV (Online Fig. 3f) at R30. H_2O_2PoC also improve the activities of complex III (Online Fig. 3e) at R30,but with no statistical significance.

Moderate H_2O_2PoC activated mitochondrial STAT3 during early reperfusion

To further confirm moderate H_2O_2PoC on activation of STAT3 in cardiomyocytes, the endogenous expression pattern of mitochondrial pS-STAT3 and STAT3 was examined in cardiomyocytes with or without I/R and moderate H_2O_2PoC. I/R suppressed the pS-STAT3

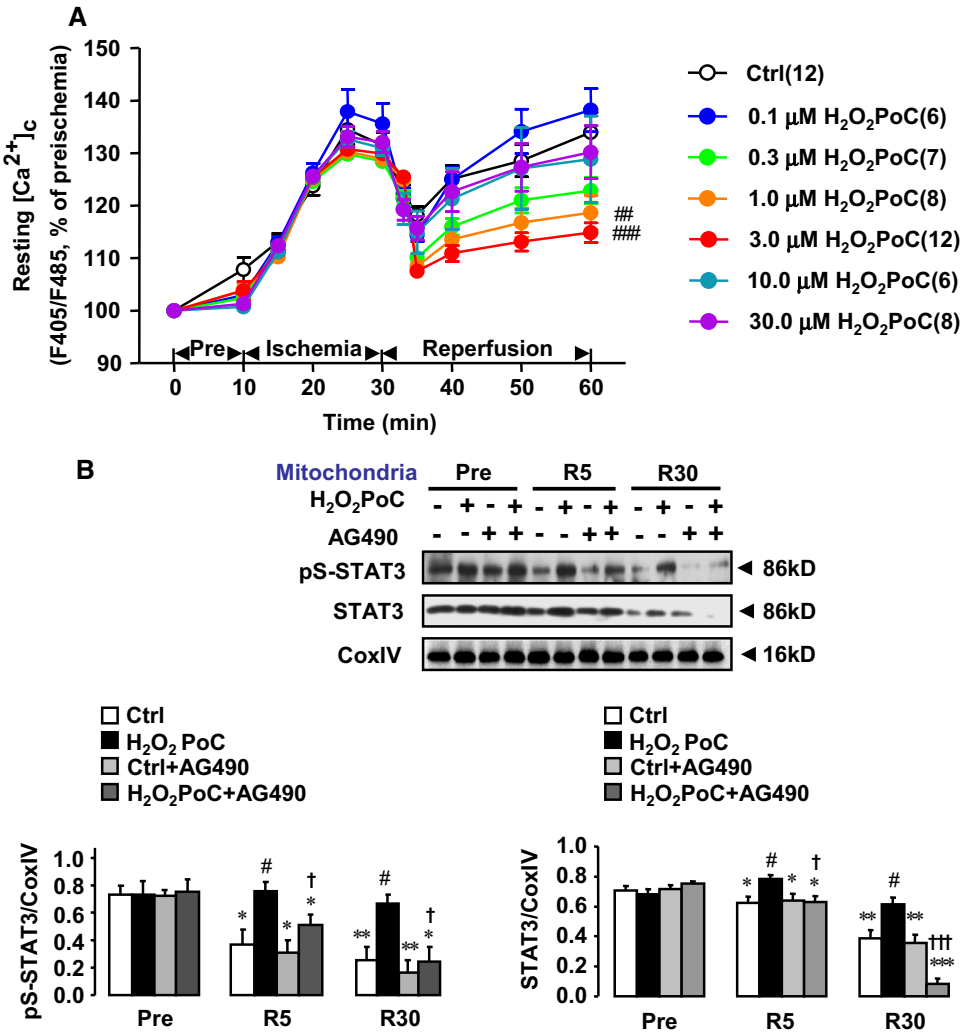

Fig. 2 Moderate H_2O_2PoC (3 μmol/L) alleviated Ca^{2+} overload and improved the expression of mitochondrial phospho-STAT3 and STAT3 during I/R. **a** Cytosolic free calcium concentration $[Ca^{2+}]_c$ in different concentrations (0.1, 0.3, 1, 3, 10, 30 μmol/L) of H_2O_2PoC. The number of myocytes is indicated in parentheses. **b** Expression of mitochondrial pS-STAT3 (left) and STAT3 (right) in isolated cardiomyocytes from control or H_2O_2PoC group with or without AG490, as detected by western blot analysis. COXIV, cytochrome c oxidase IV, was used as a housekeeping protein of mitochondria. $n = 3–8$ each. AG490, 300 nmol/L; Pre, preischemia; R5, 5 min of reperfusion; and R30, 30 min of reperfusion. Statistical significance was achieved by one-way repeated ANOVA followed by Tukey–Kramer multiple comparison: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.01$ vs. corresponding preischemic values; Statistical significance was achieved by muti-way ANOVA followed by Tukey–Kramer multiple comparison: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. corresponding control values. $^{\dagger}P < 0.05$, $^{\dagger\dagger\dagger}P < 0.001$ vs. corresponding H_2O_2PoC values

and STAT3 levels, although the COXIV level was still unchanged during reperfusion. Therefore, I/R decreased the ratios of pS-STAT3 to COXIV (Fig. 2b left) and STAT3 to COXIV (Fig. 2b right) at R5 and R30. The pS-STAT3, STAT3, and COXIV were comparable during preischemia among each group. Moderate H_2O_2PoC (3 μmol/L) further recovered the I/R-suppressed pS-STAT3 and STAT3 at R5 and R30, although moderate H_2O_2PoC did not affect COXIV. Interestingly, the mitochondrial pS-STAT3 and STAT3 expression in I/R cardiomyocytes was not changed by AG490 (300 nmol/L) treatment during reperfusion for the first 5 min, but the effects of H_2O_2PoC on the levels of mitochondrial pS-STAT3 and STAT3 were weakened by AG490 at both R5 and R30 (Fig. 2b). The aforementioned results demonstrated that mitochondrial STAT3 was required in moderate H_2O_2PoC-conferred cardioprotection.

Moderate H_2O_2PoC protected the post-ischemic $[Ca^{2+}]_c$, $[Ca^{2+}]_m$, $\Delta\Psi m$, Ca^{2+} transients and cell shortening through activated STAT3

To examine whether activated STAT3 contributed to moderate H_2O_2PoC-induced calcium homeostasis, normal cell contraction and normal $\Delta\Psi m$ during reperfusion, $[Ca^{2+}]_c$ in myocyes, $[Ca^{2+}]_m$ in myocytes, $\Delta\Psi m$, Ca^{2+} transients and cell shortening of LV myocytes were detected during simulated I/R (20-min/30-min). The preischemic and ischemic levels of $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ were similar in all groups. However, moderate H_2O_2PoC remarkably alleviated resting $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ overload due to I/R at R30. Such a protection was abolished by knocking down STAT3 with adenovirus delivery of shRNA (AdSTAT3shRNA, Online Fig. 4a), although the resting $[Ca^{2+}]_c$ (Online Fig. 5a) and $[Ca^{2+}]_m$ (Fig. 3a) of the control AdScramble group were not

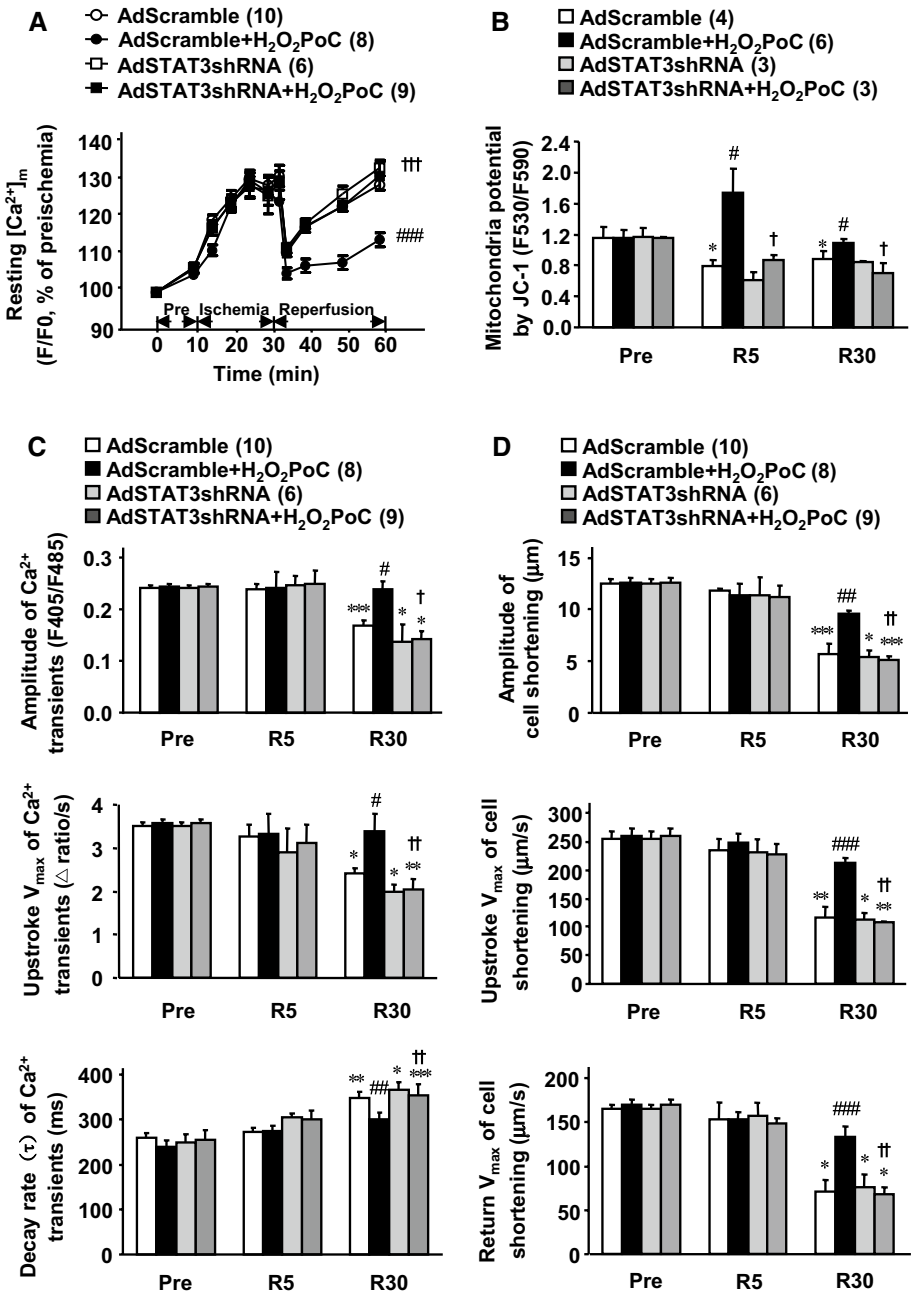

Fig. 3 Effects of H_2O_2PoC (3 μmol/L) during simulated I/R (20 min/30 min) on post-ischemic $[Ca^{2+}]_m$, $\Delta\Psi m$, Ca^{2+} transients and cell shortening in cardiomyocytes with or without down-regulation of STAT3. **a** Mitochondrial free calcium concentration $[Ca^{2+}]_m$. **b** Mitochondrial membrane potential ($\Delta\Psi m$). Amplitude, maximum upstroke velocity, and half-decay time or maximum return velocity of Ca^{2+} transients (**c**) and cell shortening (**d**). Adscramble, adenoviruses carrying the shRNA scramble; AdSTAT3shRNA, adenoviruses carrying the short hairpin RNA specific for STAT3. The number of myocytes is indicated in parentheses. Statistical significance was achieved by one-way repeated ANOVA followed by Tukey–Kramer multiple comparison: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs corresponding preischemic values; Statistical significance was achieved by muti-way ANOVA followed by Tukey–Kramer multiple comparison: $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. corresponding control values; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ vs. corresponding H_2O_2PoC values

affected by the interventions (Online Fig. 4b). Moreover, I/R-induced decreases in $\Delta\Psi m$ (Fig. 3b) at R5 and R30 were completely reversed by moderate H_2O_2PoC, while these protective effects were abrogated by AdSTAT3shRNA (Fig. 3b).

Consistently, moderate H_2O_2PoC obviously reversed the I/R-altered parameters, including amplitude, upstroke V_{max}, and decay rate τ or return V_{max} of Ca^{2+} transients (Fig. 3c) and cell shortening (Fig. 3d) at R30. No difference in these parameters was detected among all groups before ischemia. This protection was abolished by AdSTAT3shRNA (Fig. 3c, d), while the aforementioned parameters of the control AdScramble group were not affected by the interventions (Fig. 3). These results implied that STAT3 plays a vital role in H_2O_2PoC-induced maintenance of Ca^{2+} homeostasis and enhancement of Ca^{2+} transients and cell shortening during I/R.

Moderate H_2O_2PoC protected the post-ischemic $[Ca^{2+}]_c$, $[Ca^{2+}]_m$, $\Delta\Psi m$, Ca^{2+} transients and cell shortening via the inhibition of MCU

To explore whether inhibiting MCU contributed to the cardioprotective effects of H_2O_2PoC during reperfusion, $[Ca^{2+}]_c$, $[Ca^{2+}]_m$, $\Delta\Psi m$, Ca^{2+} transients, and cell shortening with or

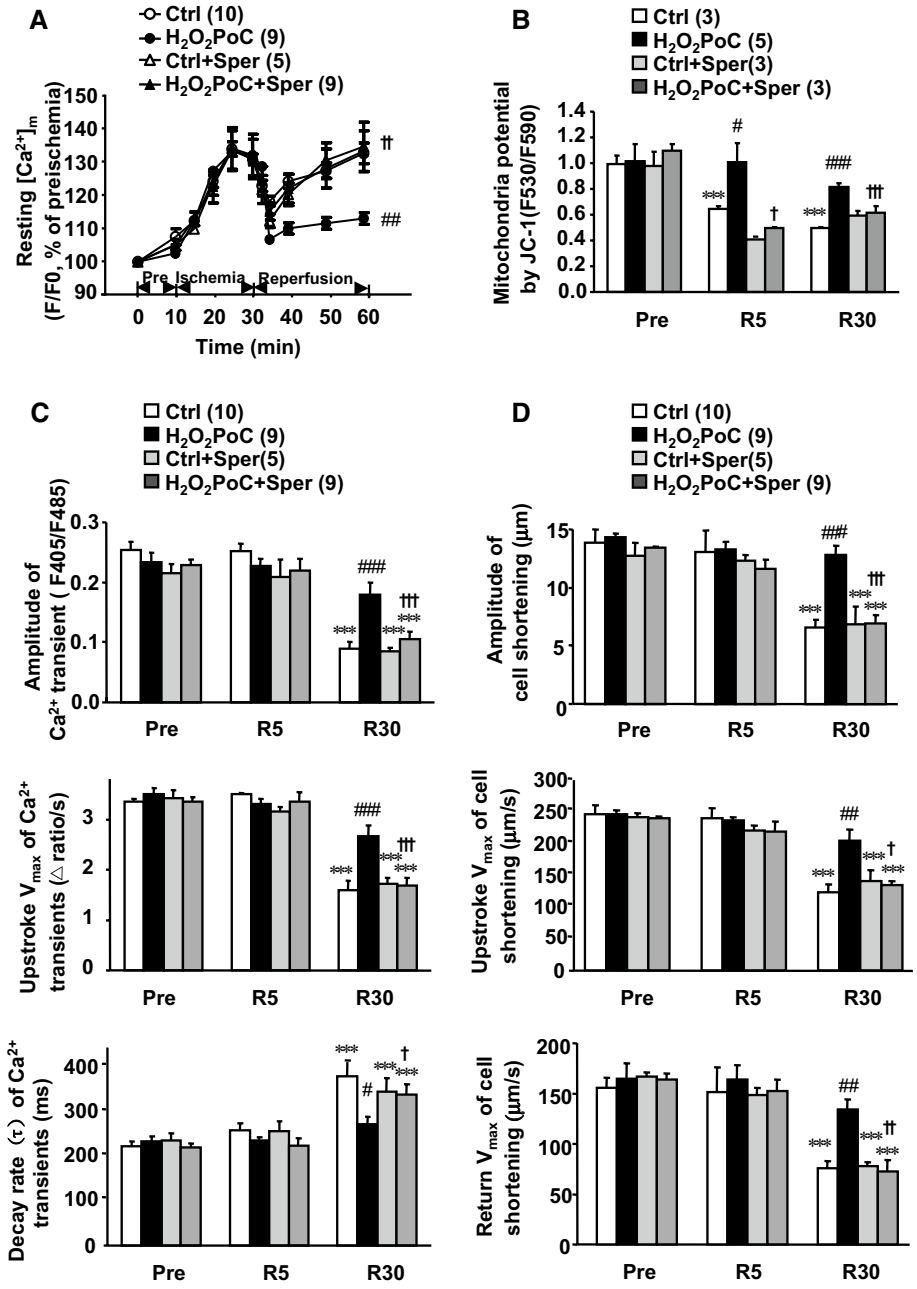

Fig. 4 Effects of H_2O_2PoC (3 μmol/L) during simulated I/R (20 min/30 min) on $[Ca^{2+}]_m$, $\Delta\Psi m$, Ca^{2+} transients and cell shortening in cardiomyocytes with or without opening of MCU. **a** Mitochondrial free calcium concentration $[Ca^{2+}]_m$. **b** Mitochondrial membrane potential ($\Delta\Psi m$). Amplitude, maximum upstroke velocity, and half-decay time or maximum return velocity of Ca^{2+} transients (**c**) and cell shortening (**d**). Sper: spermine, the opener of MCU. The number of myocytes is indicated in parentheses. Statistical significance was achieved by one-way repeated ANOVA followed by Tukey–Kramer multiple comparison: $^{***}P<0.001$ vs. corresponding preischemic values; Statistical significance was achieved by muti-way ANOVA followed by Tukey–Kramer multiple comparison: $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. corresponding control values; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ vs. corresponding H_2O_2PoC values

without the MCU agonist spermine (2 μmol/L) were examined during I/R (Fig. 4, Online Fig. 4b). The preischemic and ischemic levels of $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ were similar in all groups, whereas moderate H_2O_2PoC (3 μmol/L) reduced $[Ca^{2+}]_c$ (Online Fig. 5b) and $[Ca^{2+}]_m$ (Fig. 4a) overload and preserved the reduced $\Delta\Psi m$ (Fig. 4b) due to I/R during reperfusion. These attenuations and preservations were abrogated by spermine added during reperfusion for the first 5 min. Similarly, the dynamics of Ca^{2+} transients and cell shortening were altered by I/R and improved by moderate H_2O_2PoC at R30, but such improvement was also totally inhibited by spermine (Fig. 4c, d). These parameters of the control group were not affected by the interventions (Fig. 4). These results indicated that inhibiting MCU played an important role in moderate H_2O_2PoC-induced maintenance of Ca $^{2+}$ homeostasis and enhancement of Ca^{2+} transients and cell shortening during I/R.

STAT3 inhibited the opening of MCU in moderate H_2O_2PoC-afforded cardioprotection

$[Ca^{2+}]_m$ overload is caused by the opening of MCU during I/R and the activation of STAT3 in moderate H_2O_2PoC-alleviated $[Ca^{2+}]_m$ overload. Hence, the role of

MCU in STAT3-mediated moderate H_2O_2PoC-induced cardioprotection was clarified through simultaneously examining the time course of $[Ca^{2+}]_c$, $[Ca^{2+}]_m$, $\Delta\Psi m$, Ca^{2+} transients, and cell shortening with spermine (2 μmol/L) in cardiomyocytes transfected with recombinant adenoviruses expressing rat STAT3 (AdSTAT3, Online, Fig. 6a) in H_2O_2PoC (Fig. 5, Online Fig. 4b). No significant differences were observed in preischemic resting $[Ca^{2+}]_c$ (Online Fig. 5c), $[Ca^{2+}]_m$ (Fig. 5a), $\Delta\Psi m$ (Fig. 5b) and the dynamics of Ca^{2+} transients (Fig. 5c) and cell shortening (Fig. 5d) among all groups. However, moderate H_2O_2PoC and STAT3 overexpression by adenoviruses significantly rescued I/R-changed resting $[Ca^{2+}]_c$ (Online Fig. 5c), $[Ca^{2+}]_m$ (Fig. 5a), $\Delta\Psi m$ (Fig. 5b) and the parameters of Ca^{2+} transients (Fig. 5c) and cell shortening (Fig. 5d). Moderate H_2O_2PoC-conferred cardioprotection was mimicked by AdSTAT3 but AdSTAT3-induced cardioprotection was blocked by spermine (Fig. 5a–d, Online Fig. 5c). Besides, the cardioprotective effects of H_2O_2PoC remained unchanged with spermine in cardiomyocytes transfected with AdSTAT3 (Fig. 5a–d, Online Fig. 5c). Therefore, it was concluded that STAT3 mediates the cardioprotection of H_2O_2PoC through inhibiting the opening of MCU.

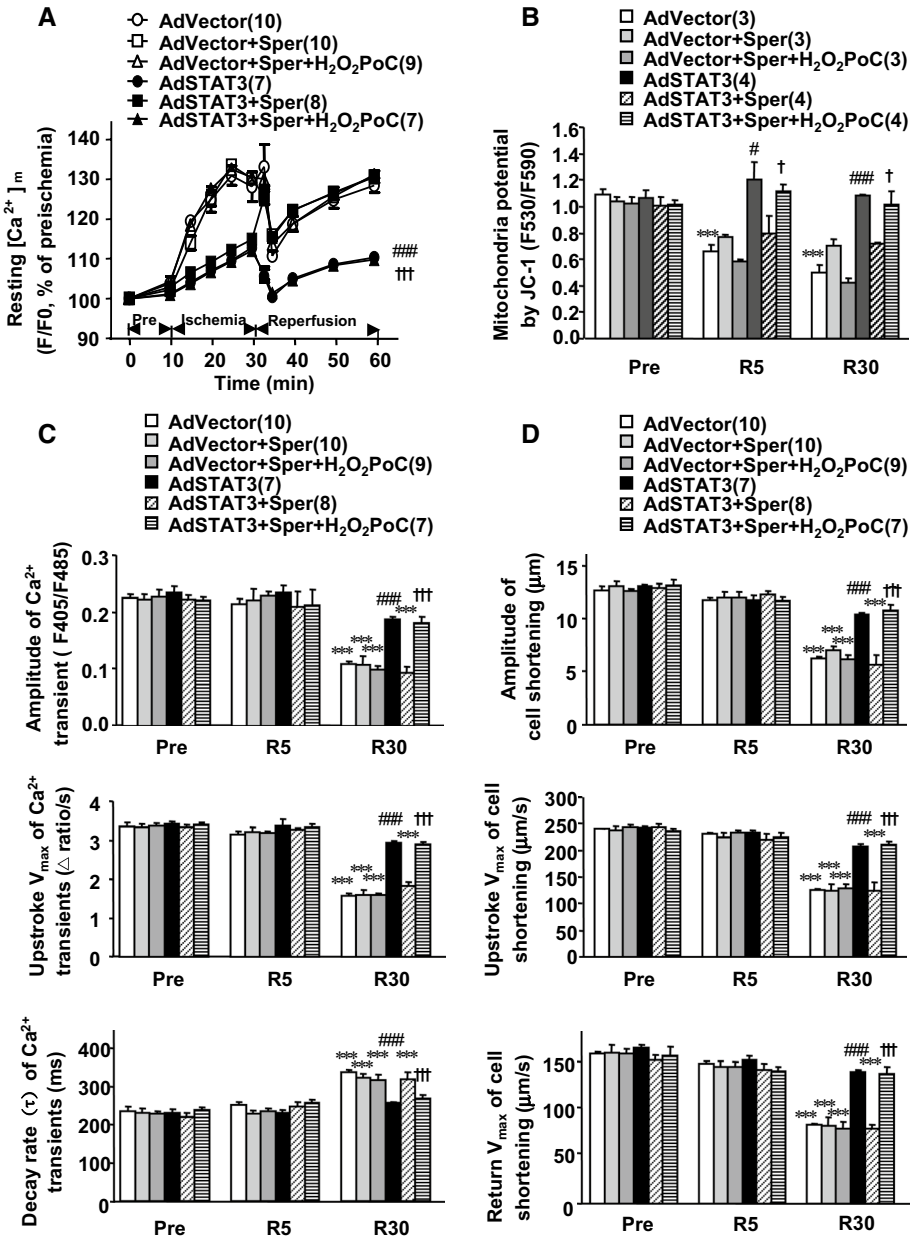

Fig. 5 Effects of H_2O_2PoC (3 μmol/L) and spermine during simulated I/R (20-min/30-min) on $[Ca^{2+}]_m$, $\Delta\Psi m$, Ca^{2+} transients and cell shortening in cardiomyocytes with or without the overexpression of STAT3. **a** Mitochondrial free calcium concentration $[Ca^{2+}]_m$. **b** Mitochondrial membrane potential ($\Delta\Psi m$). Amplitude, maximum upstroke velocity, and half-decay time or maximum return velocity of Ca^{2+} transients (**c**) and cell shortening (**d**). AdVector, adenoviruses carrying the vector; AdSTAT3, adenoviruses carrying the rat STAT3. The number of myocytes is indicated in parentheses. Statistical significance was achieved by one-way repeated ANOVA followed by Tukey–Kramer multiple comparison: $^{***}P<0.001$ vs. corresponding preischemic values; Statistical significance was achieved by muti-way ANOVA followed by Tukey–Kramer multiple comparison: $^{\#}P<0.05$, $^{\#\#\#}P<0.001$ vs. corresponding control values; $^{\dagger}P<0.05$, $^{\dagger\dagger\dagger}P<0.001$ vs. corresponding H_2O_2PoC + Sper values

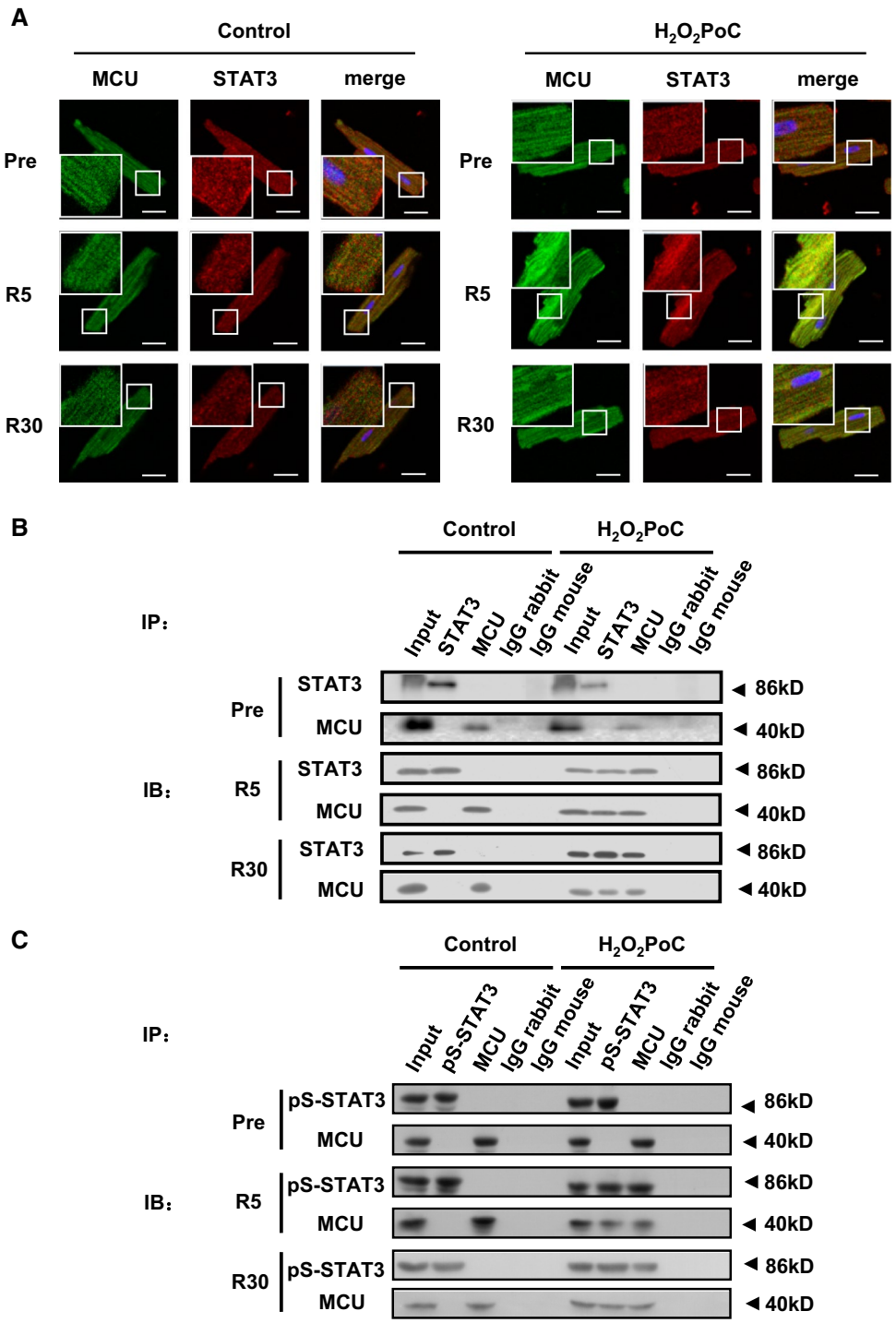

Fig. 6 Co-localization and interaction of STAT3 with MCU in rat cardiomyocytes. **a** Representative images of cardiomyocytes with dual immunostaining in the control and H_2O_2PoC groups at Pre, R5 and R30. Red represents STAT3, green represents MCU, and blue represents nuclei. Scale bars, 25 μm. **b** Co-immunoprecipitation analysis for the interaction between STAT3 and MCU in the control and H_2O_2PoC groups at Pre, R5 and R30. **c** Co-immunoprecipitation analysis for the interaction between pS-STAT3 and MCU in the control and H_2O_2PoC groups at Pre, R5 and R30. The interaction of STAT3 or pS-STAT3 with endogenous MCU in the immunoprecipitates was detected by Western blot analysis with anti-STAT3, anti-pS-STAT3 or anti-MCU antibodies. $n = 3$

Moderate H_2O_2PoC facilitated the interaction between STAT3 or pS-STAT3 and MCU during reperfusion

Subsequently, whether MCU was the direct target of STAT3 was examined. The specific immunofluorescent images were used. STAT3 and MCU seem to be co-localized in the moderate- H_2O_2PoC cardiomyocytes at R5 and R30, whereas no co-localization of the two proteins was found in control simulated I/R cells and before ischemia (Fig. 6a). Next, co-immunoprecipitation was performed to check the protein interaction between STAT3 or pS-STAT3 and MCU. MCU was detected in the immunoprecipitates from cardiomyocytes of moderate-H_2O_2PoC groups with anti-STAT3 (Fig. 6b) or anti-pS-STAT3 (Fig. 6c). STAT3 (Fig. 6b) or pS-STAT3 (Fig. 6c) was detected in the immunoprecipitates

from cardiomyoctes of moderate-H_2O_2PoC groups with anti-MCU at R5 and R30 but not before ischemia. The interaction of STAT3 or pS-STAT3 and MCU was not seen in the control I/R cadiomyocytes (Fig. 6b, c). These data were consistent with the effect on the expression of mitochondrial STAT3 and regulation of $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ by activated STAT3 through the inhibition of MCU by H_2O_2PoC.

To further explore the interacting domains of MCU and STAT3, mutant MCU with NTD deletion MCU$\Delta_{75–165}$ (Flag-MCU$\Delta_{75–165}$) was expressed as a Flag fusion protein. Wild-type MCU (Flag-MCU WT) also expressed as a Flag fusion protein (Fig. 7a).Co-immunoprecipitation was performed using lysates harvested from myocytes transfected with recombinant adenoviruses expressing rat Flag-MCU WT (AdFlag-MCU WT) or Flag-MCU$\Delta_{75–165}$ (AdFlag-MCU$\Delta_{75–165}$) subjected to simulated I/R with moderate H_2O_2PoC. Immobilized Flag-MCU$\Delta_{75–165}$ failed to interact with STAT3, whereas Flag-MCU WT still interacted with STAT3 at R5 (Fig. 7b). Immunoblotting with Flag antibody revealed the same amount of Flag-MCU WT or Flag-MCU$\Delta_{75–165}$ in the immunoprecipitates (Online, Fig. 6b). Thus, STAT3 probably prevented MCU activity via the interaction of STAT3 with MCU NTD.

Discussion

This study demonstrated that (1) moderate H_2O_2PoC protected the heart from I/R injury via activating STAT3; (2) moderate H_2O_2PoC significantly activated mitochondrial STAT3; (3) moderate H_2O_2PoC alleviated I/R-induced calcium overload and contraction suppression in ventricular myocytes via activating STAT3 and inhibiting MCU; (4) STAT3 inhibited the opening of MCU in moderate H_2O_2PoC-alleviated I/R-induced $[Ca^{2+}]_m$ overload through its interaction with MCU; (5) STAT3 could interact with MCU at NTD, which might be involved in the uptake of cytosolic calcium into mitochondria. These findings extended previous findings, demonstrated a vital role of STAT3 in the post-ischemic moderate ROS-induced cardioprotection, and provided new insights into the mechanisms underlying the cardioprotection of STAT3.

The role of ROS in cardioprotection or I/R injury depends on different kinds of ROS, ROS concentrations and availability of ROS reaction partners. Superoxide and H_2O_2 are produced normally and may have protective actions via signaling for preconditioning activating

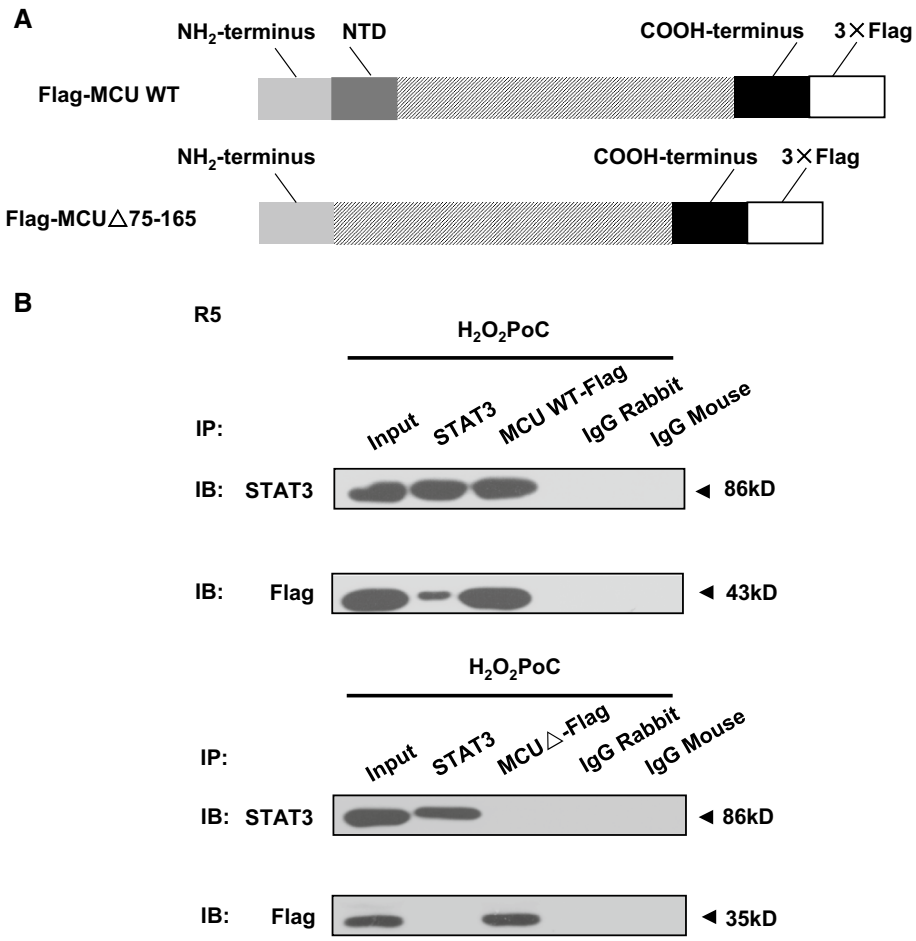

Fig. 7 Interacting regions of MCU with STAT3 in rat cardiomyocytes. **a** Schematic representation of 3×Flag-tagged wild-type MCU (Flag-MCU WT) and 3×Flag-tagged mutant MCU with the N-terminal domain (NTD) deletion (Flag-MCUΔ75-165) fusion proteins. **b** Co-immunoprecipitation analysis for the interacting regions between STAT3 and MCU at R5 in H_2O_2PoC. The interaction of STAT3 with exogenous Flag-MCU WT or Flag-MCUΔ75-165 in the immunoprecipitates was detected by Western blot analysis with anti-STAT3 or anti-Flag antibodies. $n=3$

multiple groups of protective proteins. However, with I/R the normal balance is lost and hydroxyl radicals can be produced direct cellular injury [4]. Low levels of H_2O_2 aggravate contractile dysfunction through endoplasmic reticulum stress, moderate levels of H_2O_2 are associated with increased postischemic LV performance through protein kinase B (PKB/Akt) and protein kinase C epsilon (PKCε) activation. However, high amounts of H_2O_2 aggravate contractile dysfunction through oxidant stress [53]. In the models of cardioprotection by RIPC, IPC and TNF-α preconditioning, mitochondrial ROS was reduced [17, 24, 26, 49]. Myocardial I/R injury was effectively reduced after administering melatonin, vitamin C, resveratrol, N-acetylcysteine or N-mercaptopropionylglycine [7]. However, RIPC protected the heart from I/R injury through the ROS-dependent mechanism because ROS scavengers attenuated infarct size reduction by RIPC [19]. Besides, ROS signaling at the onset of reperfusion play an important role in a model of cardioprotection by IPoC because ROS scavenger administered during the whole reperfusion phase abolished its effects [7]. Excessive ROS production during early reperfusion is a critical cause of calcium overload in cardiomyocytes [1, 49]. However, enhanced ROS during early reperfusion in IHH significantly reduced calcium overload [55]. These findings suggested that ROS generation should be quantified at a moderate level to achieve calcium homeostasis. As the ROS production in the IHH model is endogenous and it is hard to quantified, the H_2O_2PoC model was designed to represent the exact concentration of ROS during early reperfusion in an exogenous manner. The data confirmed the previous findings that moderate H_2O_2PoC (20 µmol/L) promoted the recovery of post-ischemic myocardial contractile function and limited the infract size reported recently [53]. Moreover, this study demonstrated that moderate H_2O_2PoC (3 µmol/L) reduced calcium overload due to I/R in cardiomyocytes. This was consistent with the observation that moderate H_2O_2 (25 µmol/L) given at the beginning of reoxygenation alleviated calcium accumulation in isolated adult rat hypoxia/reoxygenation cardiomyocytes [46].

Although several signal pathways interacting with mitochondria such as the reperfusion injury salvage kinases (RISKs), including PKB/Akt and PKCε, are implicated in IPC, IPoC, RIPC, IHH and H_2O_2PoC [20, 21, 26, 33, 47, 52, 53], the role of the survivor activating factor enhancement (SAFE) pathway, including JAK2/STAT3, remains unclear in H_2O_2PoC. Similar to an increase of STAT3 phosphorylation in a ROS-dependent manner in the IHH rat heart [55], an increase in I/R-decreased mitochondrial STAT3 expression in moderate-H_2O_2PoC cardiomyocytes implied a crucial role of STAT3 in the moderate H_2O_2PoC-conferred cardioprotection against I/R insult. This was further supported by the disappeared better post-ischemic cardiac contraction and more infarct size of H_2O_2PoC rat hearts treated with AG490. Accordingly, IPC-, IPoC-, IHH-induced cardioprotection was attenuated by AG490 in rat/mouse hearts [5, 18, 55]. It has been also reported that along with reduced infarct size, the ratio of pY-STAT3/total STAT3 protein at early reperfusion was significantly increased by IPC, but only trendwise by IPoC and RIPC and the expression of pY-STAT3 was increased at late reperfusion by RIPC in pig heart [23, 25]. The possible reason for the difference is different animal model with different treatment. Moreover, moderate H_2O_2PoC attenuated I/R-induced $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ overload and improved I/R-altered Ca^{2+} transients and cell shortening depending on STAT3. This was also consistent with the observation that IHH-induced attenuation of $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ overload and enhancement of Ca^{2+} transients and cell shortening was cancelled by AG490 in cardiomyocytes [55]. These data revealed that STAT3 participated in ROS signal transduction during cardioprotection as an important down-stream mediator.

Mitochondria are one of the major effectors that regulate Ca^{2+} homeostasis in cardiomyocytes. Calcium enters the mitochondria primarily through the MCU [14]. Previous studies found that the cardioprotective effects of B-type natriuretic peptide (BNP) postconditioning were through preventing the opening of MCU partly related to recovering mitochondrial membrane potential and attenuating ROS generation in I/R cardiomyocytes [50]. However, inhibiting MCU was found to play a crucial role in post-ischemic moderate ROS-induced cardioprotection with the model of moderate H_2O_2PoC. This was similar to the finding on perfusing the heart with spermine, and the cardioprotective effects of IPC and IPoC were abolished [57, 60]. Although previous studies also found that ROS production was reduced in the mice with a dominant-negative form of MCU [45], the present study showed that inhibiting MCU contributed to moderate ROS-induced attenuation of $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ overload. This result was consistent with the previous results that Ru360 or ruthenium red, at certain concentrations, abolished $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ overload in cardiac I/R injury [1, 10]. These data demonstrated that inhibiting MCU was one of the targets of post-ischemic moderate ROS during cardioprotection.

Activated STAT3 is involved in TNF-α preconditioning and postconditioning [28, 29], and TNF-α preconditioning attenuates Ca^{2+} overload in rat organotypic hippocampal cultures [2]. Thus, activated STAT3 during reperfusion would act as an important mediator for the attenuation of calcium overload in cardiomyocytes as observed in IHH [55]. Moreover, diazoxide preconditioning against transient focal cerebral ischemia was attenuated by spermine in rats [58] and protected spinal cord from I/R insult by significantly increasing the phosphorylation of STAT3 [56]. The

present study found that mitochondrial STAT3 during early reperfusion reduced Ca^{2+} overload via inhibiting the opening of MCU in H_2O_2PoC-induced cardioprotection. This was consistent with the finding that inhibiting MCU contributed to TNF-α preconditioning in isolated rat I/R hearts [16]. Drugs or RNAi-induced inhibition of MCU abrogated cell death in mouse heart during I/R, presumably by reducing Ca^{2+} influx into mitochondria and inhibiting the opening of mitochondrial permeability transition pore (mPTP) [27]. Inhibiting the opening of mPTP in cardiomyocytes isolated from preconditioning rats and skeletal muscle isolated from postconditioning rats significantly attenuated $[Ca^{2+}]_m$ overload [35, 61]. pS-STAT3 inhibited the opening of mPTP via interacting with cyclophilin D in rat cardiomyocytes [6, 48]. This raised the question whether STAT3 inhibited the opening of MCU through the regulation of mPTP. However, no cardioprotective effect was found in MCU knockout mice I/R heart treated with the mPTP inhibitor [14]. Therefore, other mechanisms involved in the direct regulatory effect of STAT3 on MCU were explored.

pS-STAT3 is crucial for interacting with 14-3-3ζ, a regulatory signaling molecule in mitochondria that phosphorylates other proteins in an interaction-dependent manner and acts as a chaperone [59]. The 14-3-3 protein isoforms υ, β, ζ have been demonstrated to interact with uncoupling protein 2 (UCP2), a member of mitochondrial ion transporters and a mitochondrial membrane protein [43]. Further, UCP2 has been shown to probably interact with MCU to regulate the process of transferring calcium from the cytoplasm to mitochondria in cardiomyocytes [38]. The data revealed an interaction between the STAT3 protein and the MCU protein using co-immunoprecipitation and immunofluorescence. STAT3 has been proved to regulate ROS generation and mitochondrial respiration for its interacting with mitochondrial complex I and complex II [51, 54]. This study was novel in proving that STAT3 inhibited the opening of MCU via interacting with MCU in cardioprotection. STAT3 is imported into mitochondria and recruited into complex I located in the inner mitochondrial membrane [54] and MCU is an ion channel in mitochondria composed of transmembrane proteins with N- and C-termini facing the matrix [34]. Hence, the possible interaction domains of STAT3 and MCU need investigation. A previous study showed that the NTD of MCU was an essential domain of MCU interacting with the regulators to modulate the mitochondrial calcium uptake [30]. Thus, it was confirmed that STAT3 could not interact with mutant MCU with NTD deletion ($MCU\Delta_{75\text{-}165}$) but interact with MCU WT in an exogenous-overexpression manner in cardiomyocytes, indicating that STAT3 might serve as a new regulator for MCU.

In conclusion, this study demonstrated that moderate H_2O_2PoC-mimicked IHH protected the heart/cardiomyocytes from I/R-induced calcium overload and contractile dysfunction via mitochondrial STAT3 interaction with the NTD of MCU to achieve the inhibition of MCU opening (Online Fig. 7). These findings suggested that moderate H_2O_2PoC might be a proper approach to study the quantity of post-ischemic ROS during early reperfusion and its cardioprotective mechanism.

Acknowledgements This work was supported by National Natural Sciences Foundation of China (81600246), Shanghai Excellent Young Teachers Funding Project (zzjkyx16004) and Shanghai Municipal Education Commission (Plateau Disciplinary Program for Medical Technology of SUMHS, 2018-2020).

Compliance with ethical standards

Conflict of interest The authors have no conflict of interest.

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