Treatment with metformin prevents myocardial ischemia–reperfusion injury via STEAP4 signaling pathway

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Abstract

Objective: The aim of the present study was to investigate the underlying mechanism of metformin in reducing myocardial apoptosis and improving mitochondrial function in rats and H9c2 cells subjected to myocardial ischemia–reperfusion (I/R) or hypoxia–reoxygenation (H/R) injuries, respectively.

Methods: Following pretreatment with metformin, male Sprague–Dawley rats were used to establish an I/R model in vivo. Serum creatinine kinase-MB and cardiac troponin T levels were examined by enzyme-linked immunosorbent assay. Infarct size and apoptosis were measured by triphenyl tetrazolium chloride staining and terminal deoxynucleotidyl transferase dUTP nick end labeling assay. Pathological changes were evaluated by hematoxylin and eosin staining. H9c2 cells were used to establish an H/R model *in vitro*. Cell apoptosis and mitochondrial membrane potential (MMP) were examined by flow cytometry and Rhodamine 123. The expression levels of six-transmembrane epithelial antigen of prostate 4 (STEAP4), B-cell lymphoma 2, Bcl-2-associated X protein, and glyceraldehyde 3-phosphate dehydrogenase in both myocardial tissues and H9c2 cells were determined by western blotting.

Results: We found that metformin decreased infarct size, increased STEAP4 expression, mitigated myocardial apoptosis, and increased MMP when the models were subjected to H/R or I/R injuries. However, STEAP4 knockdown significantly abrogated the beneficial effect of metformin.

Conclusion: We further demonstrated the protective effect of metformin on cardiomyocytes, which might be at least partly attributable to the upregulation of STEAP4. Therefore, STEAP4 might be a new target to decrease apoptosis and rescue mitochondrial function in myocardial I/R injury. *(Anatol J Cardiol 2019; 21: 261-71)*

Keywords: metformin, myocardial ischemia–reperfusion injury, apoptosis, STEAP4

Introduction

Myocardial infarction (MI) greatly threatens human health and remains a leading cause of mortality worldwide (1). There are serious complications, including heart failure, malignant ventricular arrhythmia, and even sudden cardiac death following MI (2). Early restoration of cardiac perfusion is necessary to restore perfusion of an ischemic heart (3), but the restored blood supply may trigger severe myocardial ischemia–reperfusion (I/R) injury (4). Although several therapeutic strategies, such as ischemic post-conditioning and remote ischemic preconditioning, have been suggested to mitigate myocardial I/R injury (5, 6), these measures are limited in clinical practice because of medical ethical issues. Therefore, the search for novel therapeutic agents and therapeutic strategies for I/R-stimulated injury is important.

Metformin, an orally administered biguanide drug, is widely used to lower blood glucose concentration in patients with type 2 diabetes (7). Recent studies have suggested that metformin also has a cardiovascular protective effect, which can significantly lessen a patient's cardiovascular events (8, 9). Independent of the reduction in blood glucose, metformin may also improve endothelial cell function, inhibit oxidative stress, have an anti-apoptotic effect, and improve myocardial remodeling (10-12). Increasing studies have demonstrated that some of the beneficial glucose-lowering effects of metformin might be mediated via the activation of 5′ adenosine monophosphate-activated protein kinase (AMPK), which is considered an important regulatory factor in myocardial energy metabolism (13). Metformin was reported to exert cardioprotective effects by activating the phosphatidylinositol 3-kinase–protein kinase B (AKT) pathway to attenuate myocardial cell injury (14). However, the pharmacological action of metformin on myocardial I/R injury is extremely complex. The molecular mechanisms for cardioprotective effect of metformin are not fully understood.

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The six-transmembrane epithelial antigen of prostate 4 (STEAP4) belongs to the STEAP protein family and the metalloreductases family (15). Previous studies clarified that STEAP4 is an important modulator of inflammation and nutrition and is implicated in systemic metabolic homeostasis and obesity-related insulin resistance in a manner similar to AMPK (16, 17). Experimental results showed that monoclonal antibodies to STEAP4 promote apoptosis and inhibit proliferation and glucose uptake in human adipocytes (18). Di Salvo et al. (19) found that STEAP4 is related to cardiac adaptations and may become a myocardial biomarker of heart failure. They found that the expression of STEAP4 in patients with heart failure is lower than that in normal people. Furthermore, STEAP4 was upregulated in human $CD34^+$ cells induced by hypoxia after the addition of metformin (20). Whether metformin prevents myocardial apoptosis and reverses myocardial I/R injury via STEAP4 in cardiomyocytes has not been previously reported.

The aim of the present study was to investigate the anti-apoptotic effects of metformin using Sprague–Dawley (SD) rats and cultured H9c2 cells and to explore whether the underlying mechanisms were due to the activation of STEAP4. Our results potentially provided a new perspective on understanding the cardioprotective effects of metformin.

Methods

Animals

A total of 60 healthy adult male SD rats, weighing 280–320 g, were purchased from Liaoning Changsheng Biotechnology Co. (Shenyang, China). Animals were housed in a temperature- (22 °C–25 °C) and light-controlled (a 12-hour light: dark cycle) room and given free access to water and a standard rat diet. All rats were treated and used according to the Guide for the Care and Use of Laboratory Animals (Federal Register Doc. 2011-11490; National Institutes of Health, Bethesda, MD, USA). The experimental protocol was approved by the Institutional Ethics Committee of China Medical University (Shenyang). Cervical dislocation was the method used to provide the rats with a fast and painless death.

Animal experimental protocol

The experimental animals were randomly divided into three groups with 20 rats per group as follows: (1) control group, (2) I/R group, and (3) I/R+metformin (Met) group. To achieve the I/R model, rats were anesthetized with intraperitoneal injection of pentobarbital sodium at a dose of 30 mg/kg. After the chest hair was removed, a left lateral thoracotomy was performed between the fourth and fifth intercostal space to scissor pericardium and expose the heart. Then, the left anterior descending artery (LAD) was ligated with a nylon suture for 30 min of ischemia, followed by 2 h of reperfusion. In the control group, all the steps were followed except for ligation of the LAD. Metformin, purchased from Sigma (St. Louis, MO, USA), was dissolved in saline and administered through an intraperitoneal injection at a dose of 250 mg/kg (21, 22) in a final volume of 100 µL (once daily) for 14 days (23) before I/R. The control group and I/R group received intraperitoneal injections of saline at the same volume.

ELISA

After reperfusion, blood samples were collected by 2 ml Eppendorf tube, and the serum was separated by centrifugation at 3000 rpm for 10 min at 4 °C. Enzyme-linked immunosorbent assay (ELISA) kits were used to measure creatinine kinase-MB isoenzyme (CK-MB; Nanjing Jiancheng, Nanjing, China) and serum cardiac enzymes cardiac troponin T (cTnT; Nanjing Jiancheng) according to the manufacturer's instructions. Absorbance at 450 nm was determined using a microplate reader (BioTek Instruments, Inc., Winooski, VA, USA).

Measurement of infarct size

Infarct size was estimated by triphenyl tetrazolium chloride (TTC) staining. The hearts were excised and incubated with 10% KCl solution. After freezing at −20 °C, the hearts were sectioned into slices and stained with 1% TTC solution (Solarbio Science & Technology Co., Beijing, China) at 37 °C for 10–15 min in the dark. The images of these slices were obtained using a digital camera. The infarct area and total area were analyzed by Image-Pro Plus software 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

H&E staining

The hearts were removed from the perfusion apparatus at the end of reperfusion. The heart tissues were dehydrated in ethanol and embedded in paraffin. Then, the obtained 5 µm thick sections were dewaxed, rehydrated, and stained with hematoxylin and eosin (H&E) dye. The pathological changes in the heart tissues were observed under a light microscope (×400 magnification; Olympus, Tokyo, Japan).

Measurement of apoptosis

Myocardial apoptosis was detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay using an In Situ Cell Death Detection kit (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's protocol. Myocardium tissues were embedded in paraffin and cut into 5 µm thick sections. After that step, the sections were dewaxed in xylene rehydrated in ethanol and permeabilized with 0.1% Triton X-100. Then, 3% H_2O_2 was used to block endogenous peroxidase activity. Sections were incubated with 50 µL TUNEL reaction mixture at 37 °C for 1 h and with 50 µL converter POD for 30 min. Finally, the sections were treated with diaminobenzidine (DAB) substrate. The stained sections were visualized via an Olympus microscope (×400 magnification; Olympus).

Cell culture and treatments

Rat cardiomyocyte H9c2 cells were purchased from the cell bank at the Chinese Academy of Sciences (Shanghai, China).

H9c2 cells were treated with hypoxia–reoxygenation (H/R) to mimic the I/R model *in vitro*. Specifically, the cells were treated when their confluence was 80%–90%. The culture medium (90% DMEM+10% FBS) was removed and changed to Earle's medium ($CaCl_2$ 0.18 mmol/L, $MgSO_4 \cdot 7H_2O$ 0.08 mmol/L, KCl 0.05 mmol/L, NaCl 11.43 mmol/L, $NaHCO_3$ 2.62 mmol/L, and NaH_2PO_4 0.10 mmol/L) without glucose and FBS. Thereafter, the cells were cultured in an incubator with 94% N_2, 5% CO_2, and 1% O_2 at 37 °C for 6 h to induce hypoxia. Earle's medium was removed, and the cells were cultured for 4 h with normal medium in incubators with 5% CO_2 at 37 °C to re-oxygenate the samples. In the first stage, the cells were incubated with fresh medium containing various concentrations (0, 10, 20, 40, or 80 μmol/L) of metformin 12 h prior to H/R. Metformin at a dose of 40 μM was used to additionally explore the mechanisms of action according to the cell viability analysis of the first phase. H9c2 cells were then divided into five groups as follows: (1) control group, no treatment; (2) H/R group, hypoxia (6 h) and reoxygenation (4 h); (3) H/R+Met group, adding metformin 12 h prior to H/R; (4) H/R+small interfering RNA negative control (siNC)+Met group; and (5) H/R+siSTEAP4+Met group. For both the H/R+siNC+Met and H/R+siSTEAP4+Met groups, the cells were transfected 36 h prior to H/R with 30 pmol siRNA (GenePharma, Shanghai, China) before adding metformin 12 h prior to H/R.

CCK-8 assay

To determine cell activity, H9c2 cells (5×10^3 cells/well) were plated in 96-well plates at 37 °C, followed by exposure to the aforementioned treatments. Cell viability was measured using a Cell Counting Kit-8 (CCK-8) assay kit according to the manufacturer's instructions. After treatment with 10 μL CCK-8 solution for 1 h, absorbance was measured at 450 nm using a spectrophotometer (Olympus).

Annexin V–FITC/PI

To confirm the cell rate of apoptosis, H9c2 cells were collected from 6-well plates and washed twice with phosphate-buffered saline (PBS). The cells were resuspended in binding buffer. After adding 5 μL Annexin V–fluorescein isothiocyanate (FITC) and 5 μL propidium iodide (PI) (KeyGen, Nanjing, China), the cells were analyzed after an incubation period at 18-25°C for 15 min (BD Biosciences, Franklin Lakes, NJ, USA).

Western blotting

Cardiac tissues and cellular proteins were lysed using radioimmunoprecipitation assay lysis buffer and quantified by bicinchoninic acid. After collecting the lysate supernatant, equal amounts of proteins were boiled for 5 min with loading buffer. Protein samples (30 μg per lane), separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, were transferred to polyvinylidene difluoride membranes (EMD Millipore, Bedford, MA, USA) and blocked with buffer (5% nonfat milk in Tris-buffered saline with Tween-20 (TBST) buffer) for 1 h. The membranes were incubated with primary antibodies against STEAP4 (1:500 dilution; Novus Biological, CO, USA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:1000 dilution; Proteintech, Wuhan, China), B-cell lymphoma 2 (Bcl-2; 1:500 dilution; Proteintech), and Bcl-2-associated X protein (Bax; 1:500 dilution; Proteintech). Following washing 3 times (15 min each time) with TBST, the membranes were incubated with the corresponding secondary antibody (horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit) for 1 h at 18-25°C. After washing 3 times, protein bands were displayed by using enhanced chemiluminescence reagent and quantified by Gel-Pro analyzer version 4.0 software (Media Cybernetics). GAPDH was used as a standard control.

Measurement of MMP

Rhodamine 123 (Solarbio, Beijing, China) was used in the present study to detect changes in mitochondrial membrane potential (MMP). In brief, H9c2 cells were incubated in media containing 5 mg/mL Rhodamine 123 for 30 min in darkness at 37 °C. Then, the cells were washed twice with PBS. Ultimately, the cells were analyzed under a fluorescence microscope (Thermo Scientific, CO, USA).

Statistical analysis

Data were obtained from at least three individual experiments.

The experimental results were expressed as mean±SD. The Kolmogorov–Smirnov test was applied to test the normality of distributions.

Differences among the three groups were first evaluated using a one-way analysis of variance, and if the differences were significant, multiple comparison analysis was further performed using Fisher's least significant difference test. The Kruskal–Wallis H test was used for skewed distribution (≥3 groups), and the Wilcoxon rank sum test was applied for multiple comparisons between the groups. All statistical analyses were conducted using SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA). A p value <0.05 was considered to be statistically significant.

Results

Metformin-attenuated myocardial injury following I/R in rat hearts

To examine whether metformin improves myocardial injury in rat hearts after I/R, serum levels of CK-MB and cTnT were measured by ELISA. The results showed that serum CK-MB (I/R group vs. control group: 8.337±1.055 vs. 4.102±0.838, p=0.023) and cTnT (I/R group vs. control group: 486.272±27.636 vs. 169.778±10.641, p=0.012) levels were higher in the I/R group than in the control group. When adding metformin, CK-MB (I/R+Met group vs. I/R group: 6.554±1.241 vs. 8.337±1.055, p=0.048) and cTnT (I/R+Met

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group vs. I/R group: 375.806±15.380 vs. 486.272±27.636, p=0.041) levels markedly decreased (Fig. 1a and 1b, Table 1). Meanwhile, as illustrated in Figure 2a (Table 2), the extent of MI was then evaluated at 2 h of reperfusion. There was no MI in the rats of the control. Compared with the I/R group, fewer infarct areas were observed following the addition of metformin (I/R+Met group vs. I/R group: 12.53±3.10% vs. 22.46±4.77%, p=0.011). The results of the H&E staining indicated that myocardial cells did not show any pathological changes in the control group while abnormal changes in the I/R group. Following treatment with metformin after I/R, as evidenced by less vacuolation in the cytoplasm and a decreased number of cells exhibiting nuclear condensation, fragmentation, dissolution, myocardial interstitial edema and inflammatory cell infiltration compared with the I/R group (Fig. 2b).

Table 1. CK-MB and cTnT levels in serum (mean±SD, n=5)

Group	CK-MB (ng/mL)	cTnT (pg/mL)
Control	4.102±0.838	169.778±10.641
I/R	8.337±1.055#	486.272±27.636#
I/R+Met	6.554±1.241*	375.806±15.380*

#*P*<0.05 versus control group, **P*<0.05 versus I/R group.
I/R - ischemia–reperfusion; Met - metformin; CK-MB - creatinine kinase-MB; cTnT - cardiac troponin T

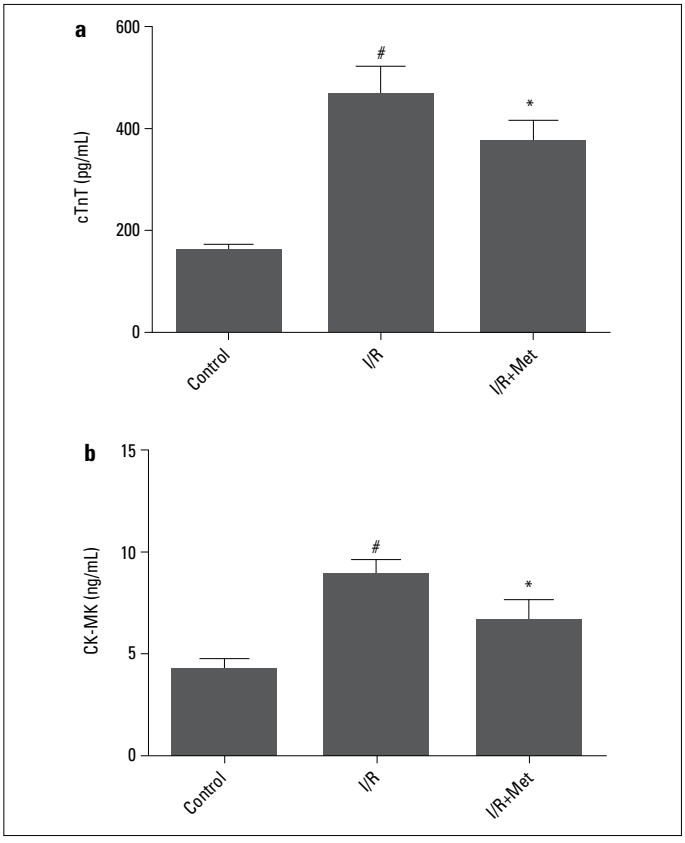

Figure 1. Metformin reverses the degree of myocardial infarction induced by I/R in rats. (a) cTnT levels were measured using a commercial ELISA kit. (b) CK-MB levels were detected using a commercial ELISA kit. Data are presented as mean±SD; n=5; #*P*<0.05 versus control, **P*<0.05 versus I/R treatment group.
I/R - ischemia–reperfusion; ELISA - enzyme-linked immunosorbent assay; Met - metformin; CK-MB - creatinine kinase-MB; cTnT - cardiac troponin T

Effect of metformin on the expression levels of STEAP4, Bax, Bcl-2, and cell apoptosis in rat hearts subjected to I/R

As illustrated in Figure 3a (Table 4), western blot analysis confirmed that protein expression levels of the pro-apoptosis proteins (Bax) significantly increased (I/R group vs. control group: 3.356±0.675 vs. 1.000±0, p=0.024) and the anti-apoptotic proteins (Bcl-2) decreased (I/R group vs. control group: 0.248±0.039 vs. 1.000±0, p=0.017) after I/R compared with the control group, which were significantly abrogated by metformin at a concentration of 250 mg/kg. The protein expression level of STEAP4 was also significantly decreased in rats subjected to I/R injury (I/R group vs. control group: 0.334±0.046 vs. 1.000±0, p=0.036) but was effectively promoted by metformin (I/R+Met group vs. I/R group: 0.756±0.088 vs. 0.334±0.046, p=0.029). Meanwhile, cell apoptosis was evaluated by the TUNEL assay (Fig. 3b, Table 3). TUNEL-positive cells in the I/R group were significantly higher than those in the control group (I/R group vs. control group: 19.87±2.07% vs. 4.44±1.76%, p=0.003). The addition of metformin significantly decreased the number of apoptotic cells (I/R+Met group vs. I/R group: 9.73±1.89% vs. 19.87±2.07%, p=0.009).

Table 2. TTC staining is performed to assess infarct size (mean±SD, n=5)

	Control	I/R	I/R+Met
Mean±SD	0.00±0%	22.46±4.77%#	12.53±3.10%*

#*P*<0.05 versus control group, **P*<0.05 versus I/R group

Table 3. Apoptosis rate is detected by TUNEL assay in the heart (mean±SD, n=5)

	Control	I/R	I/R+Met
Mean±SD	4.44±1.76%	19.87±2.07%##	9.73±1.89%**

##*P*<0.01 versus control group, ***P*<0.01 versus I/R group

Table 4. Relative Bcl-2 and Bax and STEAP4 protein expression are detected by western blot in the heart (mean±SD, n=5)

	Control	I/R	I/R+Met
STEAP4	1.000±0	0.334±0.046#	0.756±0.088*
Bcl-2	1.000±0	0.248±0.039#	0.692±0.076**
Bax	1.000±0	3.356±0.675#	2.521±0.451*

#*P*<0.05 versus control group, **P*<0.05 and ***P*<0.01 versus I/R group

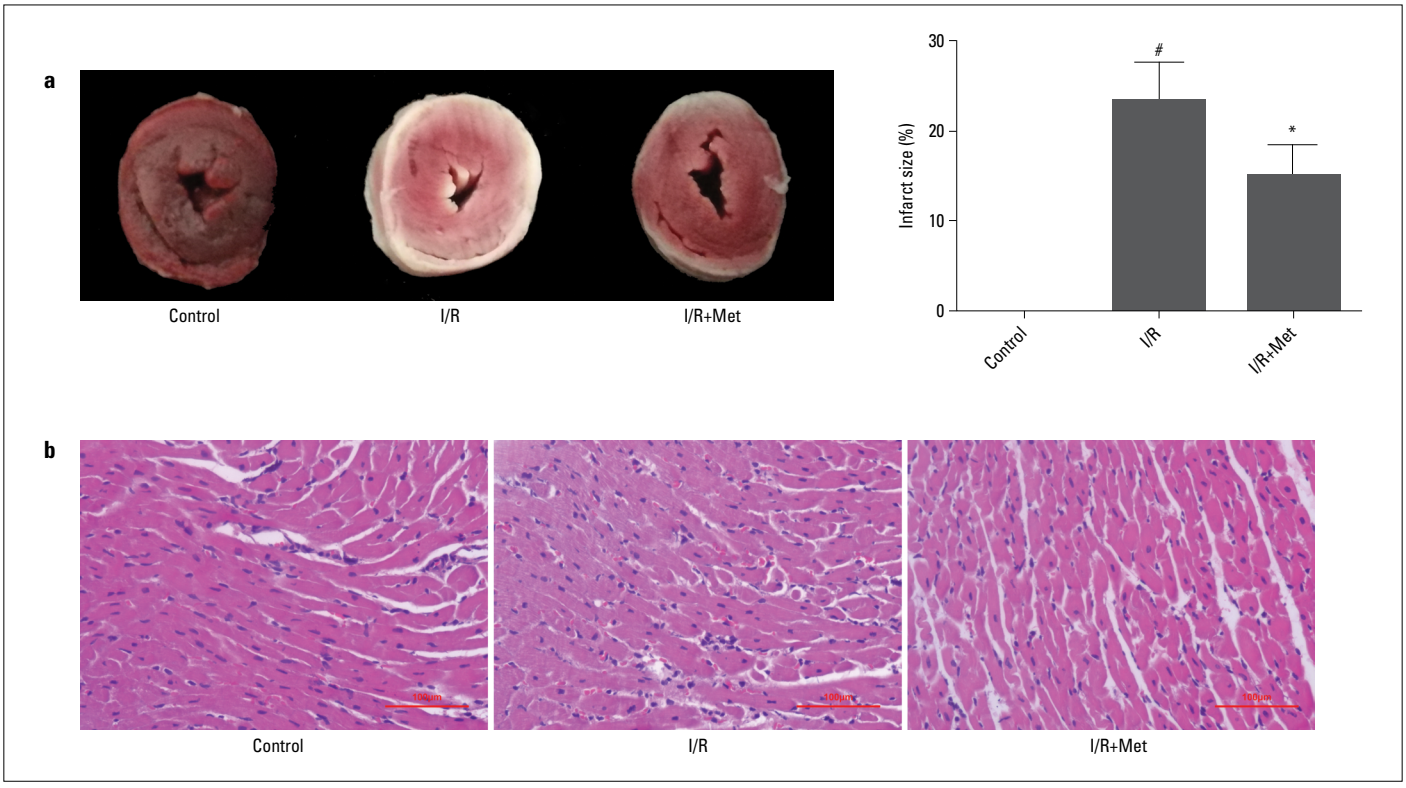

Figure 2. Protective effect of metformin on infarct size and pathological changes. (a) TTC staining was performed to assess infarct size. (b) H&E staining was performed to evaluate the pathological changes in myocardial tissues. Scale bars=50 μm. Data are presented as mean±SD; n=5; $^{\#}P<0.05$ versus control, $^{*}P<0.05$ versus I/R treatment group

I/R - ischemia–reperfusion; H&E - hematoxylin and eosin; TTC - triphenyl tetrazolium chloride

Table 5. Relative cell activity is detected by Counting Kit-8 assay in H9c2 cell exposure to concentrations (0, 10, 20, 40, or 80 μmol/L) of metformin 12 h prior to H/R (mean±SD, n=6)

	Control	H/R	H/R+Met (10 μmol/L)	H/R+Met (20 μmol/L)	H/R+Met (40 μmol/L)	H/R+Met (80 μmol/L)
Mean±SD	1.00±0	0.42±0.11#	0.58±0.17*	0.69±0.07**	0.83±0.14**	0.81±0.08**

$^{\#}P<0.05$ versus control group, $^{*}P<0.05$ and $^{**}P<0.01$ versus H/R group.
H/R - hypoxia–reoxygenation; Met - metformin

Metformin mitigated the H/R-induced apoptosis of H9c2 cardiomyocytes

To determine the suitable concentration of metformin and confirm its influence on the viability of H9c2 cells, the cells were treated with various concentrations (0, 10, 20, 40, or 80 μmol/L) before H/R injury (Fig. 4a, Table 5). Metformin was observed to improve cell viability of H9c2 cardiomyocytes in a concentration-dependent manner. Eventually, we selected the 40 μmol/L concentration of metformin to perform the subsequent experiments. CCK-8 assay indicated that metformin treatment significantly increased the cell survival of H9c2 cells in a dose-dependent, and the 40 μmol/L metformin significantly augmented the viability of H9c2 cells after H/R injury [H/R+Met (10 μmol/L) group vs. H/R group: 0.58±0.17 vs. 0.42±0.11, p=0.038; H/R+Met (20 μmol/L) group vs. H/R group: 0.69±0.07 vs. 0.42±0.11, p=0.009; H/R+Met (40 μmol/L) group vs. H/R group: 0.83±0.14 vs. 0.42±0.11, p=0.002; and H/R+Met (80 μmol/L) group vs. H/R group: 0.81±0.08 vs. 0.42±0.11, p=0.005]. As a marker of pathological cardiac apoptosis, the protein levels of Bax and Bcl-2 were also detected to determine the optimum concentration of metformin (Fig. 4b, Table 6). We determined that metformin increased the Bcl-2/Bax ratio in a dose-dependent manner [H/R+Met (10 μmol/L) group vs. H/R group: 0.41±0.09 vs. 0.22±0.06, p=0.043; H/R+Met (20 μmol/L) group vs. H/R group: 0.63±0.15 vs. 0.22±0.06, p=0.007; H/R+Met (40 μmol/L) group vs. H/R group: 0.78±0.11 vs. 0.22±0.06, P=0.002; and H/R+Met (80 μmol/L) group vs. H/R group: 0.73±0.04 vs. 0.22±0.06, p=0.004]. Thus, incubation with 40 μmol/L metformin was selected to intervene in subsequent experiments.

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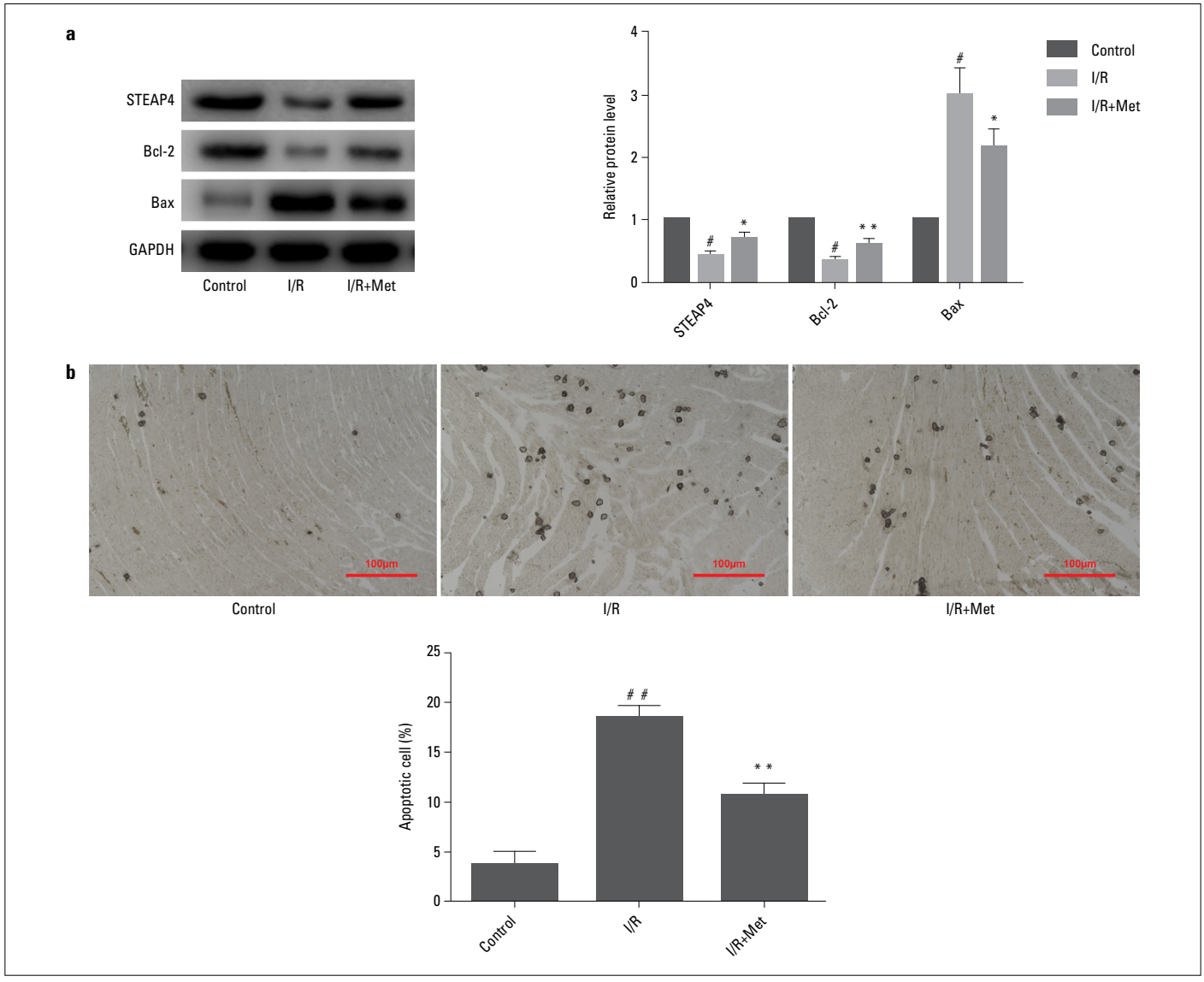

Figure 3. Metformin promoted STEAP4 expression and decreased cell apoptosis in myocardial tissues. (a) The apoptotic marker Bcl-2 and Bax and STEAP4 protein expression were detected by western blot. GAPDH was used as a loading control. (b) TUNEL assay of infarcted border zone. Scale bars=50 μm. Values are presented as mean±SD; n=5; $^{\#}P<0.05$ and $^{\#\#}P<0.01$ versus control, $^{*}P<0.05$ and $^{**}P<0.01$ versus I/R treatment group
STEAP4 - six-transmembrane epithelial antigen of prostate 4; Bcl-2 - B-cell lymphoma 2; Bax - Bcl-2-associated X protein; GAPDH - glyceraldehyde 3-phosphate dehydrogenase; I/R - ischemia–reperfusion; Met - metformin; TUNEL - terminal deoxynucleotidyl transferase dUTP nick end labeling

Table 6. Bcl-2/Bax rate is detected by western blot in H9c2 cell exposure to concentrations (0, 10, 20, 40, or 80 μmol/L) of metformin 12 h prior to H/R (mean±SD, n=6)

	Control	H/R	H/R+Met (10 μM)	H/R+Met (20 μM)	H/R+Met (40 μM)	H/R+Met (80 μM)
Bcl-2/ Bax	1.00±0	0.22±0.06$^{\#}$	0.41±0.09*	0.63±0.15**	0.78±0.11**	0.73±0.04**

$^{\#}P<0.05$ versus control group, $^{*}P<0.05$ and $^{**}P<0.01$ versus H/R group.
Bcl-2 - B-cell lymphoma 2; Bax - Bcl-2-associated X protein; H/R - hypoxia–reoxygenation; Met - metformin

Knockdown of STEAP4 abrogated the beneficial effects of metformin

Similar to the animal experiment results, metformin treatment also markedly downregulated the protein expression of Bax (H/R+Met group vs. H/R group: 1.537±0.254 vs. 2.549±0.782, p=0.022), upregulated the protein expression of Bcl-2 (H/R+Met group vs. H/R group: 0.865±0.047 vs. 0.324±0.067, p=0.035), and enhanced the expression of STEAP4 (H/R+Met group vs. H/R

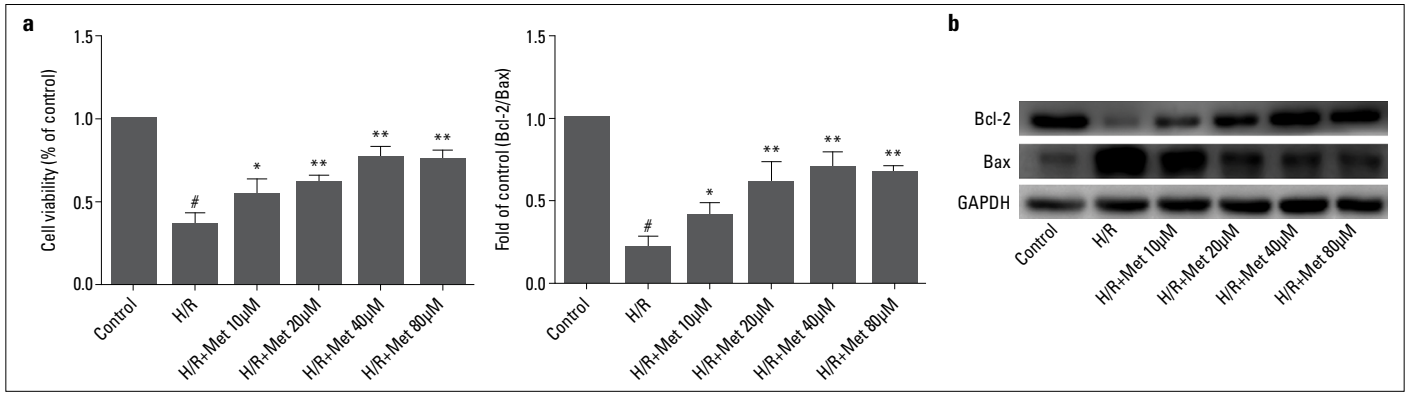

Figure 4. Metformin improved the reduced cell viability in H9c2 cardiomyocytes. (a) CCK-8 assay. (b) Western blot analysis of Bcl-2 and Bax expression. The Bcl-2/Bax ratio was calculated and compared. GAPDH served as an internal control. Data are presented as mean±SD; n=6; $^{\#}P<0.05$ versus control, $^{*}P<0.05$ and $^{**}P<0.01$ versus H/R treatment group

CCK-8 - Cell Counting Kit-8; Bcl-2 - B-cell lymphoma 2; Bax - Bcl-2-associated X protein; GAPDH - glyceraldehyde 3-phosphate dehydrogenase; H/R - hypoxia–reoxygenation; Met - metformin

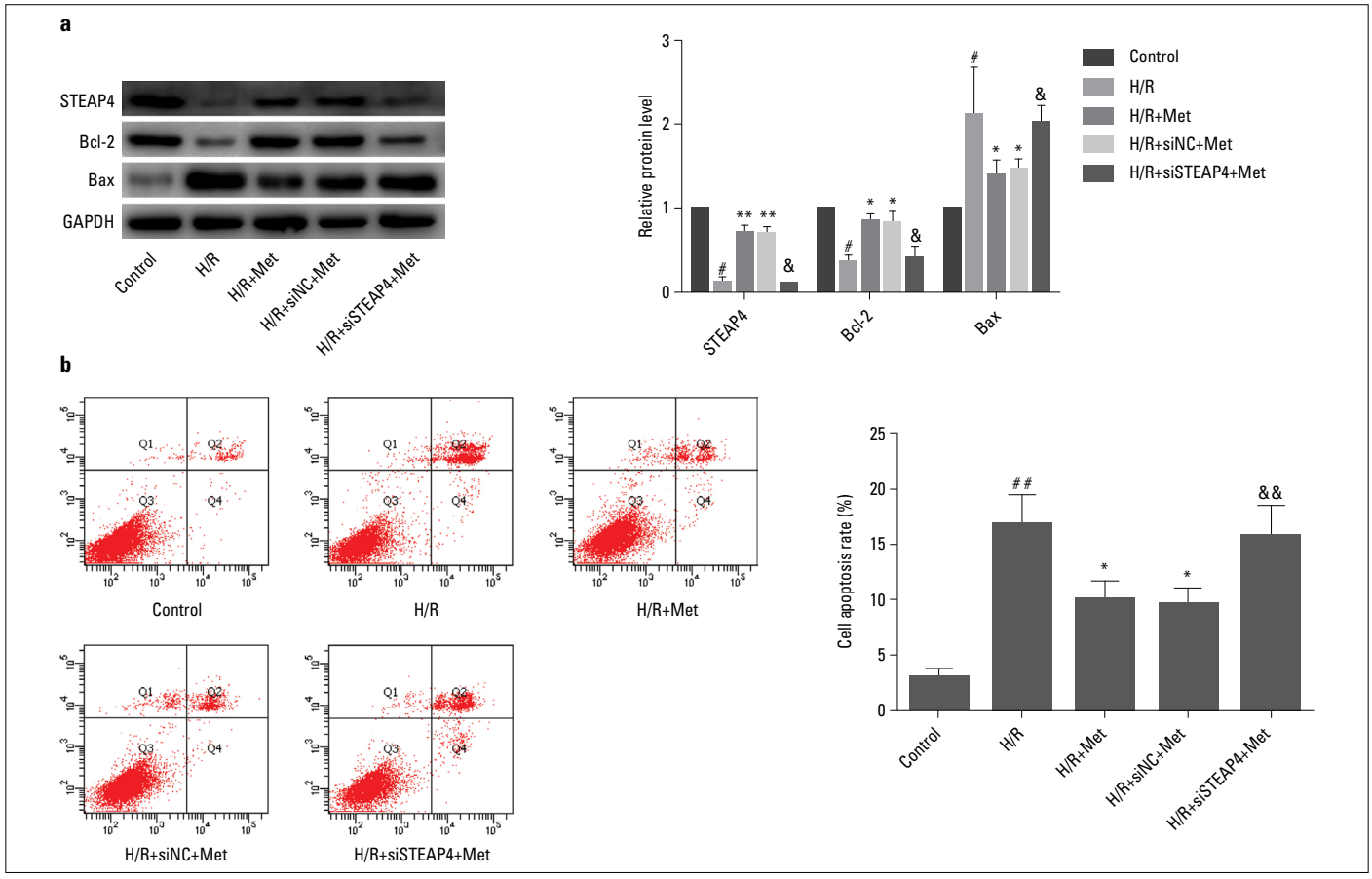

Figure 5. Knockdown of STEAP4 deteriorated cell survival in H9c2 cardiomyocytes treated with metformin after H/R. (a) The Bcl-2, Bax, and STEAP4 protein expression were detected by western blot. (b) Myocardial cell apoptosis was examined by Annexin V–FITC/PI assay. Data are presented as mean±SD; n=5; $^{\#}P<0.05$ and $^{\#\#}P<0.01$ versus control, $^{*}P<0.05$ and $^{**}P<0.01$ versus H/R treatment group, $^{\&}P<0.05$ and $^{\&\&}P<0.01$ versus H/R+siNC+Met group

Bcl-2 - B-cell lymphoma 2; Bax - Bcl-2-associated X protein; NC - negative control; siRNA - small interfering RNA; FITC - fluorescein isothiocyanate; PI - propidium iodide; Met - metformin; STEAP4 - six-transmembrane epithelial antigen of prostate 4; H/R - hypoxia–reoxygenation

group: 0.715±0.103 vs. 0.172±0.034, p=0.004) when cells were treated with H/R (Fig. 5a, Table 7). To further determine the involvement of STEAP4 in the anti-apoptotic effects of metformin, H9c2 cardiomyocytes were transfected with STEAP4 siRNA. Then, siSTEAP4 transfection partially abrogated the enhancement in the Bcl-2 (H/R+siSTEAP4+Met group vs. H/R+siNC+Met group: 0.431±0.162 vs. 0.832±0.145, p=0.034) induced by metformin compared with the siNC-transfected cells. Similarly, flow

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Table 7. Relative Bcl-2 and Bax and STEAP4 protein expression is detected by western blot in H9c2 cells (mean±SD, n=5)

	Control	H/R	H/R+Met	H/R+siNC+Met	H/R+siSTEAP4+Met
STEAP4	1.000±0	0.172±0.034#	0.715±0.103**	0.697±0.126**	0.114±0.089&
Bcl-2	1.000±0	0.324±0.067#	0.865±0.047*	0.832±0.145*	0.431±0.162&
Bax	1.000±0	2.549±0.782#	1.537±0.254*	1.681±0.193*	2.471±0.324&

#*P*<0.05 versus control group, **P*<0.05 and ***P*<0.01 versus H/R group, &*P*<0.05 versus H/R + siNC+Met group.
NC - negative control; siRNA - small interfering RNA; Met - metformin; STEAP4 - six-transmembrane epithelial antigen of prostate 4; H/R - hypoxia–reoxygenation

Table 8. Myocardial cell apoptosis rate is examined by Annexin V–FITC/PI assay (mean±SD, n=5)

	Control	H/R	H/R+Met	H/R+siNC+Met	H/R+siSTEAP4+Met
Mean±SD	4.31±0.78%	17.42±2.47%##	9.62±0.94%*	8.83±0.73%*	16.58±1.76%&&

##*P*<0.01 versus control group, **P*<0.05 versus H/R group, &&*P*<0.01 versus H/R+siNC+Met group

Table 9. The mitochondrial membrane potential of each group is detected by Rhodamine 123 (mean±SD, n=5)

	Control	H/R	H/R+Met	H/R+siNC+Met	H/R+siSTEAP4+Met
Mean±SD	1.00±0	0.71±0.12#	0.84±0.09*	0.81±0.08*	0.73±0.05&

#*P*<0.05 versus control, **P*<0.05 versus H/R group, &*P*<0.05 versus H/R+siNC+Met group

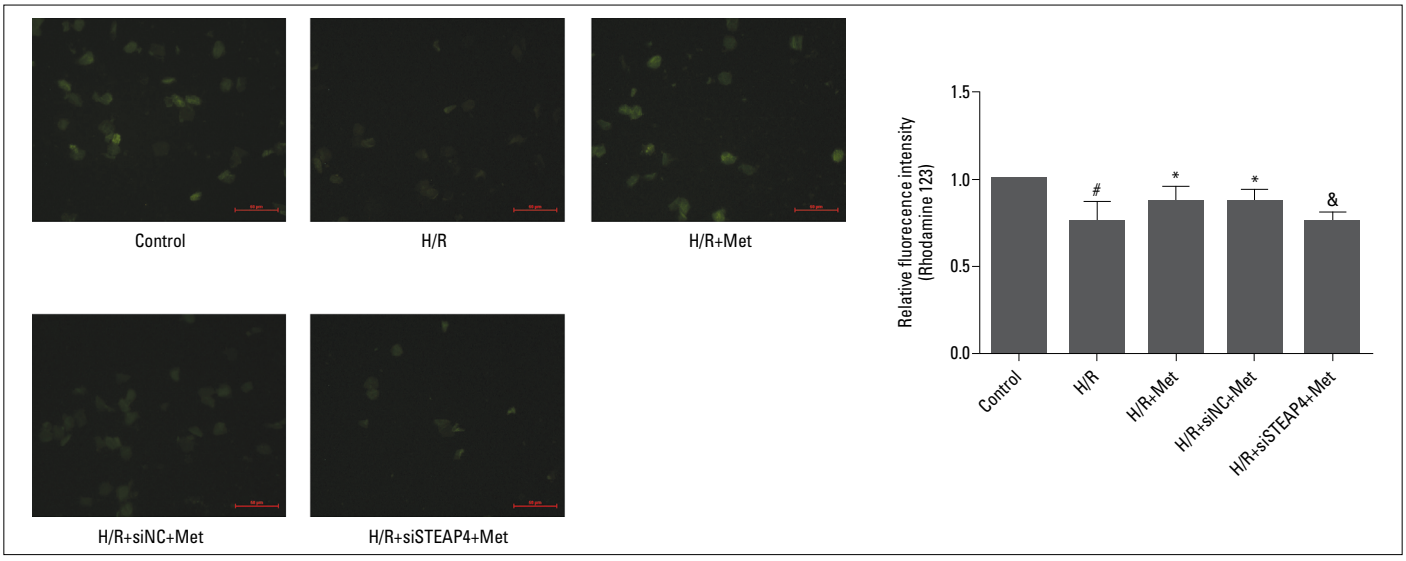

Figure 6. STEAP4 knockdown impaired the function of the mitochondrion. The MMP of each group was detected by Rhodamine 123 (green). Scale bars=25 μm. Data are presented as mean±SD; n=5; #*P*<0.05 versus control, **P*<0.05 versus H/R treatment group, &*P*<0.05 versus H/R+siNC+Met group

MMP - mitochondrial membrane potential; STEAP4 - six-transmembrane epithelial antigen of prostate 4; I/R - ischemia–reperfusion; Met - metformin; NC - negative control; siRNA - small interfering RNA; H/R - hypoxia–reoxygenation

cytometric analysis showed that H9c2 cells undergoing early and late apoptosis decreased to 9.62±0.94% from 17.42±2.47% after metformin treatment following H/R injury (H/R+Met group vs. I/R group: 9.62±0.94% vs. 17.42±2.47%, p=0.039) (Fig. 5b, Table 8). However, the protective effects of metformin on cell apoptosis were weakened by transfection of exogenous STEAP4 (H/R+siSTEAP4+Met group vs. H/R+siNC+Met group: 16.58±1.76% vs. 8.83±0.73%, p=0.001).

STEAP4 knockdown abrogated the beneficial effects of metformin on mitochondrial function

MMP changes are characteristic features of mitochondrial function. Therefore, to determine the effect of STEAP4 on mitochondrial function in H9c2 cardiomyocytes, the MMP (Fig. 6, Table 9) was detected. Cells with an MMP impairment due to H/R have a lower fluorescence intensity of Rhodamine 123 than normal cells (H/R group vs. control group: 0.71±0.12 vs. 1.00±0,

p=0.014). These MMP changes were reversed by the addition of metformin (H/R+Met group vs. I/R group: 0.84±0.09 vs. 0.71±0.12, p=0.045). However, these protective effects of metformin on the mitochondria in cardiomyocytes were attenuated by downregulation of STEAP4 (H/R+siSTEAP4+Met group vs. H/R+siNC+Met group: 0.73±0.05 vs. 0.81±0.08, p=0.037).

Discussion

Clinical studies have revealed that MI is the major cause of morbidity and mortality worldwide. Restoration of the blood supply to the heart is considered to be the most effective treatment for patients with MI (24). Nevertheless, reperfusion itself can deteriorate the extent of myocardial injury, a phenomenon known as myocardial I/R injury (25). Therefore, effective therapeutic targets are urgently needed to alleviate I/R injury in the heart.

The majority of the clinical benefits associated with metformin are a direct result of its glucose-lowering function. Metformin also has pleiotropic effects on various aspects of cardiovascular disease (26). Previous studies suggested that metformin not only reduced blood glucose but also had anti-inflammatory, antioxidative stress, and cardioprotective properties (27). Furthermore, clinical studies indicated that metformin pretreatment protected against I/R injury and might improve myocardial function after an ischemic insult (28). Several studies also showed that metformin exerts cardioprotective effects by reducing myocardial cell apoptosis, which are key features of cardiac function (29). In an experimental study, metformin significantly repressed the number of TUNEL-positive myocytes (30). It has been hypothesized that the inhibition of the pathways associated with cell apoptosis may be a potential therapeutic approach. Similarly, we also found that metformin diminished apoptosis of H9c2 cardiomyocytes and rat cardiac cells after I/R or H/R injury and increased the Bcl-2/Bax protein expression level ratio. These *in vitro* and in vivo results further confirmed the positive effect of metformin on myocardial I/R injury by mitigating cellular apoptosis. However, the molecular mechanisms of this anti-apoptotic function of metformin have not been clarified.

The mechanisms of action of metformin on the cardioprotective signaling pathways have been previously reported to activate the AMPK pathway (31). AMPK was considered a key molecule for cardioprotection via modulation of several signaling pathways involved in glucose metabolism and energy homeostasis (32). Similarly, proper expression of STEAP4 was also suggested to be important for maintenance of energy homeostasis (33). Experimental results showed that monoclonal antibodies specific for STEAP4 promoted apoptosis and inhibited proliferation and glucose uptake in human adipocytes (18). Recent studies suggested that STEAP4 was upregulated in human $CD34^+$ cells subjected to hypoxia after metformin treatment (20). Therefore, we assumed that STEAP4 participates in the protective effects of metformin, and its involvement was studied in the present study.

Several studies have identified the pivotal roles of STEAP4 in the control of cellular proliferation, differentiation, metabolism, inflammation, and many other responses in a variety of tissues (34). It has been demonstrated that there is a correlation between STEAP4 and heart failure (19). In the present study, the protein expression level of STEAP4 was significantly decreased in H9c2 cells after H/R. When these cells were cotransfected with a STEAP4 siRNA, the upregulation induced by metformin was abrogated after H/R. Knockdown of STEAP4 also inhibited the protective effects of metformin and led to an increase of apoptosis levels and decreased the Bcl-2/Bax ratio. These results showed that STEAP4 might play an important role in the metformin-associated attenuation of the apoptosis induced by I/R or H/R injury *in vitro* and in vivo. Bcl-2 and Bax are known as mitochondria-related apoptosis proteins. Thus, knockdown of STEAP4 might influence the mitochondrial energy metabolism in I/R-injured cardiomyocytes.

Recently, cell energy metabolism has attracted significant attention. It is well-established that the mitochondria play key roles in the regulation of energy metabolism (35). STEAP4 can affect mitochondrial energy metabolism (36, 37). In addition, mitochondrial function is associated with MMP dissipation. In the present study, to explore the function of STEAP4 in cardiomyocytes, we measured MMP. We found that metformin increased MMP in H9c2 cells compared with the H/R group after H/R. Knockdown of STEAP4 significantly reversed this condition. STEAP4 may play a protective role by regulating mitochondrial metabolism.

Taken together, we further identified the protective effects of metformin on H9c2 cardiomyocytes by inhibiting apoptosis and rescuing the MMP. STEAP4 serves as a master regulator in pathological myocardial damage by decreasing apoptosis and improving mitochondrial metabolic function. The beneficial effects of metformin might be at least partly attributable to the upregulation of STEAP4. Accordingly, the signaling pathway associated with STEAP4 might be a novel target for pleiotropic effects of metformin. STEAP4 might be a new target to rescue mitochondrial function and reduce apoptosis in cardiac injury.

Study limitations

First, we did not use STEAP4 gene knockdown rats; thereby, the effect of metformin on STEAP4 was not investigated in intact organism. Second, studies on the effect of STEAP4 on mitochondrial function were insufficient.

Conclusion

The results of our current study are consistent with those of previous studies in which metformin decreases I/R myocardial lesions. Moreover, we found new evidence and insight that metformin protects against these myocardial lesions by regulating the STEAP4 pathway.

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