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Reduction of SIRT1 blunts the protective effects of ischemic post-conditioning in diabetic mice by impairing the Akt signaling pathway

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ABSTRACT

Ischemic post-conditioning (IPO) activates Akt signaling to confer cardioprotection. The responsiveness of diabetic hearts to IPO is impaired. We hypothesized that decreased cardiac SIRT1, a positive regulator of Akt, may be responsible for the impaired responsiveness of diabetic hearts to IPO-mediated cardioprotection. High-fat diet and streptozotocin-induced diabetic mice were subjected to myocardial ischemia/reperfusion (MI/R, 30 min ischemia and 180 min reperfusion) or IPO (three cycles of 10 s of reperfusion and ischemia at the onset of reperfusion). Adenoviral vectors encoding GFP or SIRT1 (Ad-SIRT1) were administered by direct injection into the left ventricular. Our results showed that IPO activated the Akt signaling pathway and reduced MI/R injury in non-diabetic hearts but not in diabetic hearts, in which reduced expression of SIRT1 and increased Akt acetylation were observed. Delivery of Ad-SIRT1 into the diabetic hearts reduced Akt acetylation and restored the cardioprotective effects of IPO by modulating Akt signaling pathway. In contrast, cardiac-specific SIRT1 knockout increased Akt acetylation and blunted the cardioprotective effects of IPO. In in vitro study, transfection with wild-type SIRT1 but not inactive mutant SIRT1 reduced the expression of Akt acetylation and restored the protective effects of hypoxic post-conditioning in high glucose-incubated cardiomyocytes. Moreover, the cardiomyocytes transfected with constitutive Akt acetylation showed repressed Akt phosphorylation and blunted protective effects against hypoxia/reoxygenation injury. These findings demonstrate that the reduction of SIRT1 blunts the protective effects of IPO by impairing Akt signaling pathway and that SIRT1 up-regulation restores IPO-mediated cardioprotection in diabetic mice via deacetylation-dependent activation of Akt signaling pathway.

1. Introduction

Acute myocardial infarction is one of the major causes of morbidity and mortality around the world. Urgent reperfusion therapy is the most effective strategy but may paradoxically induce myocardial damage, termed reperfusion injury [1]. Ischemic post-conditioning (IPO), a series of repetitive cycles of brief reperfusion and ischemia at the immediate onset of reperfusion, is a promising strategy aimed at reducing myocardial ischemia-reperfusion (MI/R) injury in animal studies [2–4]. An updated meta-analysis has indicated that IPO improves cardiac function and reduces the incidence of heart failure and serious arrhythmia in patients with ST-segment elevation myocardial infarction undergoing primary percutaneous coronary intervention [5]. It is well known that the activation of the PI3K/Akt pathway is essential for the cardioprotection of IPO. Inhibiting PI3K/Akt pathway at reperfusion with wortmannin or LY294002 completely abolishes IPO-induced protection [6,7]. Activated Akt phosphorylates its downstream targets such as glycogen synthase kinase-3β (GSK-3β), endothelial nitric oxide synthase (eNOS) and BAD, which prevent mitochondrial permeability transition pore (mPTP) opening and reduce cell death [8,9].

Since diabetic patients are at high risk of developing ischemic heart disease, they are increasingly undergoing acute revascularization. Unfortunately, ischemic post-conditioning, known for their ability to reduce MI/R injury, appears to be ineffective in diabetic hearts.

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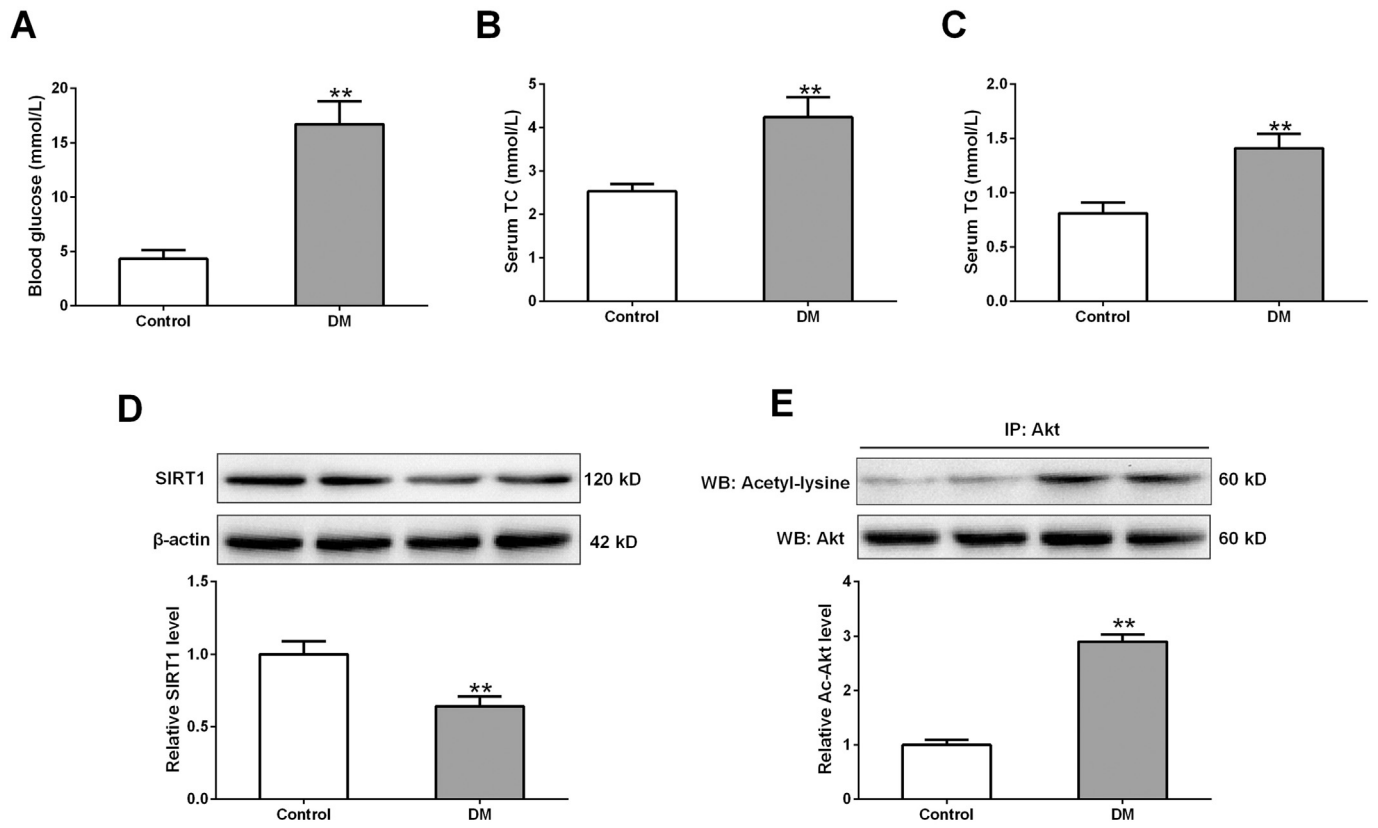

Fig. 1. Reduced SIRT1 expression and increased Akt acetylation were found in diabetic hearts. (A) Blood glucose. (B) Serum total cholesterol (TC). (C) Serum triacylglycerol (TG). (D) Representative blot images and quantitative analysis of SIRT1 expression. (E) Representative blot images and quantitative analysis of acetylated Akt (Ac-Akt). Values are presented as means ± SEM. DM, diabetes mellitus. n = 6–8 in each group. $^{**}P < 0.01$ vs. Control.

Numerous preclinical animal studies have demonstrated that the cardioprotective effects of ischemic postconditioning (IPO) are blunted in both STZ-induced type 1 animals and type 2 diabetic animals such as db/db or ob/ob [10,11]. There have been a few clinical studies investigating the interaction of diabetes with postconditioning. POST-AMI trial has found that IPO did not have the expected cardioprotective effect in ST-elevation myocardial infarction patients [12]. In this clinical trial, the proportion of diabetics was not balanced between IPO group (18%) and control group (3%), which may blunt the protective effects of IPO. In addition, Yetgin et al. have found that diabetic patients displayed higher peak creatine kinase values in the IPO analogue group [13]. These clinical findings are compatible with the observations from the preclinical setting, both of which demonstrate the resistance of the diabetic heart to IPO. Diabetes modifies myocardial responses to IPO by disturbing intracellular survival signaling. The lack of enhanced phosphorylation of Akt by IPO is critical in the loss of cardioprotection in diabetes [14,15]. However, the mechanism underlying the impaired Akt signaling in diabetic hearts is still largely unknown.

Phosphorylation is considered as the primary mechanism driving Akt signaling activation. Recent studies reveal that acetylation modification is also essential and equally important as phosphorylation for Akt signaling activation. Acetylation modification has a negative effect on the activity of Akt [16]. Protein acetylation is regulated by sirtuins (SIRTs), which are nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylase. Previous studies from our lab and others have indicated that the expression of SIRT1 is significantly reduced in diabetic heart, while the other sirtuins (SIRT2-7) are increased or unchanged [17–19], suggesting a critical role of SIRT1 in the diabetic heart. Importantly, SIRT1-dependent deacetylation of the PH domain is necessary for the activation of Akt [20]. Therefore we hypothesized that reduced SIRT1 expression in diabetic hearts may increase Akt acetylation and then blunt the cardioprotective effects of IPO via suppressing Akt signaling.

Accordingly, the aims of this study were to identify whether the decreased level of SIRT1 is responsible for the impaired responsiveness of diabetic hearts to IPO, and to investigate whether up-regulation of SIRT1 could restore the responsiveness of diabetic heart to IPO-mediated cardioprotection, and to explore the relative role of Akt signaling pathway during the process.

2. Materials and methods

2.1. Induction of diabetic mice

The experiments were carried out in compliance with the National Institutes of Health Guidelines on the Use of Laboratory Animals and the Fourth Military Medical University Ethics Committee approved all procedures. Schematic representation of the experimental protocol was available in Supplementary Fig. S1. The high-fat diet (HFD) and streptozotocin (STZ)-induced type 2 diabetic mouse model was established as previously described [21]. Male C57BL/6J mice between 8 and 10 weeks old were fed with HFD (D12492, Research Diets) for 4 weeks and then given a single intraperitoneal injection of low-dose STZ (90 mg/kg, Sigma, St. Louis, MO). One week after STZ injection, mice with 12-h fasting blood glucose level ≥ 11.1 mmol/L were considered to have developed diabetes.

2.2. MI/R and IPO operation

Mice were anesthetized with pentobarbital sodium (60 mg/kg, i.p.). Myocardial ischemia was performed by occluding the left anterior descending (LAD) coronary artery for 30 min, followed by reperfusion for 3 h. Sham-operated mice underwent the same surgical procedures without ligation. Ischemia was verified by discoloration of the ischemic

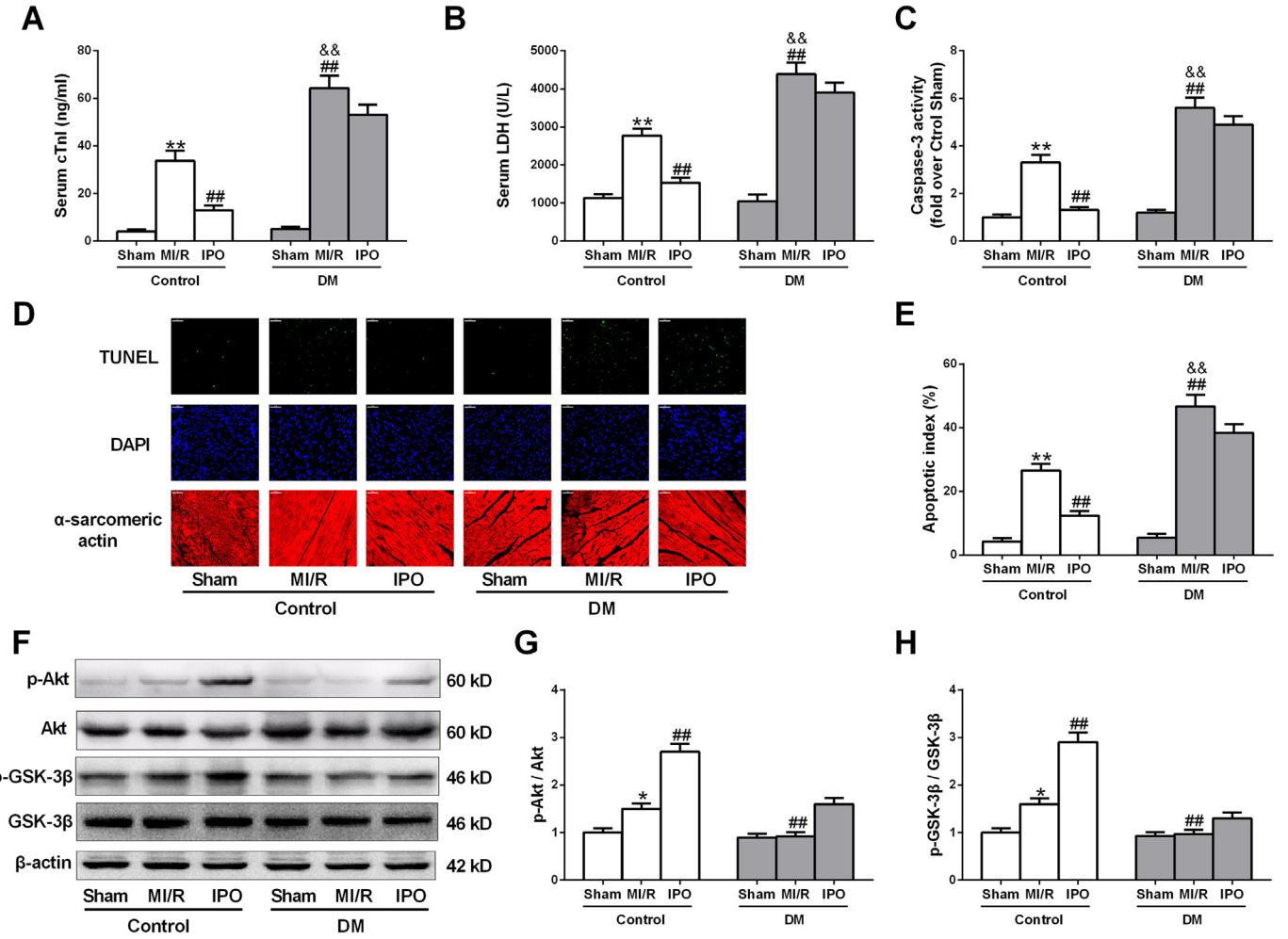

Fig. 2. IPO failed to activate the Akt/GSK-3β signaling pathway to confer cardioprotection in diabetic hearts. (A) Serum levels of cardiac troponin I (cTnI). (B) Serum levels of lactate dehydrogenase (LDH). (C) Myocardial caspase-3 activity (fold over Con). (D) Representative photomicrographs of TUNEL-stained and DAPI-stained heart sections. Original magnification × 400. (E) Percentage of apoptotic cells. (F) Representative blot images for G and H. (G) The ratio of phosphorylated to total Akt (p-Akt/Akt). (H) The ratio of phosphorylated to total GSK-3β (p-GSK-3β/GSK-3β). Values are presented as means ± SEM. DM, diabetes mellitus; MI/R, myocardial ischemia/reperfusion; IPO, ischemic post-conditioning. n = 6–8 in each group. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Control-Sham. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. Control-MI/R. $^{\&\&}P < 0.01$ vs. DM-Sham.

myocardium and elevation of ST-segment in the electrocardiograph. IPO was induced by three cycles of 10 s of reperfusion and ischemia at the beginning of reperfusion [22].

2.3. Adenoviral-mediated gene delivery

Adenovirus was constructed by Hanbio Co., Ltd. (Shanghai, China). The adenoviral-mediated myocardial gene delivery was carried out as previously described [17]. The mice were anesthetized with 2% isoflurane inhalation, and the heart was exposed at the fourth intercostal space. Adenoviral vectors (1.2×10^{10} PFU/mL) were directly injected into the left ventricular free wall with a 32-gauge needle. The adenovirus expressing SIRT1 or GFP was injected at five sites separated by about 2–3 mm around the LAD coronary artery.

2.4. Generation of cardiac-specific SIRT1-knockout ($SIRT1^{-/-}$) mice

Mice carrying a conditional allele of SIRT1 ($SIRT1^{fl/fl}$, > 99% C57BL/6 genetic background) were obtained from National Resource Center for Mutant Mice, initially from the Jackson Laboratory (stock number: 008041). $SIRT1^{fl/fl}$ mice were crossed with C57BL/6 mice expressing Mutated estrogen receptor (Mer)-Cre-Mer under the control of the cardiac-specific alpha-myosin heavy chain promoter (Myh6-MerCreMer) to generate tamoxifen-inducible cardiac-specific SIRT1-knockout mice ($csSIRT1^{-/-}$). $SIRT1^{-/-}$ mice were obtained by providing 2-week oral administration of tamoxifen to $csSIRT1^{-/-}$ mice at the dose of 30 mg/kg/d in chow as we described previously [19]. At 2 weeks after the last administration of tamoxifen, male $SIRT1^{-/-}$ mice between 8 and 10 weeks old were used in the study Age-matched male littermates ($SIRT1^{fl/fl}$ with tamoxifen) were used as controls.

2.5. Echocardiography measurements

Echocardiography was performed in M-mode with a VEVO 2100 echocardiography system (Visual Sonics, Toronto, Canada) as described previously [21]. Left ventricular end-systolic volume (LVESV), left ventricular ejection fraction (LVEF) and fraction shortening (LVFS) were measured in M-mode images using computer algorithms.

2.6. Measurement of serum cardiac troponin I and lactate dehydrogenase

Blood samples were collected from the carotid artery at the end of reperfusion. Serum cardiac troponin I (cTnI) content was measured by enzyme immunoassay using a commercial ELISA kit (Nanjing Jiancheng Reagents, China) and was presented as nanograms per milliliter (ng/mL). Lactate dehydrogenase (LDH) activity was determined by using

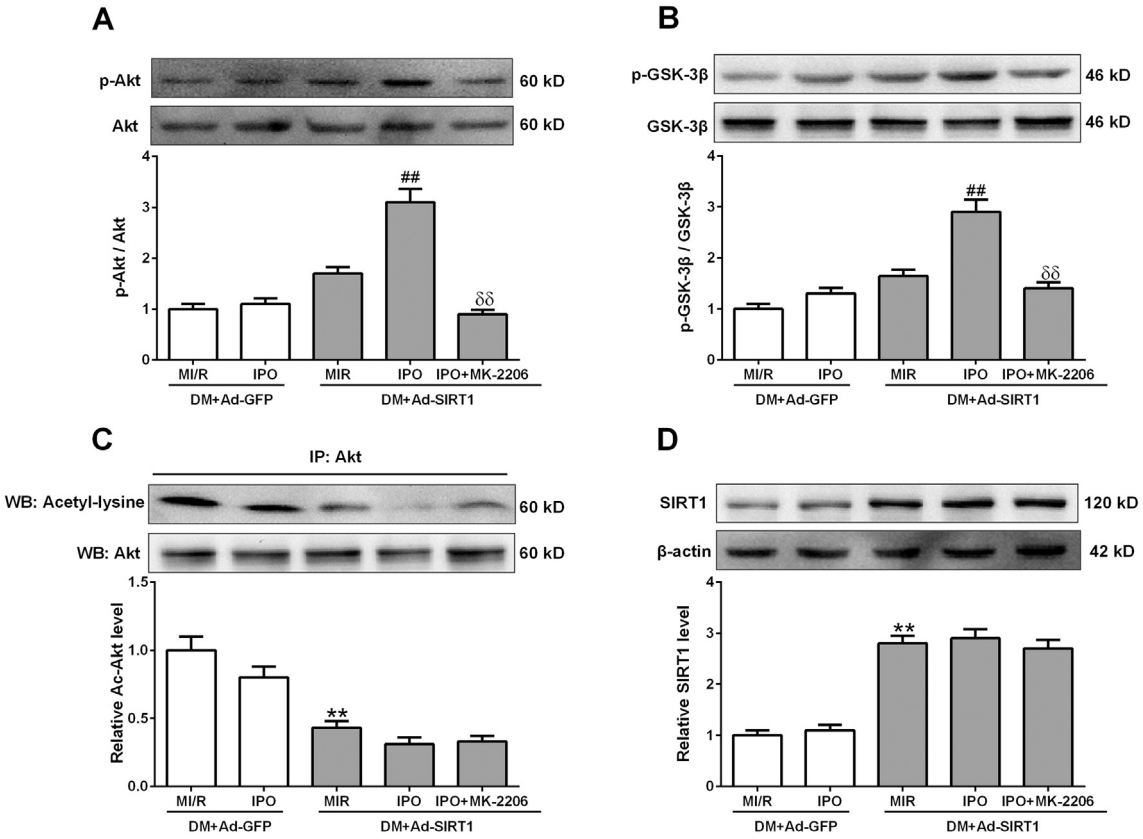

Fig. 3. SIRT1 up-regulation reduced Akt acetylation and restored IPO-mediated activation of the Akt signaling pathway. (A) Representative blot images and the ratio of phosphorylated to total Akt (p-Akt/Akt). (B) Representative blot images and the ratio of phosphorylated to total GSK-3β (p-GSK-3β/GSK-3β). (C) Representative blot images and quantitative analysis of acetylated Akt (Ac-Akt). (D) Representative blot images and quantitative analysis of SIRT1 expression. Values are presented as means ± SEM. DM, diabetes mellitus; Ad, adenoviral vectors; MI/R, myocardial ischemia/reperfusion; IPO, ischemic post-conditioning; MK-2206, Akt inhibitor. n = 6 in each group. $^{**}P < 0.01$ vs. DM + Ad-GFP-MI/R. $^{\#\#}P < 0.01$ vs. DM + Ad-SIRT1-MI/R. $^{\delta\delta}P < 0.01$ vs. DM + Ad-SIRT1-IPO.

specific commercial kits (Nanjing Jiancheng Reagents, China) and was presented as units per liter (U/L).

2.7. Quantification of cardiomyocyte apoptosis

At the end of reperfusion, cardiomyocyte apoptosis was assessed by terminal deoxynucleotidyl nick-end labeling (TUNEL) staining and myocardial caspase-3 activity measurement with the use of in situ cell death detection kits (Roche) and caspase colorimetric assay kits (Chemicon, Temecula, CA) as described in our previous study [23]. The apoptosis index was calculated as a percentage of apoptotic nuclei (TUNEL-positive staining) to total nuclei (4′,6-diamino-2-phenylindole staining).

2.8. Determination of myocardial infarct size

Myocardial infarct size was determined by Evans blue/triphenyl tetrazolium chloride (TTC) double staining and as described previously [24]. The percent of the area-at-risk (AAR) to total left ventricular area (% AAR/LV) was calculated. Myocardial infarct size was presented as a percent of the infarcted area to total area-at-risk (% INF/AAR).

2.9. Cell culture and treatment

Neonatal rat ventricular myocytes were isolated from 1 to 2-day-old Sprague-Dawley rats as previously described [25]. Cardiomyocytes were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and maintained at 37 °C in humidified air (5% CO_2). The cells were transfected with adenoviruses harboring GFP, wild-type SIRT1, catalytically inactive SIRT1 mutant (mSIRT1), Akt, Akt acetylation-defective mutant (mimics deacetylated Akt, $Lys^{20} \rightarrow$ Arg, K20R) and constitutive Akt acetylation mutant ($Lys^{20} \rightarrow$ Gln, K20Q) based on the previous study [20]. The titers of adenoviruses were nearly 1×10^{10} PFU/mL, and the multiplicity of infection (MOI) was 50:1. After 48 h of adenoviral transfection, the cardiomyocytes were treated with either normal glucose medium (5.5 mmol/L, NG) or high-glucose medium (33 mmol/L, HG) for 18 h and then subjected to hypoxia/reoxygenation (H/R) or hypoxic post-conditioning (HPO) [25]. H/R was achieved by hypoxia for 3 h, followed by 3 h of reoxygenation. Hypoxia conditions were obtained using a hypoxia chamber containing 95% N_2 and 5% CO_2 at 37 °C. HPO was performed by three cycles of 5 min of reoxygenation and hypoxia at the onset of reoxygenation [22].

2.10. Cell viability, LDH release and flow cytometry analysis

In in vitro study, cardiomyocyte viability was determined with the colorimetric CCK-8 assay (Beyotime, Nantong, China) immediately after reoxygenation. The absorbance was determined at 450 nm. Cell supernatant was collected after reoxygenation to measure LDH activity as described above. Apoptosis rate was determined by flow cytometry analysis using PE Annexin V Apoptosis Detection Kits (BD Biosciences, 559763) according to the manufacturers' instructions. Cells that are in early apoptosis are PE Annexin V positive and 7-AAD negative (Quadrant B4); and cells that are in late apoptosis or already dead are both PE Annexin V and 7-AAD positive (Quadrant B2). Quadrant B2 and B4 are presented as the percentage of apoptotic cells.

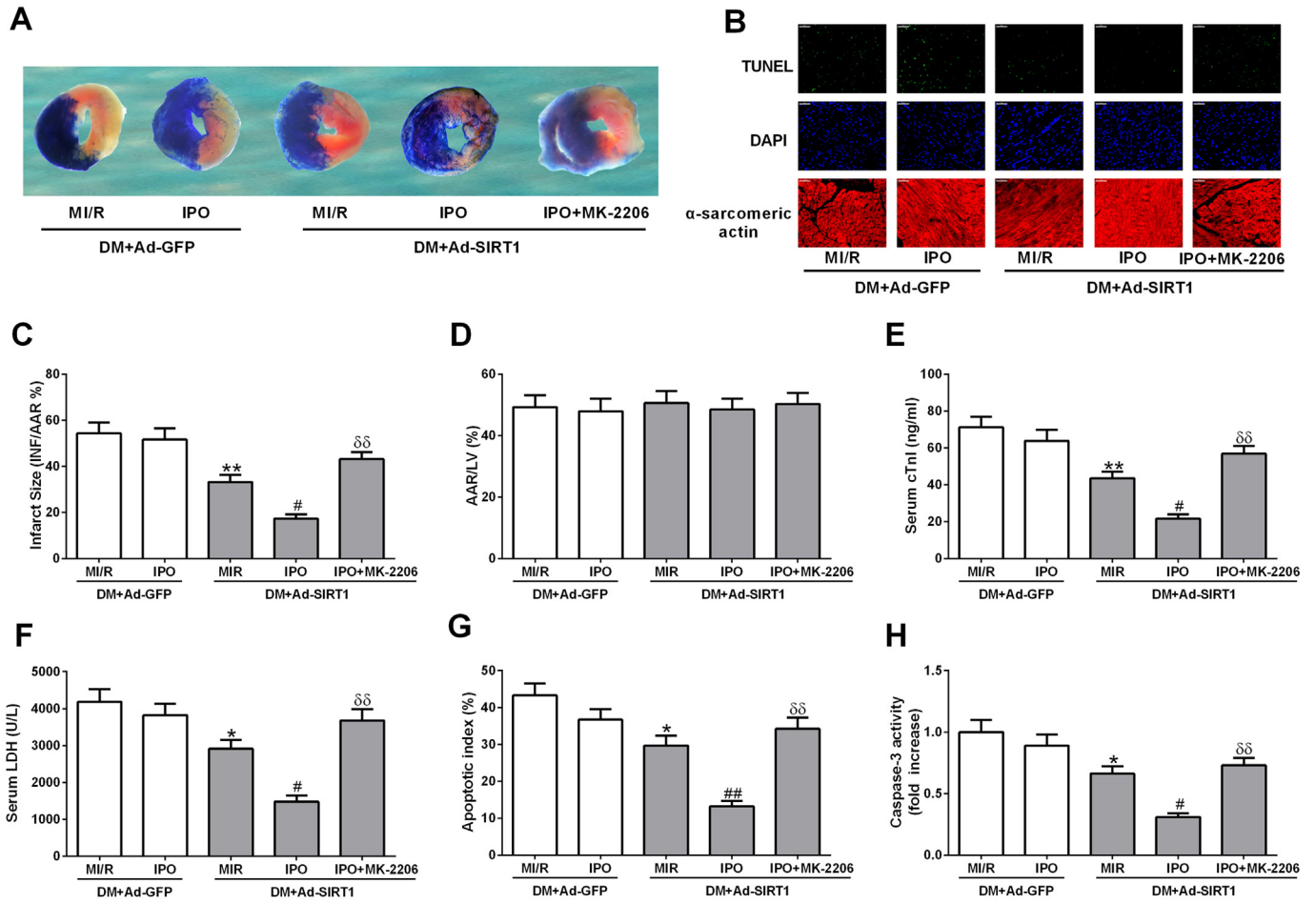

Fig. 4. SIRT1 up-regulation restored IPO-mediated reduction of MI/R injury in diabetic hearts. (A) Representative images of myocardial infarct size stained by Evans blue and TTC. (B) Representative photomicrographs of TUNEL-stained and DAPI-stained heart sections. Original magnification × 400. (C) Myocardial infarct size presented as a percent of infarct area (INF) over total area at risk (AAR). (D) Percentage of area-at-risk (AAR) to left ventricular (LV) area. (E) Serum levels of cardiac troponin I (cTnI). (F) Serum levels of lactate dehydrogenase (LDH). (G) Percentage of apoptotic cells. (H) Myocardial caspase-3 activity (fold over DM + Ad-GFP-MI/R). Values are presented as means ± SEM. DM, diabetes mellitus; Ad, adenoviral vectors; MI/R, myocardial ischemia/reperfusion; IPO, ischemic post-conditioning; MK-2206, Akt inhibitor. n = 8 in each group. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. DM + Ad-GFP-MI/R. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. DM + Ad-SIRT1-MI/R. $^{\delta\delta}P < 0.01$ vs. DM + Ad-SIRT1-IPO.

2.11. Western blotting and immunoprecipitation

Proteins from myocardial tissue (area-at-risk) or cultured cardiomyocytes were analyzed by Western blotting using the standard method as described previously [26]. The primary antibodies used were as follows: SIRT1 (Cell signaling, #8469), p-Akt (Ser473) (Cell signaling, #4060), Akt (Cell signaling, #9272), p-GSK 3β (Ser9) (Cell signaling, #5558), GSK 3β (Cell signaling, #9832), and β-actin (Proteintech, 20536-1-AP). The expression of Akt acetylation was determined by immunoprecipitation assay as previously described [17]. Akt (1:1000 crosslinked to magnetic beads for extraction) was immunoprecipitated from myocardial tissue or cardiomyocyte lysate, and the primary antibody against acetyl-lysine (Cell signaling, #9441) was employed to detect the association of the acetyl-lysine with Akt by using immunoblotting.

2.12. Statistical analysis

Statistical analysis was performed with the utilization of GraphPad Prism software version 5.0. All data were expressed as means ± standard error (SEM). Differences between the two groups were determined by an unpaired Student's *t*-test (Fig. 1 and Supplemental Fig. S4). All other data were compared by using one-way ANOVA followed by Bonferroni's multiple comparisons test for every two groups. *P* values < 0.05 were taken as statistically significant.

3. Results

3.1. Reduced SIRT1 expression and increased Akt acetylation were found in diabetic hearts

Blood glucose and serum lipid including total cholesterol (TC) and triacylglycerol (TG) were significantly increased in high-fat diet (HFD) and streptozotocin (STZ)-induced diabetic mouse (Fig. 1A–C), indicating that diabetic model was successfully created. There were no significant changes in body weight between control mice (25.7 ± 3.2 g) and diabetic mice (28.3 ± 3.9 g). Compared with control hearts, diabetic hearts showed reduced SIRT1 expression and increased Akt acetylation (Fig. 1D and E).

3.2. IPO failed to activate the Akt/GSK-3β signaling pathway to confer cardioprotection in diabetic hearts

As shown in Fig. 2A–E, compared with control mice, diabetic mice exhibited aggravated myocardial injury (serum cardiac troponin I (cTnI) and lactate dehydrogenase (LDH)) and increased cardiomyocyte

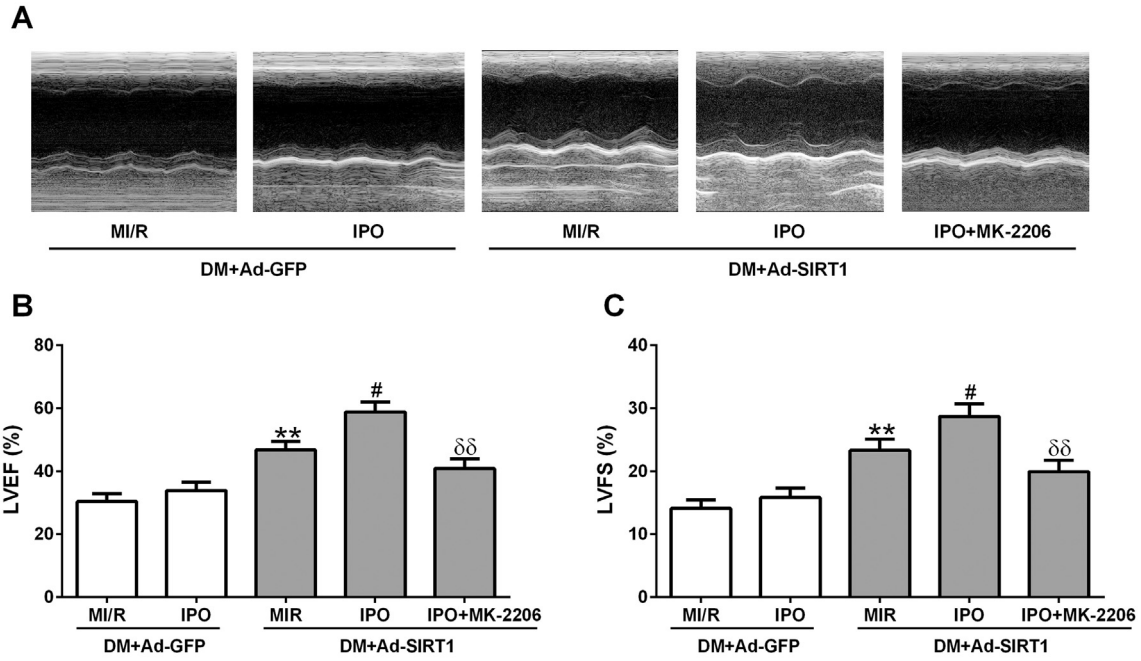

Fig. 5. SIRT1 up-regulation restored IPO-mediated improvement of cardiac function in diabetic hearts. (A) Representative echocardiography images. (B) Left ventricular ejection fraction (LVEF). (C) Left ventricular fractional shortening (LVFS). Values are presented as means ± SEM. DM, diabetes mellitus; Ad, adenoviral vectors; MI/R, myocardial ischemia/reperfusion; IPO, ischemic post-conditioning; MK-2206, Akt inhibitor. n = 8 in each group. $^{**}P < 0.01$ vs. DM + Ad-GFP-MI/R. $^{\#}P < 0.05$ vs. DM + Ad-SIRT1-MI/R. $^{\delta\delta}P < 0.01$ vs. DM + Ad-SIRT1-IPO.

apoptosis (apoptotic index and caspase-3 activity) after MI/R. IPO significantly reduced MI/R-induced myocardial injury and cardiomyocyte apoptosis in control mice but failed to exert similar protective effects in diabetic mice. These results demonstrated that diabetic mice were more vulnerable to MI/R injury, while their responsive to IPO was impaired. Activation of Akt and its downstream target GSK-3β are essential for IPO-mediated cardioprotection. As shown in Fig. 2F–H, MI/R insult increased the phosphorylation of Akt and GSK-3β in control mice, which were further increased by IPO. However, IPO failed to increase the phosphorylation of Akt and GSK-3β in diabetic mice. Taken together, these results indicated that IPO failed to activate the Akt/GSK-3β signaling pathway in diabetic hearts, in which reduced expression of SIRT1 and increased Akt acetylation were observed.

3.3. SIRT1 up-regulation restored IPO-mediated protective effects in diabetic hearts by modulating Akt signaling pathway

We next investigated whether the up-regulation of SIRT1 with adenovirus could restore the cardioprotection by IPO in diabetic mice, and whether these effects could be affected by MK-2206 (300 μg/kg, intravenously), an allosteric inhibitor of all Akt isoforms [27,28]. As shown in Fig. 3A and B, in diabetic mice receiving Ad-GFP, IPO had no significant effects on the phosphorylation of Akt and GSK-3β. Delivery of Ad-SIRT1 into the diabetic hearts significantly increased SIRT1 expression and reduced Akt acetylation (Fig. 3C and D). Moreover, IPO increased the phosphorylation of Akt and GSK-3β in diabetic mice receiving Ad-SIRT1 (Fig. 3A and B).

As shown in Figs. 4 and 5, IPO failed to reduce MI/R injury (infarct size and serum cTnI and LDH) and cardiomyocyte apoptosis (apoptotic index and caspase-3 activity) and improve cardiac function (LVEF and LVFS) in diabetic mice receiving Ad-GFP. In diabetic mice receiving Ad-SIRT1, IPO reduced MI/R-induced myocardial injury and cardiomyocyte apoptosis and improved cardiac function, and these effects were abolished by MK-2206. The percentage of AAR/LV was comparable in all the groups (Fig. 4D). These results indicated that SIRT1 up-regulation restored the protective effects of IPO in diabetic hearts by modulating Akt signaling pathway.

3.4. Cardiac-specific SIRT1 knockout blunted the cardioprotective effects of IPO by blocking the activation of the Akt signaling pathway

Cardiac-specific SIRT1-knockout ($SIRT1^{-/-}$) mice were used to further verify the pivotal role of SIRT1 in IPO-mediated protective effects. There were no significant changes in left ventricular ejection fraction (LVEF) and left ventricular end-systolic volume (LVESV) between wild-type (WT) and Myh6-MerCreMer (Cre) mice with or without tamoxifen administration (Supplementary Fig. S2). Moreover, our previous study has demonstrated that there were no significant changes in LVEF and LVESV between $SIRT^{fl/fl}$ and $SIRT1^{-/-}$ mice at 2 weeks after tamoxifen treatment [19]. These data indicate that a 2-week protocol of oral tamoxifen administration at the dose of 30 mg/kg/d did not result in cardiac dysfunction. Almost no SIRT1 protein expression was observed in the hearts of $SIRT1^{-/-}$ mice, while the protein levels of SIRT1 were not changed in other tissues such as the brain, liver, and muscle (Supplementary Fig. S3 and Fig. 6D). IPO increased the phosphorylation of Akt and GSK-3β in $SIRT^{fl/fl}$ mice (Fig. 6A and B). In $SIRT1^{-/-}$ mice, IPO had no significant effects on the phosphorylation of Akt and GSK-3β (Fig. 6A and B). The expression of Akt acetylation was significantly increased in the hearts of $SIRT1^{-/-}$ mice (Fig. 6C).

As shown in Figs. 7 and 8, IPO reduced myocardial injury (infarct size and serum cTnI and LDH) and cardiomyocyte apoptosis (apoptotic index and caspase-3 activity) and improved cardiac function (LVEF and LVFS) in $SIRT^{fl/fl}$ mice. Nevertheless, IPO failed to reduce MI/R injury and cardiomyocyte apoptosis and improve cardiac function in $SIRT1^{-/-}$ mice. The percentage of AAR/LV was comparable in all the groups (Fig. 7D). Interestingly, delivery of Ad-Akt into the hearts of $SIRT1^{-/-}$ mice significantly increased the expression of total Akt, phosphorylated Akt and acetylated Akt, and reduced MI/R injury and cardiomyocyte apoptosis (Supplementary Fig. S4). These results suggested that cardiac-specific SIRT1 knockout mimics an important molecular characteristic of diabetic hearts and SIRT1 deficiency alone is sufficient to blunt

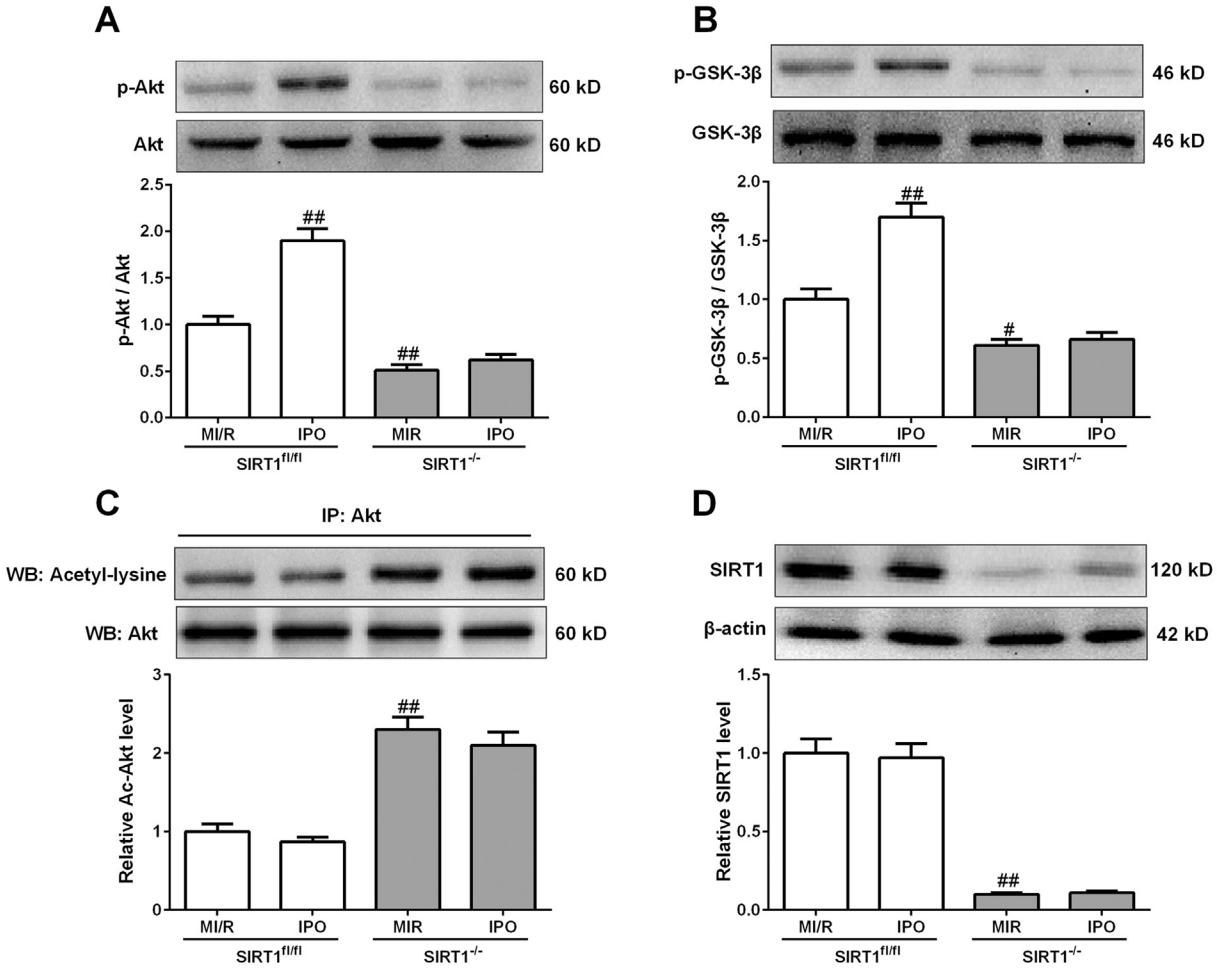

Fig. 6. Cardiac-specific SIRT1 knockout increased Akt acetylation and blunted IPO-mediated activation of Akt signaling pathway. (A) Representative blot images and the ratio of phosphorylated to total Akt (p-Akt/Akt). (B) Representative blot images and the ratio of phosphorylated to total GSK-3β (p-GSK-3β/GSK-3β). (C) Representative blot images and quantitative analysis of acetylated Akt (Ac-Akt). (D) Representative blot images and quantitative analysis of SIRT1 expression. Values are presented as means ± SEM. MI/R, myocardial ischemia/reperfusion; IPO, ischemic post-conditioning. n = 6 in each group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. SIRT1$^{fl/fl}$-MI/R.

protective effects of IPO by suppressing the activation of Akt.

3.5. Up-regulation of wild-type SIRT1 but not catalytically inactive mutant SIRT1 restored the protective effects of HPO

As shown in Supplementary Fig. S5, the expression of SIRT1 was significantly reduced in the cardiomyocytes after exposure to high glucose (HG) for 18 h. There was no further reduction in the expression of SIRT1 after 30 h exposure of HG compared with 18 h exposure of HG. Thus we chose 18 h as the time for HG exposure in the cardiomyocytes. To explore the role of SIRT1-mediated deacetylation in the protective effects of post-conditioning, primary cardiomyocytes were transfected with adenoviruses harboring GFP, wild-type SIRT1 and catalytically inactive SIRT1 mutant (mSIRT1). As shown in Figs. 9A, B and 10, in normal glucose medium (NG)-treated cardiomyocytes transfected with Ad-GFP (NG + Ad-GFP), hypoxic post-conditioning (HPO) group showed enhanced phosphorylation of Akt and GSK-3β, increased cell viability, reduced LDH release and cardiomyocyte apoptosis compared with hypoxia/reoxygenation (H/R) group. In HG-treated cardiomyocytes transfected with Ad-GFP (HG + Ad-GFP, in which the expression of SIRT1 has already been reduced), HPO failed to enhance the phosphorylation of Akt and GSK-3β and protect against H/R-induced cellular injury. The expression of Akt acetylation was increased in HG + Ad-GFP-treated cardiomyocytes compared with NG + Ad-GFP-treated cells in H/R or HPO conditions (Fig. 9C). Both Ad-SIRT1 and Ad-mSIRT1 significantly increased the expression of SIRT1 (HG + Ad-SIRT1 or HG + Ad-mSIRT1, Fig. 9D), while Ad-SIRT1 but not Ad-mSIRT1 efficiently reduced Akt acetylation in HG-treated cardiomyocytes (HG + Ad-SIRT1 vs. HG + Ad-mSIRT1, Fig. 9C). In HG-treated cardiomyocytes transfected with Ad-SIRT1 (HG + Ad-SIRT1), HPO group showed enhanced phosphorylation of Akt and GSK-3β, increased cell viability, reduced LDH release and cardiomyocyte apoptosis compared with H/R group (Figs. 9A, B and 10). However, in HG-treated cardiomyocytes transfected with Ad-mSIRT1 (HG + Ad-mSIRT1), HPO has no significant effects on the phosphorylation of Akt and GSK-3β and failed to protect against H/R-induced cellular injury. These results suggested that the deacetylation but not the expression of SIRT1 is necessary for HPO to confer protective effects.

3.6. Acetylation modification inhibited the phosphorylation of Akt and blunted its protective effects against H/R injury

To further explore the effect of Akt acetylation modification on its phosphorylation and protective effects, primary cardiomyocytes were transfected with adenoviruses harboring Akt, Akt acetylation-defective mutant (Lys^{20} → Arg, K20R, mimics deacetylated Akt) and constitutive Akt acetylation mutant (Lys^{20} → Gln, K20Q). As shown in Fig. 11A and D, transfection with Akt, Akt acetylation-defective mutant (K20R) and

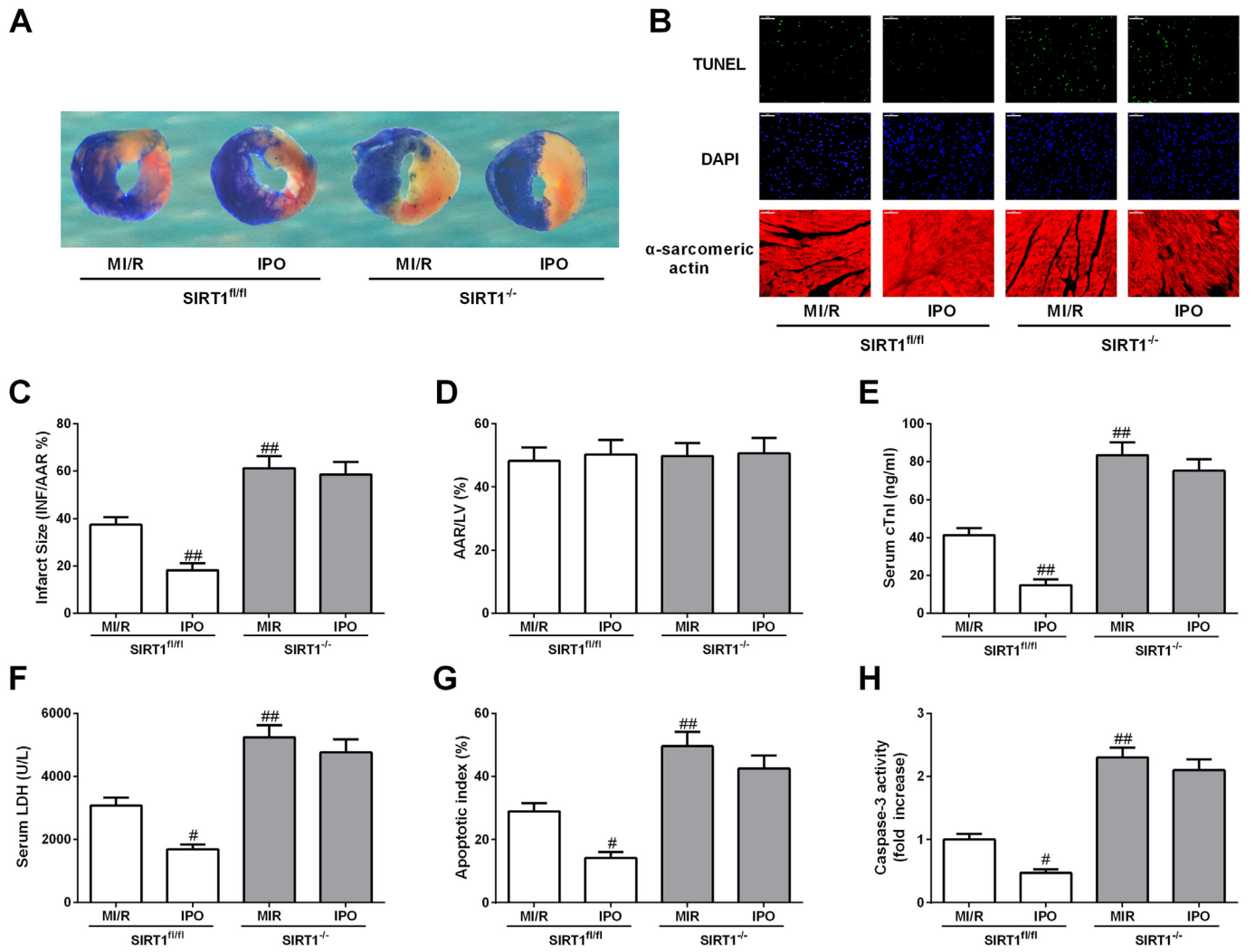

Fig. 7. Cardiac-specific SIRT1 knockout blunted IPO-mediated reduction of MI/R injury in diabetic hearts. (A) Representative images of myocardial infarct size stained by Evans blue and TTC. (B) Representative photomicrographs of TUNEL-stained and DAPI-stained heart sections. Original magnification × 400. (C) Myocardial infarct size presented as a percent of infarct area (INF) over total area at risk (AAR). (D) Percentage of area-at-risk (AAR) to left ventricular (LV) area. (E) Serum levels of cardiac troponin I (cTnI). (F) Serum levels of lactate dehydrogenase (LDH). (G) Percentage of apoptotic cells. (H) Myocardial caspase-3 activity (fold over SIRT1$^{fl/fl}$-MI/R). Values are presented as means ± SEM. MI/R, myocardial ischemia/reperfusion; IPO, ischemic post-conditioning. n = 8 in each group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. SIRT1$^{fl/fl}$-MI/R.

constitutive Akt acetylation mutant (K20Q) all increased the expression of Akt. Compared with Ad-Akt transfection, Ad-Akt (K20R) transfection showed increased phosphorylation of Akt and GSK-3β (Fig. 11B–D) and enhanced protective effects against H/R injury in HG-cultured cardiomyocytes as evidenced by increased cell viability and reduced LDH release and decreased cardiomyocyte apoptosis (Fig. 11E–H). In contrast, Ad-Akt (K20Q) transfection showed repressed Akt phosphorylation (Fig. 11B–D) and blunted protective effects against H/R injury in HG-cultured cardiomyocytes compared with Ad-Akt transfection (Fig. 11E–H). These results showed that acetylation modification repressed the phosphorylation of Akt and blunted its protective effects against H/R injury.

4. Discussion

In this study, we demonstrate that SIRT1-mediated deacetylation is required for IPO to activate the Akt signaling pathway to confer cardioprotection. Reduced SIRT1 and increased Akt acetylation are responsible for the loss of IPO-mediated cardioprotection in diabetes. Up-regulation of SIRT1 restores IPO-mediated protective effects in diabetic hearts via deacetylation-dependent activation of Akt. To be the best of our knowledge, this is the first study demonstrating that the reduction of SIRT1 blunts the cardioprotective effects of IPO in diabetes by impairing the Akt signaling pathway (Fig. 12).

SIRT1 is a nicotinamide adenine dinucleotide (NAD+)-dependent protein deacetylase regulating many important physiologic processes including cell survival, metabolism and energy balance [29]. Previous studies from our lab and others have demonstrated that up-regulation of SIRT1 protects the heart against I/R injury in non-diabetic and diabetic animals, while cardiac specific SIRT1 knockout exacerbates myocardial injury caused by I/R [17,30]. Nadtochiy et al. have found that ischemic preconditioning (IPC) enhances SIRT1-mediated lysine deacetylation and the protective effects of IPC is blunted by SIRT1 knockdown (SIRT1$^{+/-}$) or specific SIRT1 inhibitor splitomicin [31,32]. Nevertheless, Adam et al. have reported that IPC-mediated protection against MI/R is not prevented by SIRT1 inhibitor SIII [33]. The inconsistencies between these studies may be due to variations in the animal models and/or inhibitor specific. In addition, Potenza et al. have demonstrated that inhibition of SIRT1 abrogates the protective effects of adiponectin preconditioning against MI/R injury [34], suggesting that SIRT1 plays an important role in the cardioprotective effects of pharmacological preconditioning with adiponectin.

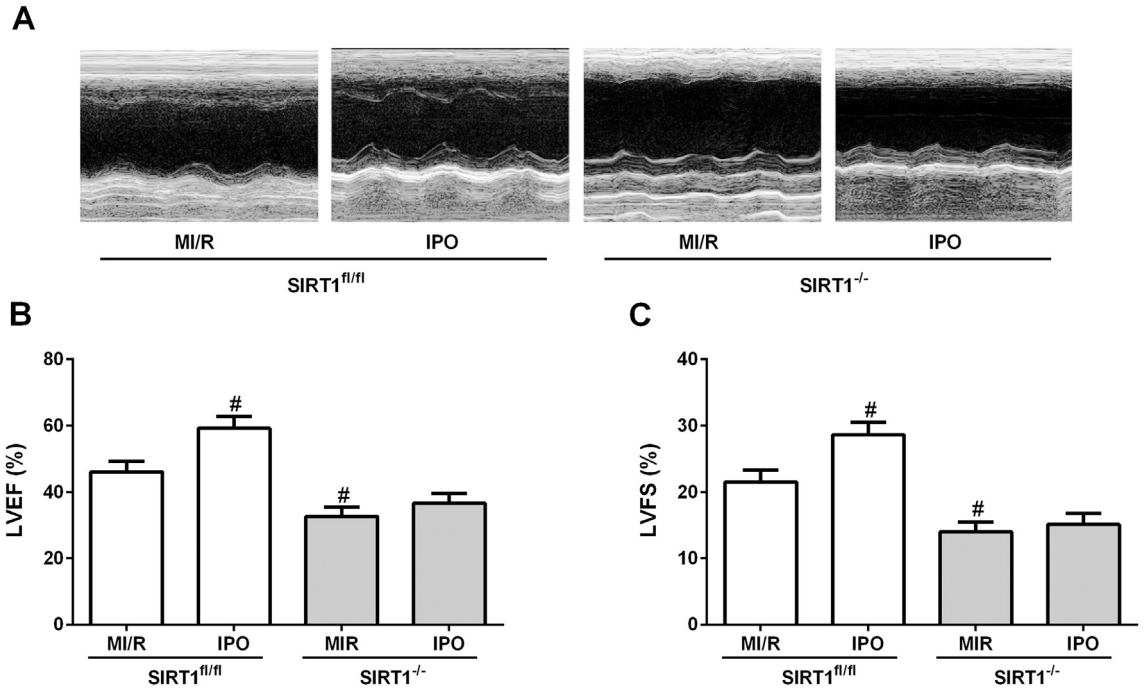

Fig. 8. Cardiac-specific SIRT1 knockout blunted IPO-mediated improvement of cardiac function in diabetic hearts. (A) Representative echocardiography images. (B) Left ventricular ejection fraction (LVEF). (C) Left ventricular fractional shortening (LVFS). Values are presented as means ± SEM. MI/R, myocardial ischemia/reperfusion; IPO, ischemic post-conditioning. n = 8 in each group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. $SIRT1^{fl/fl}$-MI/R.

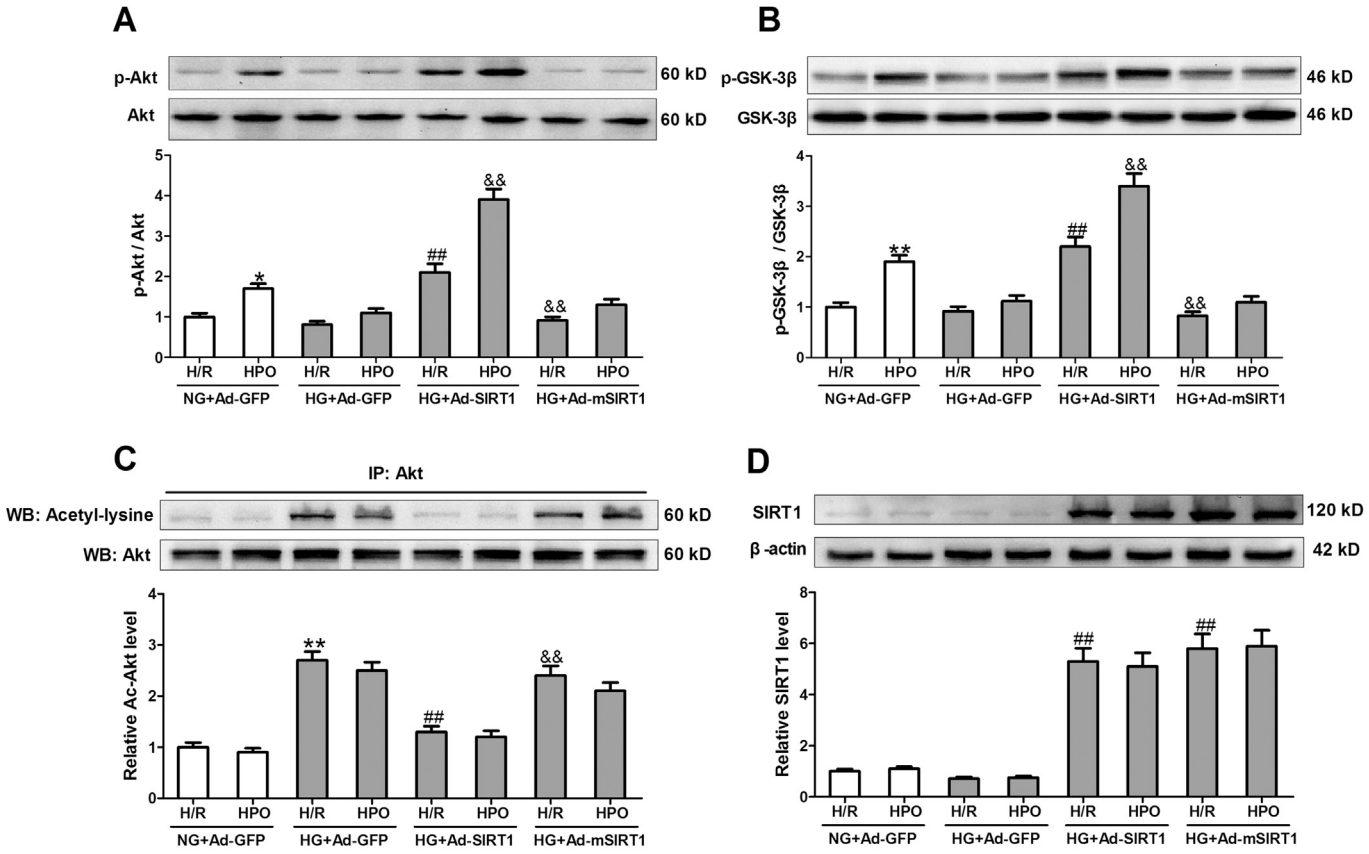

Fig. 9. Up-regulation of wild-type SIRT1 but not catalytically inactive mutant SIRT1 reduced Akt acetylation and restored HPO-mediated activation of Akt signaling pathway. (A) Representative blot images and the ratio of phosphorylated to total Akt (p-Akt/Akt). (B) Representative blot images and the ratio of phosphorylated to total GSK-3β (p-GSK-3β/GSK-3β). (C) Representative blot images and quantitative analysis of acetylated Akt (Ac-Akt). (D) Representative blot images and quantitative analysis of SIRT1 expression. Values are presented as means ± SEM. NG, normal glucose (5.5 mM); HG, high glucose (33 mM glucose); Ad, adenoviral vectors; H/R, hypoxia/reoxygenation; HPO, hypoxic post-conditioning; mSIRT1, catalytically inactive mutant SIRT1. n = 6 in each group. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. NG + Ad-GFP-H/R. $^{\#\#}P < 0.01$ vs. HG + Ad-GFP-H/R. $^{\&\&}P < 0.01$ vs. HG + Ad-SIRT1-H/R.

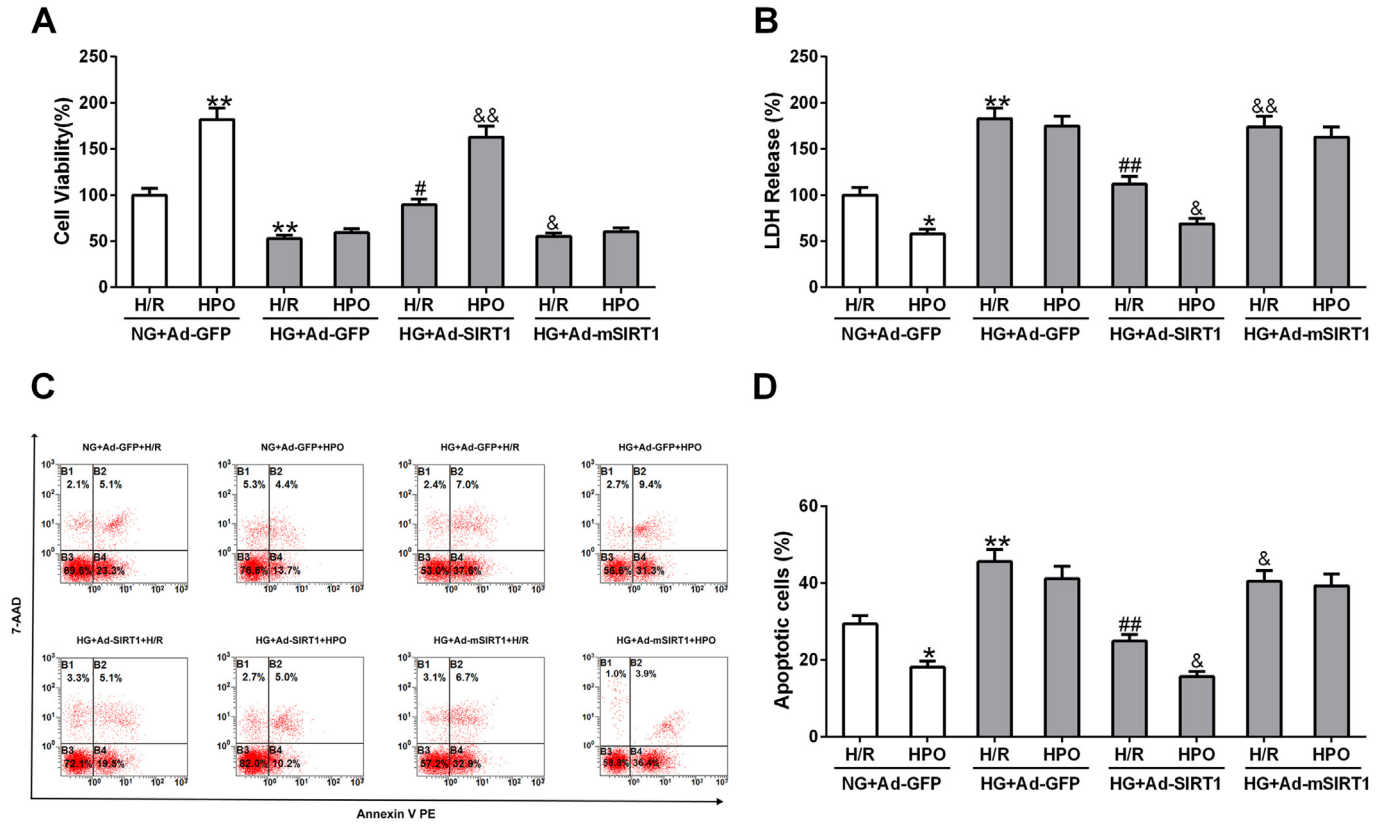

Fig. 10. Up-regulation of wild-type SIRT1 but not catalytically inactive mutant SIRT1 restored the protective effects of HPO. (A) Cell viability. (B) LDH release. (C) Representative images of flow cytometry analysis (major finding is D). (D) Percentage of apoptotic cells analyzed by flow cytometry. Quadrant B2 and B4 are presented as the percentage of apoptotic cells. Values are presented as means ± SEM. NG, normal glucose (5.5 mM); HG, high glucose (33 mM glucose); Ad, adenoviral vectors; H/R, hypoxia/reoxygenation; HPO, hypoxic post-conditioning; mSIRT1, catalytically inactive mutant SIRT1. n = 6 in each group. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. NG + Ad-GFP-H/R. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. HG + Ad-GFP-H/R. $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ vs. HG + Ad-SIRT1-H/R.

Since the onset of ischemia is an acute and unpredictable event, the clinical use of ischemic pre-conditioning is limited and current attention is focused largely on IPO to alleviate MI/R injury [35]. However, the role of SIRT1 in IPO-mediated cardioprotection, especially in blunted cardioprotection of IPO in diabetes is still largely unknown. Our study demonstrated for the first time that up-regulation of SIRT1 in diabetic hearts restored IPO-mediated protective effects. Interestingly, even without diabetes, SIRT1 deficiency alone is sufficient to blunt the protective effects of IPO. Both gain- and loss-of-function experiments indicate that the reduction of SIRT1 is responsible for the blunted protective effects of IPO in diabetic hearts.

Numerous studies have identified a number of signaling pathways are involved in the myocardial protection of IPO [36–38]. Among these pathways, the activation of Akt by phosphorylation is considered as an initial step that induces phosphorylation of downstream kinases such as GSK-3β to inhibit the opening of mitochondrial permeability transition pore and reduce the necrotic effects of reperfusion [6,39]. Loss of IPO-mediated cardioprotection has been previously described in both type 1 and type 2 diabetic animal models [10,11,40]. The complete or partial failure of IPO to reduce MI/R injury in diabetes is attributed to disrupted signaling pathways. Xue et al. showed that phosphorylated Akt was decreased in STZ-induced type 1 diabetic rats compared with that in control rats at baseline [41]. Bouhidel et al. reported that phosphorylated Akt was increased in type 2 diabetic ob/ob mice at baseline [10]. In our study, the expression of basal phosphorylated Akt was not significantly changed in HFD-STZ-induced diabetic sham hearts compared with non-diabetic sham hearts. These inconsistent findings may be due to different diabetic animal models, which may represent different situations in diabetic subjects. Our study observed that there was also no significant change in the expression of total Akt between diabetic and non-diabetic sham hearts, it is then speculated that post-translational modifications may hinder IPO-induced Akt phosphorylation in diabetes.

Acetylation is an important protein modification that negatively regulates the activity of Akt [16]. It has been showed that the level of Akt acetylation inversely correlates with Akt phosphorylation and signaling induced by IGF-1 or insulin. SIRT1 deacetylates Akt to promote its binding to PIP3 and consequent Akt activation [20]. Here we found that cardiac-specific SIRT1 knockout increased the expression of Akt acetylation and blunted IPO-induced activation of Akt. Ad-SIRT1 but not Ad-mSIRT1 reduced Akt acetylation and restored HPO-induced activation of Akt. Moreover, Akt acetylation-defective mutant (K20R) exhibited increased phosphorylation of Akt and enhanced protective effects against H/R injury, while constitutive Akt acetylation mutant (K20Q) exhibited repressed phosphorylation of Akt and blunted protective effects against H/R injury. These results demonstrate that the reduction of SIRT1 in diabetic hearts causes the increase of Akt acetylation, which represses the activation of Akt signaling and blunts the protective effects of IPO. It should be noted that complex mechanisms are involved in the impaired Akt signaling in diabetic hearts. Increased expression of PTEN is a negative regulator of Akt signaling in diabetes. Inhibition of PTEN has been shown to restore IPO-mediated cardioprotection in diabetic hearts by improving the PI3K/Akt signaling pathway [41]. Interestingly, SIRT1 has been reported to decrease PTEN acetylation and inhibit the interaction between PTEN and its receptors in COS7 cells [42]. It is speculated that the inhibitory effects of SIRT1 on PTEN may be involved in the restoration of IPO-mediated cardioprotection in diabetic hearts. Further study may be needed to clarify the interrelationship between SIRT1 and PTEN in this process.

There are still some limitations in the present study. First, our

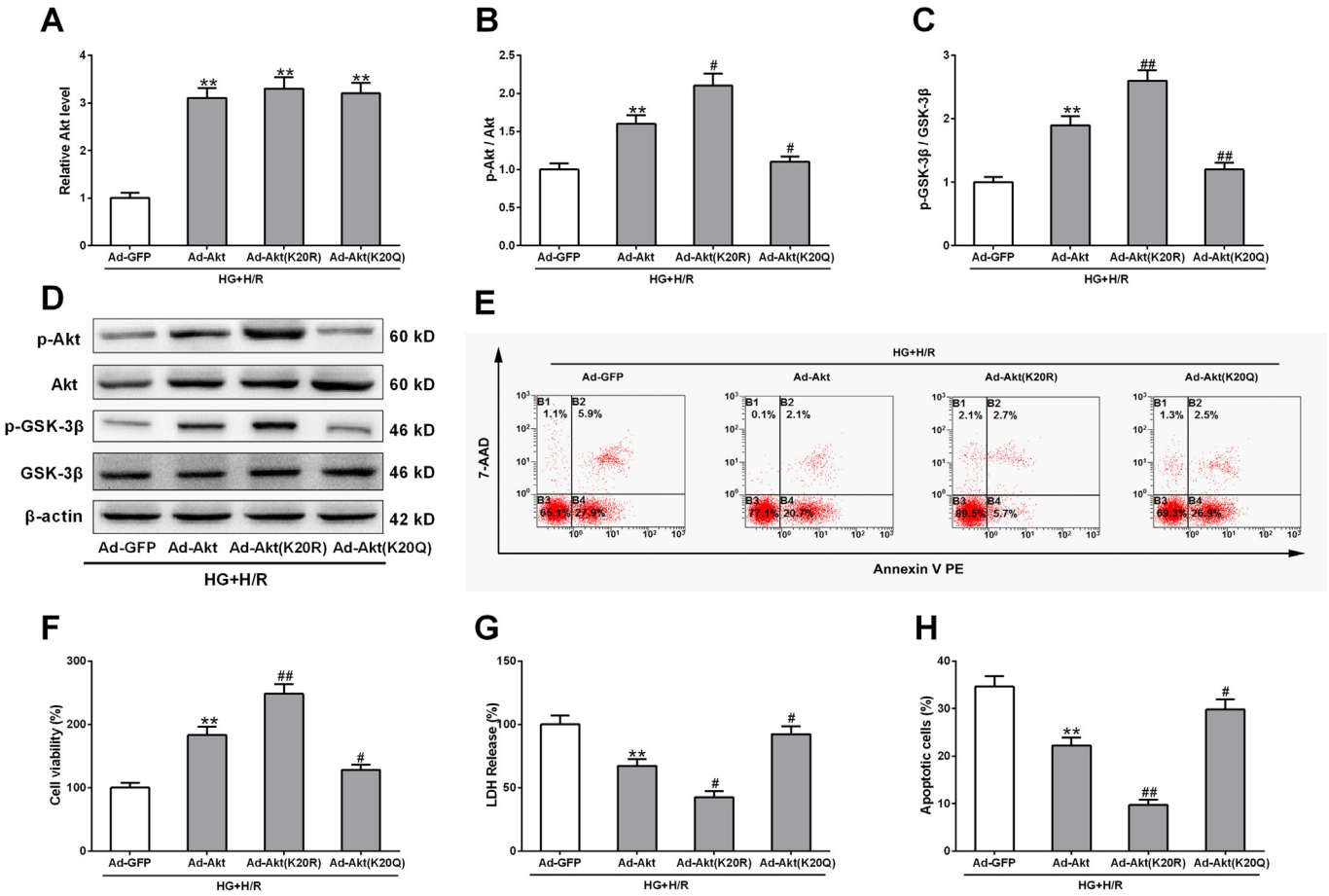

Fig. 11. Acetylation modification inhibited the phosphorylation of Akt and blunted its protective effects against H/R injury. (A) Quantitative analysis of Akt expression. (B) The ratio of phosphorylated to total Akt (p-Akt/Akt). (C) The ratio of phosphorylated to total GSK-3β (p-GSK-3β/GSK-3β). (D) Representative blot images (major findings are A, B and C). (E) Representative images of flow cytometry analysis (major finding is H). (F) Cell viability. (G) LDH release. (H) Percentage of apoptotic cells analyzed by flow cytometry. Values are presented as means ± SEM. Ad, adenoviral vectors; Akt K20R, Akt $Lys^{20} \rightarrow$ Arg (acetylation-defective mutant Akt); Akt K20Q, Akt $Lys^{20} \rightarrow$ Gln (constitutive Akt acetylation mutant); HG, high glucose (33 mM glucose); H/R, hypoxia/reoxygenation. n = 6 in each group. $^{**}P < 0.01$ vs. Ad-GFP. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. Ad-Akt.

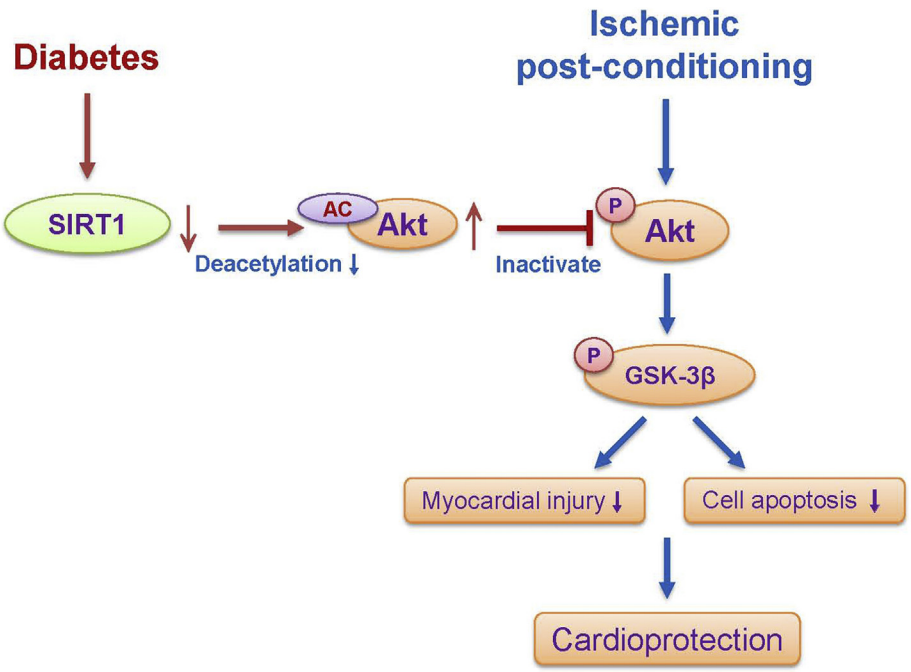

Fig. 12. Schematic figure illustrating that reduction of SIRT1 in diabetic hearts blunts the protective effects of IPO by impairing the Akt signaling pathway. IPO increases the phosphorylation of Akt and GSK-3β and then reduces MI/R-induced myocardial injury and cell apoptosis in the hearts. The expression of SIRT1 is reduced in diabetic hearts, which increases Akt acetylation and blunts the cardioprotective effects of IPO by blocking the activation of the Akt signaling pathway.

animal experiments were exclusively performed in HFD-STZ-induced diabetic mice. Whether the research findings can be applied to other diabetic models such as db/db mice needs further investigation. Second, the conclusion that SIRT1 up-regulation restores IPO-mediated protective effects in diabetic hearts by modulating Akt signaling pathway was verified by using Akt inhibitor MK-2206 rather than genetic manipulation. Third, there are huge species specific differences in the cardioprotective signaling pathways afforded by IPO [35]. The Akt signaling plays an important role in IPO-mediated cardioprotection in rodents and humans [43,44], but has no role in pigs [45]. Thus, whether our research findings can be applied to humans and other species needs further investigation. Despite these limitations, we believe that this study has established a direct link between SIRT1 and impaired Akt signaling in diabetic hearts, which may provide some insight into the problem of blunted cardioprotective effects by IPO in diabetes.

5. Conclusions

In summary, our study demonstrates that the reduction of SIRT1 in diabetic hearts blunts the cardioprotective effects of IPO in HFD-STZ-induced diabetic mice by impairing the Akt signaling pathway. Up-regulation of SIRT1 restores cardioprotection by IPO in diabetes via deacetylation-dependent activation of Akt signaling pathway. These findings identify SIRT1 as an effective therapy to restore myocardial responsiveness to IPO in diabetes.

List of abbreviations

Ad	adenoviral vectors
cTnI	cardiac troponin I
DAPI	4′,6-diamidino-2-phenylindole
DM	diabetes mellitus
GFP	green fluorescent protein
GSK-3β	glycogen synthase kinase-3β
H/R	hypoxia/reoxygenation
HFD	high-fat diet
HG	high glucose
HPO	hypoxic post-conditioning
IPO	ischemic post-conditioning
LVEF	left ventricular ejection fraction
LVFS	left ventricular fractional shortening
LDH	lactate dehydrogenase
MI/R	myocardial ischemia/reperfusion
mPTP	mitochondrial permeability transition pore
PI3K	phosphoinositide 3-kinase
PTEN	phosphatase and tensin homologue deleted on chromosome 10
STZ	streptozotocin
TUNEL	terminal deoxynucleotidyl nick-end labeling

Conflict of interest

The authors declare that they have no potential conflicts of interest.

Transparency document

The Transparency document associated with article can be found, in online version.

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Authors' contributions

F.F. and Y.L. conceived and designed the study. M.D., C.G. and J.Q. performed the animal experiments. L.H., C.G., K.Z. and M.Y. carried out the cell experiments. M.D., H.Y., C.G. and J.F. performed the molecular biology experiments. M.D., L.H., H.Y., C.G. and F.F. analyzed the data. M.D. drafted the manuscript. F.F., C.G. and Y.L. revised and edited the manuscript. All authors have read and approved the final version of this manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dummy.2019.01.002.

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