SENPR1 protects against myocardial ischaemia/reperfusion injury via a HIF1α-dependent pathway

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Aims SUMO-specific protease 1 (SENP1) removes SUMO from proteins and plays important roles in the regulation of multiple cellular signalling pathways. However, little is known about the role of SENP1 in coronary heart disease. In this study, we tested the hypothesis that SENP1 protects against myocardial ischaemia/reperfusion (I/R) injury and investigated the underlying molecular mechanisms involved.

Methods and results First, we found that SENP1 levels increased after I/R in human and mouse myocardium in vivo and in rat cardiomyocytes in vitro. We then performed coronary artery ligation to induce I/R injury in wild-type (WT) and heterozygous SENP1-knockdown (SENP1+/−) mice. Compared with WT mice, SENP1+/− mice had normal cardiac function at baseline but lower systolic function after I/R. Post-I/R myocardial infarction sizes were larger in SENP1+/− mice. Furthermore, we demonstrated that SENP1 regulates the expression of hypoxia-inducible factor 1α (HIF1α), a critical protective factor during I/R, in vivo and in vitro. Overexpression of HIF1α reversed the deteriorating effect of SENP1 knockdown on cellular death.

Conclusion Our results suggest that SENP1 deficiency exacerbates I/R injury in cardiomyocytes via a HIF1α-dependent pathway.

Keywords SENP1 • Cardiac function • Ischaemia/reperfusion • HIF1α • Apoptosis

1. Introduction

Acute myocardial infarction (AMI) is a major cause of death in modern society. Reperfusion, including percutaneous coronary intervention and coronary artery bypass graft surgery, is an essential treatment for salvaging ischaemic myocardium from necrosis. However, reperfusion itself can lead to necrosis and apoptosis.1–4 Studies in animal models of AMI suggest that lethal reperfusion injury accounts for up to 50% of the final size of a myocardial infarct. 5 Therefore, factors that mediate cardiomyocyte survival during myocardial infarction and ischaemia/reperfusion (I/R) injury are considered promising therapeutic targets. Many factors, such as Sirt1, microRNA-320, and cyclosporine, have been shown to contribute to myocardial survival in I/R, but the clinical applicability of these findings is either uncertain or far from being practical. Thus, the mechanisms involved in I/R need to be further elucidated before new, clinically significant therapeutic targets can be discovered.

SUMO, a ubiquitin-like protein, has been shown to play an important role in regulating the function and localization of many proteins.9–16 SUMOylation is the process by which SUMO modifies its downstream proteins by forming isopeptide bonds with specific lysine residues on the target proteins.14,15 This modification is a dynamic process and is catalysed by the activating (E1), conjugating (E2), and ligating (E3) enzyme functions of SUMO, and it is reversed by a family of Sentrin/SUMO-specific proteases (SEPNPs).9–13,17 SUMO-specific protease 1 (SENP1) is a member of the SENP family and functions under hypoxic conditions. It modulates the biological characteristics of cancers via deSUMOylation.18–20 and has been implicated in the development of hypoxic pulmonary hypertension.21 However, little is known about the effect of SENP1 on myocardial I/R injury.

AMI is an ischaemic heart disease characterized by acute hypoxia in the myocardium. Given the regulatory effects of SENP1 in hypoxia, we hypothesized that SENP1 may play a vital role in myocardial infarction. In this study, we investigated whether SENP1 is beneficial or maladaptive...
in acute cardiac I/R injury. We tested this hypothesis by evaluating the expression of SENP1 in human and mouse myocardium as well as in rat cardiomyocytes. We subjected heterozygous SENP1 knockout (SENP1<sup>+/−</sup>) mice to I/R injury and explored the specific mechanisms involved. Our results indicate that a reduction in SENP1 levels exacerbates I/R injury in cardiomyocytes via a hypoxia-inducible factor 1 α (HIF1α)-dependent pathway.

2. Methods

2.1 Experimental animals
Heterozygous SENP1 knockout (SENP1<sup>+/−</sup>) mice were produced as previously reported. In brief; the SENP1<sup>−/−</sup> embryonic stem (ES) cell line XG001 was obtained from BayGenomics. XG001 cells were generated by using a gene trap protocol with the trapping construct pGT1Lxf containing the intron from the engrafted-2 gene upstream of the gene encoding the β-galactosidase/neomycin-resistance fusion protein (see http://baygenomics.ucsf.edu). The vector was inserted into intron 8 of the SENP1 locus. A male chimeric mouse was generated from the ES cell line. Littermates (SENP1<sup>+/−</sup>) were used as a control. C57BL6 mice were obtained from The Jackson Laboratory. All the animal procedures were performed to conform the NIH guidelines (Guide for the care and use of laboratory animals) and Use Committee of Shanghai Jiaotong University.

2.2 In vivo I/R injury in human myocardium
I/R injury was induced by aortic cross-clamping for 30 min followed by unclamping for 15 min. Cardiac tissue specimens were obtained with informed consent from patients undergoing cardiac surgery. This study conformed to the principles of the Declaration of Helsinki and was approved by the Jiao Tong University School of Medicine Ethics Committee. All patients were scheduled for mitral valve replacement on cardiopulmonary bypass. Tissue samples from 16 patients, 8 male and 8 female, were used. The mean age of the patients was 47.81 ± 18.68 years. Atrial cells were obtained from the atrial appendage at three time points. The first time is before aortic cross-clamp, this time the cardiac tissue is normal. The second time is 30 min after aortic cross-clamp when the cardiac tissue is under ischaemia. The last time is unclamping for 15 min when the cardiac tissue is in recovering the blood supply. Individual specimens were immediately transferred to ice-cold Ca<sup>2+</sup>-free Krebs-Ringer saline solution (consisting of 10 mmol/l HEPES, 129 mmol/l NaCl, 4.7 mmol/l KCl, 1.2 mmol/l KH<sub>2</sub>PO<sub>4</sub>, 1.2 mmol/l MgSO<sub>4</sub>·7H<sub>2</sub>O, 5.0 mmol/l NaHCO<sub>3</sub>, 5.5 mmol/l glucose, and 2.0 mg/mL bovine serum albumin). The solution was further supplemented with 20 mmol/l of taurine, 2.0 mmol/l of L-carnitine, 5.0 mmol/l of creatine, and 30.0 mmol/l of 2,3-butanedione monoxime and buffered at pH 7.4. Specimens were transported to the molecular biology laboratory within 15 min of acquisition. Connective and adipose tissues were trimmed.

2.3 In vivo I/R injury in mouse myocardium
Mice were housed in a temperature-controlled environment with 12 h light/dark cycles and received food and water ad libitum. Three-month-old mice were anesthetized by isoflurane inhalation. A rodent ventilator (model 683, Harvard Apparatus, Inc.) was used with 65% oxygen during the surgical procedure. The animals were kept warm using heat lamps and heating pads. The animals were treated with epoxomicin as previously described.

2.4 Isolation and culture of neonatal mouse cardiomyocytes
Hearts were harvested from 1- to 3-day-old mice, promptly after euthanasia by decapitation, and primary cultures of neonatal mouse cardiomyocytes were performed as described previously. Briefly, the isolated hearts were cleared of connective tissue and atria, and cut into ~1 mm blocks. Then, the tissues were digested by trypsin at 37°C for 1 min. Cell suspensions were shifted out and neutralized with cell culture medium. Cardiac tissues were trypsinized until the tissues disappeared and cell suspensions were collected. Single cells were obtained by filtering the cells through a 70 mm filter. Then, all suspensions were pelleted by centrifugation at 200 g for 3 min. The isolated cells were then resuspended in DMEM (Hyclone Laboratories) supplemented with 10% foetal bovine serum (Gibco) and 1% penicillin/streptomycin. Isolated cardiomyocytes were seeded onto cell culture flask and cultivated at 37°C in humid air with 5% CO<sub>2</sub>. The culture medium was replaced with fresh DMEM medium after 48 h and cells were further cultured for 24 h.

2.5 Hypoxia/reoxygenation in cardiomyocytes
Rat cardiomyocytes from the H9C2 cell line (from ATCC, USA) or mouse neonatal cardiomyocytes were cultured in an airtight incubation tank for 2 h with a <1% oxygen concentration followed by 0.5 h of reoxygenation.

2.6 Echocardiography
Echocardiography was performed using a Vevo 770 high-resolution imaging system at 24 h after I/R as previously described. Twelve animals were anesthetized with isoflurane inhalation and placed in supine position. The chest was shaved, and the parasternal short- and long-axis views were used to obtain two-dimensional and M-mode images by an echocardiogram. At least 10 independent cardiac cycles per each experiment were obtained. Cardiac output was normalized with the animals’ weights to obtain cardiac indices.

2.7 Assessment of area at risk and infarct size
After I/R, the animals were re-anesthetized and intubated, and the chest was opened. After the heart at the diastolic phase was arrested by KCl injection, the ascending aorta was cannulated and perfused with saline to wash out the blood. The LAD was occluded with the same suture, which had been left at the site of the ligature. To demarcate the ischaemic area at risk (AAR), Alcian blue dye (1%) was perfused into the aorta and coronary arteries. Hearts were excised, and LVs were sliced into 1-mm-thick cross-sections. The heart sections were then incubated with a 1% triphenyltetrazolium chloride (TTC) solution at 37°C for 10 min. The infarct area (pale), the AAR (not blue), and the total LV area from both sides of each section were measured by two blinded observers using Adobe Photoshop (Adobe Systems, Inc.), and the values obtained were averaged. The percentage of infarcted area and AAR of each section were multiplied by the weight of the section and then totalled. AAR/LV and infarct area/AAR were expressed as a percentage.

2.8 Histopathological and immunohistochemical analyses
Human and mouse heart specimen were fixed in 10% formalin (PBS buffered), dehydrated, and embedded in paraffin. Heart architecture was determined from transverse 5-μm deparaffinized sections stained with H&E. Immunohistochemistry for SENP1 and HIF1α was performed as described previously.

2.9 RNAi
For the SENP1-siRNA experiment, SENP1-specific siRNA oligo (GGCTC AATGACGAGATCATTTT) and no-specific oligo (negative control) were
designed and synthesized by GenePharma (Shanghai). The inhibitory efficiency on SENP1 expression is about 70% (see Results 3.2 for details). H9C2 cells in six-well plates were transfected with the non-specific (40 pmol/well) or SENP1-siRNA oligonucleotide with Lipofectamine 2000 (Invitrogen).

2.10 Cellular apoptosis analysis

In situ cardiomyocyte apoptosis was examined using the TdT-mediated dUTP nick end-labelling (TUNEL) assay (Boehringer Mannheim, Indianapolis, IN, USA). Five slides from each block were evaluated for the percentage of apoptotic cells by using the TUNEL assay. Five watch fields were chosen randomly under microscopy on each section. Positive brown cells and total cells were counted by MIA 4.0 (Medical Image Analysis System, Beijing Bingyang Keji Corp., China). The five fields were averaged for each block, and the blocks were averaged for each heart to obtain one representative value for each heart. The hearts were then statistically assessed. Apoptosis index (positive cells/total cells × 100%) was used as the indicator of apoptosis. Cardiomyocyte apoptosis in vitro was evaluated by flow cytometry. Briefly, cardiomyocytes (1 × 10^6) were washed twice with cold PBS and labelled with titrated FITC-conjugated annexin-V in the dark for 10 min at room temperature. Five minutes after propidium iodide (PI) solution was added, the percentages of dead cells and cells undergoing apoptosis were evaluated by flow cytometry (Becton–Dickinson, San Jose, CA, USA).

2.11 Cellular death analysis

Cells were exposed to hypoxia for 12 h and reoxygenation for 4 h. Cell necrosis was analysed by an LDH assay and Trypan blue staining. Lactate dehydrogenase (LDH) levels were assessed with LDH assay kits (Cytotox 96 G1780 Promega). Trypan blue staining was assessed with the Trypan blue staining cell viability assay kit (Prolab AR1175). Experiments were performed according to the manufacturer’s instructions.
2.12 RNA isolation and quantitative real-time PCR

Total RNA samples were isolated using RNA sta-60 reagent (Tel-Test, Inc.), according to the manufacturer’s instructions. Samples of total RNA (500 ng) were reverse-transcribed using a Taqman reverse transcription PCR Kit (Applied Biosystems), and the resulting cDNA was used as a PCR template. The mRNA levels were determined by real-time PCR with a 7500 Fast Real-Time PCR system (Applied Biosystems) according to the manufacturer’s instructions. The mRNA levels of SENP1 were determined by quantitative real-time PCR with SYBR green detection. Tubulin RNA was amplified as an internal control. The relative gene expression level was calculated using the comparative Ct method formula: 2-ΔΔCt. The primers used in this study are listed in the table below.

2.13 Immunoprecipitation

H9C2 cells (10 cm dish) were lysed in 0.5 mL of IP buffer [20 mM Tris–HCl (pH 7.4), 0.3% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM Na3VO4, 4 mM EDTA, and 400 mM NaCl] containing 1 mM phenylmethylsulfonyl fluoride, 5 mM N-ethylmaleimide, and 50 µL of cocktail solution (One complete ULTRA Tablet, Mini, EDTA-free dissolved in a volume of 0.5 mL of DDWater). After treated with hypoxia for 2 h, cells were treated by addition of MG132 (10 mM). Cell lysis was performed for 30 min on ice. After centrifugation at 12 000 g for 15 min at 4 °C, the supernatants were added to appropriate antibody 1 µL (HIF1α Antibody—NB100-105, NOVUS, mouse) rotated for 16 h at 4 °C and coupled with 30 µL of protein G-Sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The bead suspensions were rotated for 3 h at 4 °C. Beads were then washed five times with radioimmune precipitation assay buffer. The immunoprecipitates were treated with 50 µL of 2% SDS-treating solution and analysed by western blotting.

2.14 Plasmids and western blot analysis

Flag-HIF1α wild-type (WT) and Flag-HIF1α K719T plasmids were obtained from Xunlei Kang (University of Texas Southwestern Medical Center). An antibody against HIF1α was obtained from NOVUS. Antibodies against glucose transporter-1 (GLUT-1), heme oxygenase 1 (HO-1), Caspase-3, and Tubulin were obtained from ABCAM. The antibody against SUMO-1 was purchased from Epitomics. The antibody against SENP1 was produced in our lab as previously reported. All antibodies were used at 1:1000 dilution. Protein bands on western blots were developed with an enhanced chemiluminescence substrate kit. Quantification of the blots was performed with the Image J software.

2.15 Quantification of caspase-3 activity

Caspase-3 activity was assessed using Caspase-3 Colorimetric Assay Kits (Beyotime, China). In brief, cardiomyocytes were subjected to hypoxia/reoxygenation. The cells were then washed in PBS and suspended in 100 mL of lysis buffer (20 mM HEPES, pH 7.9, 20% glycerol, 200 mM KCl, 0.5 mM EDTA, 0.5% NP-40, 0.5 mM DTT, and 1% protease inhibitor cocktail) for 30 min on ice. The cell lysates were centrifuged at 12 000 g at 4 °C for 20 min and then collected. The protein concentration was determined by the Bradford method. Supernatant samples containing 50 mg of total protein were used for the determination of caspase-3 activity. These samples were added to each well of 96-well microtiter plates with the DEVD-pNA at 37 °C for 2 h. The optical density of each well was measured at 405 nm using a microplate reader.

2.16 Statistical analysis

All values are presented as the mean ± SEM. Statistical significance of multiple treatments was determined using the GraphPad Prism Software Version 5.9 (San Diego, CA, USA) via Student’s t-test or ANOVA (one-way and two-way), followed by either a Newman–Keuls or Bonferroni post hoc test when appropriate. For all tests, a P-value of <0.05 was considered significant.

3. Results

3.1 SENP1 is up-regulated by I/R in vivo and by hypoxia/reoxygenation in vitro

SENP1 expression is known to be up-regulated in endothelial cells under hypoxic conditions. Because ischaemia causes hypoxia in the myocardium, we hypothesized that SENP1 levels are up-regulated in response to I/R. To test this hypothesis, we examined SENP1 expression in cardiomyocytes exposed to I/R. We collected cardiac tissue specimens from 16 patients undergoing cardiac surgery. I/R was achieved in these patients by aortic cross-clamping for 30 min followed by unclamping for 15 min. Heart biopsies were taken before clamping (control), before clamp removal (ischaemia 30 min), and after clamp removal (I/R). The levels of SENP1 protein were assayed by immunohistochemical staining. We found that SENP1 protein levels increased significantly after the myocardium was subjected to ischaemia. After reperfusion, SENP1 levels slightly decreased but were significantly higher than in the control group (P < 0.05, Figure 1A). Real-time PCR assays revealed similar changes in SENP1 expression at the mRNA level (Figure 1B). In the mouse model, where myocardial I/R injury was induced through LAD ligation, we found that both ischaemia and reperfusion led to elevated SENP1 mRNA levels. In addition, after an extended period of reperfusion (4–24 h), we observed a greater increase in SENP1 mRNA levels than in the ischaemia-only treatment group (Figure 1C). Unlike the results in human specimens, reperfusion was more powerful than ischaemia in stimulating SNEP1 expression in mice (Figure 1C). In the in vitro model, cells from the rat cardiomyocyte cell line H9C2 were exposed to hypoxia for 2 h followed by reoxygenation for 30 min. Consistently, an up-regulation of SENP1 mRNA was observed (Figure 1D). Taken together, these data indicate that SENP1 expression is stimulated in cardiomyocytes under I/R conditions.

3.2 SENP1 reduces myocardial infarction and alleviates cardiomyocyte death

Because the SENP1−/− embryos are severely anaemic and fatal, we used SENP1+/− mice in our study. To investigate the specific role of SENP1 in myocardial I/R injury, we compared myocardial damage in SENP1+/− and SENP1+/+ mice under I/R conditions. The reduction of SENP1 expression in SENP1+/− mice was verified by the real-time PCR assays (Figure 2E). The age and body weight of SENP1−/− and SENP1+/+ mice were set to be identical. In normal conditions, no morphological differences were observed between SENP1+/− and SENP1+/+ mouse hearts, and their heart rates were almost identical. LAD ligation surgeries were performed in SENP1+/− and SENP1+/+ mice to induce myocardium I/R injury. To delineate the AAR and the actual infarcted area, we stained the mouse hearts with Evans blue and TTC 24 h after reperfusion. The relative sizes of the white, necrotic areas (areas of infarction) within the AARs in SENP1−/− mice were significantly larger than those in SENP1+/− mice, whereas the AAR sizes were similar between SENP1+/− and SENP1+/+ mice (Figure 2A and B). These results suggest that SENP1 protects against I/R-induced myocardial infarction in vivo.

We then applied an RNAi approach in H9C2 cells to further demonstrate the protective effect of SENP1 against cell death. H9C2 cells were pretreated with SENP1-siRNA or non-specific siRNA as controls and subjected to hypoxia for 2 h followed by reperfusion for 30 min. Cell death was determined by LDH assays and Trypan blue staining. Both assays revealed modest but statistically significant increases in cell
death in the SENP1-siRNA-treated cells compared with controls (Figure 2C and D), indicating that SENP1 indeed reduces cardiomyocyte death.

3.3 Cardiac function after I/R is preserved in SENP1+/+ compared with SENP1+/− mice

Because SENP1 reduces myocardial infarct size, we postulated that SENP1 also protects cardiac function from I/R injury. To test this hypothesis, we examined the left ventricular function and cardiac morphology of SENP1+/+ and SENP1+/− mice. Echocardiography was performed under light anesthesia. No significant changes in ejection fraction (EF) and LV fractional shortening (LVFS) were observed between SENP1+/+ and SENP1+/− mice before I/R. However, 24 h after reperfusion, SENP1+/− mice had significantly reduced LVFS and EF compared with SENP1+/+ mice (Figure 3A–C). These results, together with the effects of SENP1 deficiency on myocardial infarction, suggest that activation of SENP1 is protective against I/R injury.

3.4 SENP1 protects cardiomyocytes from apoptosis

Cardiomyocyte apoptosis induced by I/R injury is a major cause of cardiac functional impairment. SENP1 deficiency has been reported to promote the apoptosis of mouse embryonic fibroblast (MEF) cells under endoplasmic reticulum stress. To elucidate whether SENP1 regulates cardiomyocyte apoptosis in I/R, we examined cellular apoptosis in vivo and in vitro. SENP1 expression in vivo and in vitro. SENP1 expression levels in SENP1+/− mice and siRNA-treated H9C2 cells was examined by real-time PCR. Hypo, hypoxia; Hypo/Reoxy, hypoxia/reoxygenation. **P < 0.01 compared with no SENP1-siRNA CON group; ***P < 0.01 compared with the SENP1-siRNA CON group. Two-way ANOVA was performed for (C and D). Student’s t-test was performed for (B, E and F). All experiments were repeated eight times.
markers Caspase-3 and Bcl-2 were analysed. Cardiomyocytes from SENP1
+/- and SENP1+/+ mice were subjected to hypoxia/reoxygenation. SENP
knockdown resulted in a stimulation of Caspase-3 (Figure 4E). Cardiomyocytes
from SENP1+/- and SENP1+/+ mice were infected with HIF1a overexpression
plasmids, HIF1a WT or K719T (see Supplementary material online, Figure 5I). HIF1a WT
and SENP1+/- mice were subjected to I/R. Then, echocardiography was performed
for (C and D). These data suggest that a HIF1a-dependent pathway may be involved in the
cardioprotective activity of SENP1.

To further demonstrate that HIF1a is involved in the cardioprotective function
of SENP1, we performed a rescue study. H9C2 cells were transfected with HIF1a
overexpression plasmids, HIF1a WT or K719T (see Supplementary material online, Figure 5I). HIF1a WT and SENP1+/- mice were subjected to I/R injury, although the latter one to a less extent, the level of necrosis (Figure 6A) and apoptosis (Figure 6B and C) induced by SENP1 knockdown. Overexpression of HIF1a also reverses the expression level of GLUT-1 and HO-1 (Figure 6D and E). Thus, taking all these results together, we conclude that SENP1 protects cardiac function in I/R via a HIF1a-dependent pathway.

4. Discussion

Our results indicate that (i) SENP1 is activated by I/R of the myocardium in
vitro, (ii) activation of SENP1 protects cardiac function against I/R injury, (iii) SENP1 plays an important anti-necrotic and anti-apoptotic role in cardiomyocytes during I/R, and (iv) the protective effects of

**Figure 3** SENP1 preserves cardiac function after I/R in vivo. (A) SENP1+/- and SENP1+/- mice were subjected to I/R. Then, echocardiography was performed. (B and C) Compared with SENP1+/- mice, SENP1+/- mice showed deterioration of EF% and FS%. **p < 0.01 compared with the SENP1+/- CON group, ##p < 0.01 compared with the SENP+/- CON group. Two-way ANOVA was performed for (C and D). All experiments were repeated six times.
SENP1 in myocardial I/R injury are, at least partly, mediated by the activation of a HIF1α-dependent pathway.

In our study, enhanced SENP1 expression was observed in cardiomyocytes subjected to ischaemia or I/R. We demonstrated that SENP1 expression is increased at both the transcriptional and translational levels in human myocardium during I/R. The greatest weakness of this study is that we used only atrial myocardium, as we did not have access to ventricular myocardium. Considering the difference in morphology and function (e.g. electrophysiological and contractile properties) between atrial cardiomyocytes and their ventricular counterpart, additional studies in two other model systems (the mouse I/R model in vivo and H9C2 cell hypoxia/reoxygenation model in vitro) were performed. However, we must admit that these two model systems still have limitations. The mouse cardiomyocytes we used are neonatal cardiomyocytes rather than adult, and H9C2 cells are myoblasts but not true cardiomyocytes. Despite of these limitations, our data from three different model systems are consistent. Both the mouse vivo model and the rat vitro model exhibited a significant up-regulation of SENP1 down-regulates cardiomyocyte apoptosis in vivo and in vitro. (A and B) SENP1+−− and SENP1++/ mice were stimulated with I/R. TUNEL assays were performed to analyse the level of apoptosis. (C and D) Cells from the rat cardiomyocyte cell line H9C2 were pretreated with siRNA to knockdown SENP1. Cells were then stimulated with hypoxia/reoxygenation. Cells were stained with Annexin V and PI before apoptosis was analysed by FACS. (E–H) Expression and activity of apoptotic markers Caspase-3 and Bcl-2 were also analysed. Cardiomyocytes from SENP1+−− and SENP1++/ mice were subjected to hypoxia/reoxygenation. Whole cellular proteins were extracted. Bcl-2 and active Caspase-3 expression were examined by western blotting (E–G). Caspase-3 activity was assessed using Caspase-3 Colorimetric Assay Kits (H). *P < 0.05 compared with no SNP1-siRNA CON group; **P < 0.01 compared with the SENP1-siRNA CON group; ^P < 0.05 compared with the SENP−/− CON group; #P < 0.05 compared with the SENP+/− CON group; ## P < 0.01 compared with the SENP+/− CON group. Student’s t-test was performed for (B). Two-way ANOVA was performed for (C, F–H). All experiments were repeated seven times.
Collectively, these results demonstrate that SENP1 expression is enhanced in cardiomyocytes with I/R injury. Although the mechanism involved in this up-regulation activity is still not clear, it is a logically reasonable finding based on earlier studies. Activation of HIF1α induces SENP1 expression. Myocardial ischaemia leads to tissue hypoxia and the concomitant activation of HIF1α. Thus, it is easy to postulate that SENP1 expression is enhanced under I/R via activating HIF1α. However, the mechanism involved in SENP1 up-regulation in...
Our results demonstrate that hearts from SENP1+/− mice display more severe myocardial injury and lower systolic function after I/R. However, as SNEP1 knockdown in this model is not cardiomyocyte-specific, it is difficult to determine whether the protective effects of SENP1 on cardiac I/R injury result directly from its activity in myocardium rather than its effects in tissues. To explore this issue, we studied the effects of SENP1 knockdown on hypoxia/reoxygenation injury in cells from the rat cardiomyocyte cell line H9C2. We found that SENP1 knockdown in these cardiomyocytes results in an increase in cellular apoptosis and necrosis under hypoxia/reoxygenation conditions.

Figure 6  Overexpression of HIF1α reduces the myocardial deterioration effect of SENP1 knockdown. All data are acquired in H9c2 cells. (A) Overexpression of HIF1α reverses the effect of SENP1-siRNA on necrosis. Cells were pretreated with or without SENP1-siRNA. Then, cells were transfected with Flag-HIF1α plasmid or Flag-HIF1α K719T plasmid and stimulated with hypoxia/reoxygenation. After hypoxia/reoxygenation, cellular necrosis was assayed by Trypan blue staining. (B and C) Overexpression of HIF1α reverses the effect of SENP1-siRNA on apoptosis. Cells were pretreated with or without SENP1-siRNA. Then, cells were transfected with Flag-HIF1α plasmid or Flag-HIF1α K719T plasmid and stimulated with hypoxia/reoxygenation. After hypoxia/reoxygenation, cells were stained with Annexin V and PI. Cellular apoptosis was assayed by FACS. (D and E) Overexpression of HIF1α restores the levels of HIF1α and its downstream targets. After H9C2 cells were pretreated with or without SENP1-siRNA, cells were transfected with Flag-HIF1α plasmid or Flag-HIF1α K719T plasmid. Then, cells were subjected to hypoxia. The extents of apoptosis were tested by FACS. The protein levels of HIF1α, GLUT-1, and HO-1 were assayed by western blotting. **P < 0.01 compared with the SENP1-siRNA CON group; *P < 0.05 compared with no SENP1-siRNA CON group; *P < 0.01 compared with the SENP1-siRNA + Flag-HIF1α WT group; $P < 0.01$ compared with the SENP1-siRNA + Flag-HIF1α K719T group. Two-way ANOVA was performed for (A, B, and E). All experiments were repeated eight times.
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We therefore hypothesized that the reduction in cardiac function in response to hypoxia is mediated by a HIF-dependent pathway. In our study, we found that SENP1 knockdown decreased HIF1α expression in cardiomyocytes by enhancing SUMOylation and resulted in enhanced cellular necrosis and apoptosis. Meanwhile, the expressions of GLUT-1 and HO-1 were reduced. More importantly, our rescue study showed that both HIF1α K719T and HIF1α WT significantly reduced, although HIF1α WT to a less extent, the induction of necrosis and apoptosis caused by SENP1 knockdown. Thus, we concluded that the impairment of cardiac function in SENP1−/− mice is mediated, at least in part, through the reduction in HIF1α stability and activity.

In conclusion, we show that SENP1 is activated by I/R and plays a protective role in the heart by stimulating HIF1α-dependent factors. Stimulation of SENP1 may represent a novel strategy for protecting the heart from I/R injury.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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