# HIF-1α protein SUMOylation is an important protective mechanism of action of hypothermia in hypoxic cardiomyocytes

HUAQIN LIU<sup>1\*</sup>, XIYUN BIAN<sup>2,3\*</sup>, MEILI XU<sup>1</sup>, XIAOFANG MA<sup>2,3</sup>, CHUNYAN ZHANG<sup>3,4</sup>, JINJIN JIANG<sup>1</sup>, JIANFENG FU<sup>1</sup> and XIAOZHI LIU<sup>2,3</sup>

<sup>1</sup>Department of Anesthesiology, The Fourth Hospital of Hebei Medical University, Shijiazhuang, Hebei 050011; <sup>2</sup>Central Laboratory, <sup>3</sup>Tianjin Key Laboratory of Epigenetics for Organ Development in Preterm Infants, The Fifth Central Hospital of Tianjin; <sup>4</sup>Department of Pharmacy, Tianjin Binhai New Area Hospital of Traditional Chinese Medicine, Tianjin 300450, P.R. China

Received February 17, 2020; Accepted June 23, 2020

DOI: 10.3892/mmr.2021.12115

Abstract. Different degrees of myocardial ischemia-reperfusion injury during open-heart surgery are inevitable. Therapeutic hypothermia is an important technique for reducing ischemia-reperfusion injury; however, there are numerous potential adverse effects. Furthermore, the underlying molecular mechanisms of action of therapeutic hypothermia remain unclear. In the present study, rat hearts were perfused for 30 min and subjected to 30 min of regional ischemia, followed by 120 min of reperfusion. Animals received intraperitoneal injection of spectomycin B1 at 30 min prior to the start of surgery. Total myocardial area, infarct area, myocardial injury, and apoptosis were assessed. H9C2 cells were incubated for 24 h at 34°C with 5% CO<sub>2</sub> to simulate therapeutic hypothermic stress, and cell viability and mitochondrial injury were evaluated. The levels of protein SUMOylation, hypoxia-inducible factor (HIF)-1 $\alpha$  and vascular endothelial growth factor (VEGF) were determined by western blot analysis. It was demonstrated that hypoxia significantly increased the overall modification by the small ubiquitin-related modifier protein (SUMO) of various proteins in cardiomyocytes, both in vitro and ex vivo. In turn, this increased the protein levels of HIF-1 $\alpha$ , continuously stimulated downstream VEGF expression. Therapeutic

#### \*Contributed equally

Key words: myocardial ischemia-reperfusion, therapeutic hypothermia, SUMOylation, hypoxia-inducible factor- $1\alpha$ , angiogenesis

hypothermia further increased protein SUMOylation, whereas inhibiting the SUMOylation pathway reduced the protective effect of therapeutic hypothermia on hypoxic cardiomyocytes. Overall, these data suggested that increasing SUMOylation of HIF-1 $\alpha$  may be an important molecular mechanism underlying the protective effects of therapeutic hypothermia following hypoxia in myocardial cells. These findings may aid in the use of therapeutic hypothermia for treatment of myocardial ischemia-reperfusion and help avoid excessive side effects.

#### Introduction

Bigelow et al (1) first applied therapeutic hypothermia in the clinic in 1950, and this technique remains one of the most important measures of myocardial protection during myocardial ischemia-reperfusion injury. Reperfusion of the ischemic myocardium itself can conversely worsen myocardial injury (2). Therapeutic hypothermia can reduce myocardial oxygen consumption and inhibit myocardial metabolism, thereby enhancing myocardial tolerance to hypoxia (3). However, therapeutic hypothermia can also cause several adverse side effects. For example, hypothermia can deactivate the Na<sup>+</sup>-K<sup>+</sup>-ATPase and Ca<sup>2+</sup>-ATPase of the sarcolemma and sarcoplasmic reticulum, which may cause cell volume dysregulation and cell swelling (4,5). Furthermore, therapeutic hypothermia has been reported to reduce cell membrane potential and substance transport (6). As such, the optimal treatment of myocardial ischemic perfusion with therapeutic hypothermia remains unclear. A greater understanding of the molecular and cellular mechanisms that are active during therapeutic hypothermia in the clinic is required; this includes determining the indications for treatment and to reduce potential side effects.

Small ubiquitin-related modifier protein (SUMO) is a member of the ubiquitin family of proteins, all of which possess similar structures, but different functions. SUMO can covalently modify numerous proteins to regulate their stability, function and localization (7). Similar to ubiquitin modification, SUMO modification (8) (termed SUMOylation) is a dynamic process catalyzed by a small number of E1-activating enzymes, a single E2 binding enzyme (ubiquitin carrier protein 9; Ubc9)

*Correspondence to:* Professor Jianfeng Fu, Department of Anesthesiology, The Fourth Hospital of Hebei Medical University, 12 Jiankang Road, Shijiazhuang, Hebei 050011, P.R. China E-mail: fffjf2008@sohu.com

Professor Xiaozhi Liu, Central Laboratory, The Fifth Central Hospital of Tianjin, 41 Zhejiang Road, Tanggu Street, Binhai New Area, Tianjin 300450, P.R. China E-mail: lxz7997@126.com

and multiple E3 ligases. Furthermore, SUMOylated proteins can be de-SUMOylated through SUMO-specific proteases (8).

Hypoxia-inducible factor (HIF)-1 is an important factor that mediates the response of cells to hypoxia (9). HIF-1 is comprised of two subunits termed  $\alpha$  and  $\beta$  (10). The transcriptional activity and protein stability of HIF-1α are regulated by intracellular oxygen concentration. Under normoxic conditions, HIF-1 $\alpha$  is rapidly degraded by the ubiquitin-proteasome pathway. However, under hypoxic conditions, the stability increases and it enters the nucleus to combine with HIF-1ß to form a dimer that regulates hypoxia-induced gene transcription, including vascular endothelial growth factor (VEGF), glucose transporter 1 (Glut-1), matrix metalloproteinases and multidrug transporter (11-14). Notably, HIF-1a can be modified by SUMO1, which can affect the stability of HIF-1 $\alpha$  (15). Nevertheless, the role and regulatory mechanisms of HIF-1 $\alpha$ SUMOylation in normal physiological function remain unclear (16). Therapeutic hypothermia can enhance the binding capacity of SUMO2/3 to its target proteins, and can serve a role in inhibiting apoptosis and improving neural function in the early stage of ischemia (17,18). However, it remains unclear whether regulation of HIF-1a protein by SUMOylation is involved in the protective mechanism underlying the effects of therapeutic hypothermia on myocardial ischemia.

In the present study, the SUMOylation of HIF-1 $\alpha$  was examined to determine if it participated in the protective mechanism of therapeutic hypothermia against myocardial ischemia. The present results indicated that SUMOylation of HIF-1 $\alpha$  had an indispensable role in the protective mechanism of therapeutic hypothermia on the ischemic myocardium. This new knowledge on the mechanism of therapeutic hypothermia in myocardial protection may aid in the rational application of therapeutic hypothermia to treat myocardial ischemia-reperfusion.

# Materials and methods

Cell culture and hypoxia or hypothermia exposure. Rat cardiomyocyte H9C2 cells were obtained from the American Type Culture Collection, and were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100  $\mu$ g/ml penicillin and 50  $\mu$ g/ml streptomycin sulfate (all from Gibco; Thermo Fisher Scientific, Inc.) at 37°C with 5%  $CO_2$ . To evaluate the effects of hypoxia on H9C2 cells, culture medium without glucose, L-aspartic acid, L-glutamic acid or sodium pyruvate was equilibrated overnight in an anoxic chamber with 85% N<sub>2</sub>, 10% H<sub>2</sub> and 5% CO<sub>2</sub>. The cultures were transferred to an anoxic chamber and washed three times with anoxic medium. After 24 h of oxygen-glucose deprivation at 37°C, the cells were transferred back to the incubator at 37°C with 5% CO<sub>2</sub> for an additional 24 h. To simulate therapeutic hypothermic stress, the culture dishes loaded with H9C2 cells were placed in incubators for 24 h at 34°C with 5% CO<sub>2</sub>. Cells cultured in normal conditions (37°C with 5% CO<sub>2</sub>) were used as controls.

Blockade of the SUMOylation pathway. H9C2 cells were seeded in 6-well plates at a density of  $1 \times 10^5$  cells and incubated overnight at 37°C in normoxic conditions. Spectomycin B1 (20  $\mu$ M; Nanjing Chemlin Chemical Co., Ltd.) was added to

the culture medium 30 min prior to reperfusion for 24 h to block the effects of Ubc9, as previously reported (18).

Western blotting. Total protein was extracted from cells or tissues using RIPA buffer (Beijing Solarbio Science & Technology Co., Ltd.) supplemented with 1 mM phenylmethane sulfonyl fluoride and 20 mM N-ethylmaleimide, as previously reported (19). The protein homogenate (40  $\mu$ g per lane; 2  $\mu$ g/ $\mu$ l) was resolved by SDS-PAGE using 10% gels, blotted onto Immobilon PVDF membranes (EMD Millipore), blocked with 5% skimmed milk for 90 min at room temperature, and incubated with the appropriate primary antibody at 4°C overnight. The β-actin antibody was re-probed after stripping. Information on all antibodies is shown in Table I. Primary antibodies were recovered and the membrane was incubated with the appropriate secondary antibodies for 1 h at room temperature. The membranes were visualized with enhanced chemiluminescence reagents (EMD Millipore). Densitometric semi-quantification analysis of the western blot bands was performed using image analysis software (ImageJ v1.48; National Institutes of Health).

Lactate dehydrogenase (LDH) detection. H9C2 cells were seeded into 96-well plates  $8x10^4$  cells/ml and incubated under hypoxic and/or hypothermic conditions with or without spectomycin B1. The cells were then collected, sonicated twice at interval setting 0.5 with a UP-200S sonicator and centrifuged at 13,680 x g at 4°C for 10 min. The LDH content in the supernatant obtained from centrifugation of the sonicated cells was measured using an ELISA (cat. no. YM-S0351; Shanghai Yuanmu Biotechnology Co., Ltd.), in accordance with the manufacturer's instructions.

Detection of apoptosis using flow cytometry. H9C2 cells (1x10<sup>5</sup> cells) were collected following EDTA-trypsin digestion and washed twice with cold PBS. Apoptosis was then assessed within 1 h using an Annexin V fluorescein isothiocyanate apoptosis detection kit (BD Biosciences) and a FACSCalibur flow cytometer (BD Biosciences), according to the manufacturer's protocol. The samples cells were analyzed using the FlowJo software version 9 (FlowJo LLC).

Rats and experimental grouping. Animal experiments were performed according to the regulations and guidelines approved by the Animal Ethics Committee of The Fifth Central Hospital of Tianjin (Tianjin, China; approval no. TJWZX2018044). Animal studies were conducted according to the Guide for the Care and Use of Laboratory Animals (8th edition, 2011) and the guidelines for Animal Research Reporting In Vivo Experiments guidelines (https://grants.nih.gov/grants/olaw/guide-for-the-care-and-useof-laboratory-animals.pdf). A total of 48 6-week-old Sprague-Dawley female rats (mean weight, 200 g) were purchased from the Animal Center of Nanjing University (Nanjing, China. Animals were housed in the Experimental Animal Center of The Fifth Central Hospital of Tianjin, and maintained under a controlled temperature (22-24°C), stable humidity (40-60%) and a 12 h-light/dark cycle with ad libitum access to food and water. Animals were randomly divided into four groups (n=6 each): i) Control group, the

ntibody target Supplier		Catalogue number	Dilution	
SUMO1	Abcam	ab11672	1:1,000	
HIF-1α	Abcam	ab216842	1:500	
VEGF	Abcam	ab69479	1:500	
Ubc9	Abcam	ab75854	1:2,000	
Caspase 3	Abcam	ab13847	1:500	
Cleaved-caspase 3	Abcam	ab2302	1:1,000	
Bax	Abcam	ab32503	1:5,000	
Bcl2	Abcam	ab692	1:500	
β-actin	Abcam	ab8227	1:1,000	
Goat anti-rabbit IgG	Jackson ImmunoResearch	111-035-003	1:2,000	
Goat anti-mouse IgG	Jackson ImmunoResearch	111-035-003	1:2,000	

Table I. Annouv mornation	Table I.	Antibody	infor	mation
---------------------------	----------	----------	-------	--------

SUMO, small ubiquitin-related modifier; HIF, hypoxia inducible factor; VEGF, vascular endothelial growth factor; Ubc9, ubiquitin carrier protein 9.

isolated heart of each rat was perfused at 37°C for 30 min, exposed to ischemia for 30 min and reperfusion for 120 min. The animals in the control group did not receive an injection of spectomycin B1. ii) Therapeutic hypothermia group, the isolated heart of each rat was perfused at 37°C for 30 min, then exposed to ischemia for 30 min and reperfusion at 34°C for 120 min. Animals in this group did not receive an injection of spectomycin B1. iii) Spectomycin B1 group, the isolated heart of each rat was perfused at 37°C for 30 min, then exposed to ischemia for 30 min and reperfusion for 120 min. At 30 min prior to the start of surgery, animals received intraperitoneal injection of spectomycin B1 (2 mg/kg) according to the reagent instructions. iv) Therapeutic hypothermia + spectomycin B1 group, the isolated heart of each rat was perfused at 37°C for 30 min, followed by ischemia for 30 min and reperfusion at 34°C for 120 min. At 30 min prior to the start of surgery, animals received intraperitoneal injection of spectomycin B1 (2 mg/kg), according to the reagent instructions.

Production of the isolated heart perfusion model. The rats were anesthetized with sodium pentobarbital (60 mg/kg intraperitoneally; Merial) and fixed to the operating table. Briefly, after the thorax was opened, the heart was removed and mounted on a Langendorff apparatus. All hearts were perfused with Krebs-Henseleit buffer containing 118.5 mM sodium chloride, 4.7 mM potassium chloride, 1.2 mM magnesium sulfate, 24.8 mM sodium bicarbonate, 1.8 mM calcium chloride, 1.2 mM potassium hydrogen phosphate and 10 mM glucose, which was heated to 37°C and gassed with  $95\% O_2/5\% CO_2$  to stop the hearts from beating. Subsequently, a 4-0 silk suture was placed around the left coronary artery and the ends of the suture were passed through a small piece of soft vinyl tubing to form a snare. Regional ischemia was induced by fixing the snare to the heart using a hemostat. After 30 min of ischemia, the hearts were reperfused for 120 min by releasing the hemostat. Rats were sacrificed by exsanguination during the Langendorff procedure.

Measurement of myocardial infarction (MI). After reperfusion was complete, the heart was removed. All hearts were cut into 1-mm-thick cross-sections and incubated in 1% triphenyltetrazolium chloride (Sigma-Aldrich; Merck KGaA) at 37°C for 20 min. The sections at the level of the papillary muscle were photographed with a ruler. The unstained area was considered infarcted myocardium. Total myocardial area (TMA), and infarct area (IA) at the mid-papillary muscle were measured by planimetry. The percentage of infarct area was calculated as IA/TMA x100%.

Confocal imaging of mitochondrial membrane potential. Mitochondrial membrane potential of H9C2 cells was measured using a commercial assay (JC-10 mitochondrial membrane potential assay kit; Beijing Solarbio Science & Technology Co., Ltd.). Fluorescence changes were detected with a laser scanning confocal microscope. The maximum excitation wavelength of the JC-10 monomer was 515 nm and the maximum emission wavelength was 529 nm (green). The maximum excitation wavelength of the JC-10 polymer was 585 nm and the maximum emission wavelength was 590 nm (red).

Statistical analysis. All statistical analyses were performed using SPSS 19.0 for Windows (IBM Corp.). Each experiment was performed at least three times. Data are presented as the mean  $\pm$  SD. The differences between the experimental and control group were tested using an unpaired t-test. For comparing differences among more than two experimental groups, the means were compared using one-way ANOVA followed by a Tukey-Kramer test. P<0.05 was considered to indicate a statistically significant difference.

# Results

Hypoxia can significantly increase the expression of SUMO1 in cardiomyocytes and activate the HIF- $1\alpha$ /VEGF pathway. Because HIF- $1\alpha$  has previously been identified as a target



Figure 1. Hypoxia can significantly increase the expression of SUMO1 protein(all of the SUMO detected SUMO1 conjugates), stabilize HIF-1 $\alpha$  and activate the VEGF pathway in cardiac myocytes. (A) SUMO1, HIF-1 $\alpha$  and VEGF protein expression levels were examined using western blotting. (B) Graph of experimental data. Data are presented as the mean  $\pm$  SD (n=3). \*P<0.05 vs. control group. SUMO, small ubiquitin-related modifier; HIF, hypoxia inducible factor; VEGF, vascular endothelial growth factor.



Figure 2. Inhibition of the SUMOylation pathway reduces the expression of HIF-1 $\alpha$  and VEGF in cardiomyocytes, and aggravates injury following hypoxia. (A) Ubc9, SUMO1, HIF-1 $\alpha$  and VEGF protein expression were examined using western blotting. (B) LDH content was measured using ELISA. (C) Flow cytometry was used to detect cardiomyocyte apoptosis. Data are presented as the mean  $\pm$  SD (n=3). \*P<0.05 vs. control group. \*P<0.05 vs. hypoxia group. SUMO, small ubiquitin-related modifier; HIF, hypoxia inducible factor; VEGF, vascular endothelial growth factor; Ubc9, ubiquitin carrier protein 9; LDH, lactate dehydrogenase; Spec B1, spectomycin B1.

protein of SUMO1 (15), the expression of SUMO1 in cardiomyocytes was initially examined under normal and hypoxic conditions. The present results showed that cardiomyocytes expressed low levels of SUMO1 under normal oxygen conditions, which significantly increased following exposure to hypoxic conditions (Fig. 1A and B). In addition, HIF-1 $\alpha$  and VEGF expression were examined. The protein expression levels of HIF-1 $\alpha$  and VEGF in cardiomyocytes were significantly increased under hypoxic conditions compared with normoxia. Bae *et al* (20) have demonstrated that the protein level and transcriptional activity of HIF-1 $\alpha$  can be upregulated by SUMO1. These findings validated the hypothesis that



Figure 3. Therapeutic hypothermia increases HIF-1 $\alpha$  and VEGF protein levels to antagonize myocardial cell injury following hypoxia. (A) SUMO1, HIF-1 $\alpha$  and VEGF protein expression were examined using western blotting. (B) LDH content was measured using ELISA. (C) Flow cytometry was used to detect cardiomyocyte apoptosis. Data are presented as the mean  $\pm$  SD (n=3). \*P<0.05 vs. control group. #P<0.05 vs. hypoxia group. SUMO, small ubiquitin-related modifier; HIF, hypoxia inducible factor; VEGF, vascular endothelial growth factor; LDH, lactate dehydrogenase.

hypoxia may increase the expression of SUMO1 in cardiomyocytes, increase the protein levels of HIF-1 $\alpha$  and continuously activate its downstream VEGF transcription, thereby antagonizing the actions of hypoxia (Fig. 1A and B).

Inhibition of SUMOylation can reduce signaling of the HIF-1a/VEGF pathway and aggravate cardiomyocyte injury following hypoxia. To examine whether the hypoxia-activated HIF-1a/VEGF pathway is SUMOylation-dependent, cardiomyocytes were pretreated with spectomycin B1, a specific inhibitor of the E2 binding enzyme Ubc9, in order to inhibit the binding of SUMO1 to target proteins, prior to induction of hypoxia. The results demonstrated that spectomycin B1 effectively inhibited the activity of Ubc9 (19), which reduced the amount of SUMO1 conjugation to target proteins and decreased the levels of HIF-1a and VEGF proteins in myocardial cells under hypoxia (Fig. 2A).

Subsequently, the effect of inhibition of the SUMOylation pathway on myocardial injury following hypoxia was tested. Treatment with spectomycin B1 alone did not induce significant LDH release and cell apoptosis in the cardiomyocytes (Fig. 2B and C), but caused a significant reduction in hypoxic tolerance of cardiomyocytes, characterized by higher levels of LDH released (Fig. 2B) and increased cellular late apoptotic (Q2) and early apoptotic (Q3) test by flow cytometry(Fig. 2C).

Therapeutic hypothermia further increases protein SUMOylation, stabilizes the HIF- $1\alpha$ /VEGF pathway, and enhances hypoxic tolerance of cardiomyocytes. Next, the

effects of therapeutic hypothermia were examined on protein SUMOylation in normoxic and hypoxic cardiomyocytes (Fig. 3A). Therapeutic hypothermia significantly increased the frequency of SUMO1 conjugates in normoxic cardiomyocytes, and also significantly increased protein SUMOylation in hypoxic cardiomyocytes compared with the 37°C control (Fig. 3A). By contrast, therapeutic hypothermia had a minimal effect on HIF-1 $\alpha$  and VEGF protein levels in normoxic cardiomyocytes, but significantly increased HIF-1 $\alpha$  and VEGF protein levels in hypoxic cardiomyocytes (Fig. 3A).

Next, the effect of therapeutic hypothermia on the cytotoxicity to cardiomyocytes was examined under normoxic and hypoxic conditions. Therapeutic hypothermia did not affect LDH release or cardiomyocyte apoptosis under normoxia (Fig. 3B and C). However, under hypoxic conditions, therapeutic hypothermia significantly reduced LDH release and the percentage of apoptotic cardiomyocytes (Fig. 3B and C). These data suggested that therapeutic hypothermia may increase the protein levels of HIF-1 $\alpha$  by increasing protein SUMOylation, resulting in protein levels of the downstream VEGF, thereby antagonizing hypoxia-induced cardiomyocyte injury.

Inhibition of SUMOylation can reduce the protective effect of therapeutic hypothermia on hypoxic cardiomyocytes. To assess whether the protective action of hypothermia on hypoxic cardiomyocytes was SUMOylation-dependent, cardiomyocytes were treated with spectomycin B1. Spectomycin B1 significantly reduced the protein levels of Ubc9 and conjugated SUMO1 under both normoxic and hypoxic conditions. By contrast,



Figure 4. Inhibition of the SUMO pathway can block the protective effect of therapeutic hypothermia on hypoxic cardiomyocytes. (A) Ubc9, SUMO1, HIF-1 $\alpha$  and VEGF protein expression levels were examined using western blotting and were semi-quantified. (B) LDH content was measured using ELISA. (C) Flow cytometry was used to detect cardiomyocyte apoptosis. Data are presented as the mean  $\pm$  SD (n=3). \*P<0.05 vs. control group. \*P<0.05 vs. hypoxia group.  $\Delta$ P<0.05 and  $\Delta$ P<0.01 vs. hypoxia group. SUMO, small ubiquitin-related modifier; HIF, hypoxia inducible factor; VEGF, vascular endothelial growth factor; Ubc9, ubiquitin carrier protein 9; LDH, lactate dehydrogenase; Spec B1, spectomycin B1.

therapeutic hypothermia had no effect on the myocardial cells after spectomycin B1 treatment, compared with untreated cells under the same conditions (Fig. 4A). Correspondingly, when cardiomyocytes were treated with spectomycin B1, therapeutic hypothermia did not increase the protein levels of HIF-1 $\alpha$  and VEGF (Fig. 4A) and reduce the protective effect of hyothermia (Fig. 4B and C). These data suggested that the percentage of apoptotic cardiomyocytes under hypoxia was increased following treatment with the Ubc9 inhibitor. Nevertheless, as there are an abundant number of SUMO-conjugated proteins involved in various cellular processes, treatment with a Ubc9 inhibitor will block all modification by SUMO1 of HIF-1 $\alpha$  as well as other proteins involved in cellular apoptosis, which could also be modified by SUMO2/3. Consequently, the present findings (Figs. 2C and 4C) may also involve de-SUMOylation of other proteins due to the inhibition of Ubc9.

Therapeutic hypothermia can reduce the area of MI after ischemia-reperfusion, while inhibition of the SUMO pathway can offset this protective effect. Finally, a rat model of myocardial ischemia-reperfusion was used to test whether the protective effect of therapeutic hypothermia on ischemic myocardium was SUMO-dependent. As expected, therapeutic hypothermia reduced myocardial infarct size in rats exposed to myocardial ischemia and reperfusion (Fig. 5A). Furthermore, treatment with spectomycin B1 to inhibit SUMO conjugation further increased MI in ischemia-reperfusion rats (Fig. 5A). In addition, when therapeutic hypothermia was administered to rats undergoing myocardial ischemia-reperfusion pretreated with spectomycin B1, the protective effect of therapeutic hypothermia was significantly reduced and was characterized by a large myocardial infarct area (Fig. 5A), decreased mitochondrial membrane potential (Fig. 5B), increased cleaved-caspase 3/caspase 3 ratio and a decreased Bcl2/Bax ratio (Fig. 5C).

## Discussion

Ischemia-reperfusion injury is a common pathophysiological phenomenon following treatment of ischemic heart disease, including coronary artery bypass grafting after acute MI, thrombolysis after acute MI and open-heart surgery under extracorporeal circulation (21-23). The stress response of cardiomyocytes to hypoxia is a complex and delicately regulated process, involving a series of biological response reactions that alter the proteome and genome, activate angiogenesis, anaerobic metabolism and other signaling pathways. The stress response is also known to regulate the cell cycle, cell differentiation, apoptosis and necrosis (24,25). Thus, it is important to reduce and prevent myocardial ischemia-reperfusion injury.

Therapeutic hypothermia involves reducing the core body temperature to within a suitable range. Adult patients with cardiac arrest outside the hospital can be given low temperature therapy (32-35°C) for 12-24 h, which has a protective effect against ischemia-reperfusion injury (26).



Figure 5. Therapeutic hypothermia reduces the area of myocardial infarction after ischemia-reperfusion, whereas inhibition of the SUMO pathway can offset this protective effect. (A) Representative images and analysis of myocardial infarction in rats that underwent myocardial ischemia-reperfusion in the different treatment groups. (B) Confocal imaging of mitochondrial membrane potential. Magnification, x400. (C) SUMO1, HIF-1 $\alpha$ , VEGF, cleaved-caspase 3, caspase 3, Bcl2 and Bax protein expression levels were examined using western blotting and were semi-quantified. Data are presented as the mean  $\pm$  SD (n=3). \*P<0.05 vs. control group. \*P<0.05 vs. hypoxia group. SUMO, small ubiquitin-related modifier; HIF, hypoxia inducible factor; VEGF, vascular endothelial growth factor; Spec B1, spectomycin B1.

Therapeutic hypothermia has also been reported to reduce reperfusion injury in animal models of acute MI and improve ventricular remodeling after MI (27). However, most randomized controlled clinical trials have revealed that therapeutic hypothermia does not benefit patients with acute MI, but can benefit patients with large-scale MI and rapid temperature targeting before reperfusion (28). Nevertheless, the exact molecular and cellular mechanisms of therapeutic hypothermia remain to be fully elucidated, and are important for its clinical application.

The balance of post-translational modification of proteins involving ubiquitin and SUMO is essential for eukaryotic cells to respond to hypoxic stress (29,30). Unlike the degradation of target proteins caused by ubiquitination, SUMO1, which is present in all eukaryotic cells, can regulate protein-protein interactions, transcriptional activity, enhance substrate stability and affect the target protein subcellular localization (31,32). Numerous SUMO1 substrates, including HIF-1 $\alpha$ , I $\kappa$ B $\alpha$ , poly(ADP-ribose) polymerase 1, p53, Mdm2, c-jun, Glut-1 and Glut-4, have important roles in the oxygen response (33). Furthermore, hypothermia has been shown to significantly increase the expression of SUMO1 in cells, thereby protecting downstream target proteins from enzymatic hydrolysis (34).

In the present study, protein SUMO1 modification was examined to determine if SUMO1 was involved in the protective action of therapeutic hypothermia on myocardial injury induced by ischemia-reperfusion. Using an in vitro oxygen glucose deprivation model and an in vivo model of myocardial ischemia-reperfusion in rats, it was verified that protein SUMOvlation was essential for hypoxic tolerance and the therapeutic hypothermia-mediated cytoprotection of cardiomyocytes. Specifically, blockade of SUMO conjugation by spectomycin B1 was associated with a reduction in the protective effect of therapeutic hypothermia on cardiomyocytes. These data confirmed that therapeutic hypothermia-mediated myocardial protection was dependent on protein SUMOylation. Thus, the protein SUMOylation pathway involving SUMO1 should be considered when treating myocardial ischemia-reperfusion using therapeutic hypothermia because any drug or clinical intervention that increases or inhibits protein SUMOylation may affect the protective action of therapeutic hypothermia on

the ischemic myocardium or be associated with additional complications.

# Acknowledgements

Not applicable.

## Funding

This study was supported by grant from Tianjin Natural Science Foundation of China (grant nos. 18JCQNJC12800 and 19JCZDJC35200), Tianjin Special Project of New Generation Artificial Intelligence Technology (grant no. 18ZXZNSY00260), and Binhai Health and Family Planning Commission Science and Technology Projects (grant no. 2019BWKQ030).

#### Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

# **Authors' contributions**

JFF and XZL designed the experiments. HQL, XYB, MLX, XFM, CYZ and JJJ performed the experiments, and collected and analyzed data. HQL and XYB drafted the manuscript. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors read and approved of the final version to be published.

# Ethics approval and consent to participate

Animal experiments were performed according to the regulations and guidelines approved by the Animal ethics Committee of The Fifth Central Hospital of Tianjin (Tianjin, China).

#### Patient consent for publication

Not applicable

#### **Competing interest**

The authors declare that they have no competing interests

#### References

- Bigelow WG, Lindsay WK and Greenwood WF: Hypothermia; its possible role in cardiac surgery: An investigation of factors governing survival in dogs at low body temperatures. Ann Surg 132: 849-866, 1950.
- Jennings RB: Historical perspective on the pathology of myocardial ischemia/reperfusion injury. Circ Res 113: 428-438, 2013.
- Otterspoor LC, van Nunen LX, van 't Veer M, Johnson NP and Pijls NHJ: Intracoronary hypothermia before reperfusion to reduce reperfusion injury in acute myocardial infarction: A novel hypothesis and technique. Ther Hypothermia Temp Manag 7: 199-205, 2017.
- Han Y, Rajah GB, Hussain M and Geng X: Clinical potential of pre-reperfusion hypothermia in ischemic injury. Neurol Res 41: 697-703, 2019.

- Paquot F, Huart J, Defraigne JO, Krzesinski JM and Jouret F: Implications of the calcium-sensing receptor in ischemia/reperfusion. Acta Cardiol 72: 125-131, 2017.
- Witcher R, Dzierba AL, Kim C, Smithburger PL and Kane-Gill SL: Adverse drug reactions in therapeutic hypothermia after cardiac arrest. Ther Adv Drug Saf 8: 101-111, 2017.
- 7. Zhao X: SUMO-mediated regulation of nuclear functions and signaling processes. Mol Cell 71: 409-418, 2018.
- Rosik J, Szostak B, Machaj F and Pawlik A: Potential targets of gene therapy in the treatment of heart failure. Expert Opin Ther Targets 22: 811-816, 2018.
- Wang X, Liang X, Liang H and Wang B: SENP1/HIF-1α feedback loop modulates hypoxia-induced cell proliferation, invasion, and EMT in human osteosarcoma cells. J Cell Biochem 119: 1819-1826, 2018.
- Chachami G, Stankovic-Valentin N, Karagiota A, Basagianni A, Plessmann U, Urlaub H, Melchior F and Simos G: Hypoxia-induced changes in SUMO conjugation affect transcriptional regulation under low oxygen. Mol Cell Proteomics 18: 1197-1209, 2019.
- 11. Tomasi ML and Ramani K: SUMOylation and phosphorylation cross-talk in hepatocellular carcinoma. Transl Gastroenterol Hepatol 3: 20, 2018.
- Zhou HJ, Xu Z, Wang Z, Zhang H, Zhuang ZW, Simons M and Min W: SUMOylation of VEGFR2 regulates its intracellular trafficking and pathological angiogenesis. Nat Commun 9: 3303, 2018.
- 13. Sallais J, Alahari S, Tagliaferro A, Bhattacharjee J, Post M and Caniggia I: Factor inhibiting HIF1-A novel target of SUMOylation in the human placenta. Oncotarget 8: 114002-114018, 2017.
- 14. Ciria M, García NA, Ontoria-Oviedo I, González-King H, Carrero R, De La Pompa JL, Montero JA and Sepúlveda P: Mesenchymal stem cell migration and proliferation are mediated by hypoxia-inducible factor-1α upstream of notch and SUMO pathways. Stem Cells Dev 26: 973-985, 2017.
- Han X, Wang XL, Li Q, Dong XX, Zhang JS and Yan QC: HIF-1α SUMOylation affects the stability and transcriptional activity of HIF-1α in human lens epithelial cells. Graefes Arch Clin Exp Ophthalmol 253: 1279-1290, 2015.
  Cui CP, Wong CC, Kai AK, Ho DW, Lau EY, Tsui YM, Chan LK,
- 16. Cui CP, Wong CC, Kai AK, Ho DW, Lau EY, Tsui YM, Chan LK, Cheung TT, Chok KS, Chan ACY, *et al*: SENP1 promotes hypoxia-induced cancer stemness by HIF-1α deSUMOylation and SENP1/HIF-1α positive feedback loop. Gut 66: 2149-2159, 2017.
- 17. Liu X, Ren W, Jiang Z, Su Z, Ma X, Li Y, Jiang R, Zhang J and Yang X: Hypothermia inhibits the proliferation of bone marrow-derived mesenchymal stem cells and increases tolerance to hypoxia by enhancing SUMOylation. Int J Mol Med 40: 1631-1638, 2017.
- Li G, Liu X, Su Z and Zhang D: Hypothermia exerts early neuroprotective effects involving protein conjugation of SUMO 2/3 in a rat model of middle cerebral artery occlusion. Mol Med Rep 16: 3217-3223, 2017.
- Hirohama M, Kumar A, Fukuda I, Matsuoka S, Igarashi Y, Saitoh H, Takagi M, Shin-ya K, Honda K, Kondoh Y, *et al*: Spectomycin B1 as a novel SUMOylation inhibitor that directly binds to SUMO E2. ACS Chem Biol 8: 2635-2642, 2013.
- 20. Bae SH, Jeong JW, Park JA, Kim SH, Bae MK, Choi SJ and Kim KW: Sumoylation increases HIF-1alpha stability and its transcriptional activity. Biochem Biophys Res Commun 324: 394-400, 2004.
- 21. Li R, Wei J, Jiang C, Liu D, Deng L, Zhang K and Wang P: Akt SUMOylation regulates cell proliferation and tumorigenesis. Cancer Res 73: 5742-5753, 2013.
- 22. Del Re DP, Amgalan D, Linkermann A, Liu Q and Kitsis RN: Fundamental mechanisms of regulated cell death and implications for heart disease. Physiol Rev 99: 1765-1817, 2019.
- 23. Rossello X and Yellon DM: Cardioprotection: The disconnect between bench and bedside. Circulation 134: 574-575, 2016.
- 24. Heusch G and Gersh BJ: The pathophysiology of acute myocardial infarction and strategies of protection beyond reperfusion: A continual challenge. Eur Heart J 38: 774-784, 2017.
- Aggarwal S and Natarajan G: Biventricular function on early echocardiograms in neonatal hypoxic-ischaemic encephalopathy. Acta Paediatr 106: 1085-1090, 2017.
- Spath NB, Mills NL and Cruden NL: Novel cardioprotective and regenerative therapies in acute myocardial infarction: A review of recent and ongoing clinical trials. Future Cardiol 12: 655-672, 2016.
- 27. Nosaka N, Okada A and Tsukahara H: Effects of therapeutic hypothermia for neuroprotection from the viewpoint of redox regulation. Acta Med Okayama 71: 1-9, 2017.

- 28. Kloner RA, Hale SL, Dai W and Shi J: Cardioprotection: Where to from here? Cardiovasc Drugs Ther 31: 53-61, 2017.
- 29. Wang J, Wang Y and Lu L: De-SUMOylation of CCCTC binding factor (CTCF) in hypoxic stress-induced human corneal epithelial cells. J Biol Chem 287: 12469-12479, 2012.
- 30. Chen Z, Siraj S, Liu L and Chen Q: MARCH5-FUNDC1 axis fine-tunes hypoxia-induced-mitophagy. Autophagy 13: 1244-1245, 2017.
- 31. Lee YJ, Bernstock JD, Nagaraja N, Ko B and Hallenbeck JM: Global SUMOylation facilitates the multimodal neuroprotection afforded by quercetin against the deleterious effects of oxygen/glucose deprivation and the restoration of oxygen/glucose. J Neurochem 138: 101-116, 2016.
- 32. Zhou F, Dai A, Jiang Y, Tan X and Zhang X: SENP 1 enhances hypoxia induced proliferation of rat pulmonary artery smooth muscle cells by regulating hypoxia inducible factor 1α. Mol Med Rep 13: 3482-3490, 2016.
- Wang F, Cai F, Shi R, Wei JN and Wu XT: Hypoxia regulates sumoylation pathways in intervertebral disc cells: Implications for hypoxic adaptations. Osteoarthritis Cartilage 24: 1113-1124, 2016.
- 34. Lee YJ, Mou Y, Klimanis D, Bernstock JD and Hallenbeck JM: Global SUMOylation is a molecular mechanism underlying hypothermia-induced ischemic tolerance. Front Cell Neurosci 8: 416, 2014.