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

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**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% CO₂ to simulate therapeutic hypothermic stress, and cell viability and mitochondrial injury were evaluated. The levels of protein SUMOylation, hypoxia-inducible factor (HIF)-1α 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-1α, continuously stimulated downstream VEGF expression. Therapeutic 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-1α 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⁺-K⁺-ATPase and Ca²⁺-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), HIF-1α protein SUMOylation is an important protective mechanism of action of hypothermia in hypoxic cardiomyocytes
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 α and β (10). The transcriptional activity and protein stability of HIF-1α are regulated by intracellular oxygen concentration. Under normoxic conditions, HIF-1α is rapidly degraded by the ubiquitin-proteasome pathway. However, under hypoxic conditions, the stability increases and it enters the nucleus to combine with HIF-1β to form a dimer that regulates hypoxia-inducible gene transcription, including vascular endothelial growth factor (VEGF), glucose transporter 1 (Glut-1), matrix metalloproteinases and multidrug transporter (11-14). Notably, HIF-1α can be modified by SUMO1, which can affect the stability of HIF-1α (15). Nevertheless, the role and regulatory mechanisms of HIF-1α 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-1α 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-1α 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-1α 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 µg/ml penicillin and 50 µg/ml streptomycin sulfate (all from Gibco; Thermo Fisher Scientific, Inc.) at 37˚C with 5% CO₂. 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% N₂ and 15% CO₂. 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-1α can be modified by SUMO1, which can affect the stability of HIF-1α (15). Nevertheless, the role and regulatory mechanisms of HIF-1α 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-1α protein by SUMOylation is involved in the protective mechanism underlying the effects of therapeutic hypothermia on myocardial ischemia.

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Lactate dehydrogenase (LDH) detection. H9C2 cells were seeded into 96-well plates 8x10⁵ 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 (1×10⁶ 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-use-of-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
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% O₂/5% CO₂ 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 ± 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-1α/VEGF pathway. Because HIF-1α has previously been identified as a target...
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-1α and VEGF expression were examined. The protein expression levels of HIF-1α 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-1α can be upregulated by SUMO1. These findings validated the hypothesis that
hypoxia may increase the expression of SUMO1 in cardiomyocytes, increase the protein levels of HIF-1α 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-1α/VEGF pathway and aggravate cardiomyocyte injury following hypoxia.** To examine whether the hypoxia-activated HIF-1α/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-1α 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-1α/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-1α and VEGF protein levels in normoxic cardiomyocytes, but significantly increased HIF-1α 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-1α 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,
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-1α and VEGF (Fig. 4A) and reduce the protective effect of hypothermia (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-1α 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).
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-1α, IκBα, 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 SUMOylation 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 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-1α, 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 ± 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.
the ischemic myocardium or be associated with additional complications.

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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, MXX, CYZ and JJJ performed the experiments, and collected and analyzed data. HQL and XYB drafted the manuscript. 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.

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