Cardiac-targeted PIASy Gene Silencing Mediates deSUMOylation of Caveolin-3 and Prevents Ischemia/reperfusion-induced $\text{Na}_v1.5$ Down-regulation and Ventricular Arrhythmias

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Research

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Abstract

Background: Abnormal myocardial expression and function of $\text{Na}_v1.5$ causes lethal ventricular arrhythmias during myocardial ischemia-reperfusion (I/R). PIASy mediated Caveolin-3 (Cav-3) SUMO modification affects Cav-3 binding to ligand $\text{Na}_v1.5$. PIASy activity is increased after myocardial I/R, whether or not this may be attributable to plasma membrane $\text{Na}_v1.5$ downregulation and ventricular arrhythmias remains unclear.

Methods: Using recombinant adeno-associated virus subtype 9 (AAV9), rat cardiac PIASy was silenced by intraventricular injection of PIASy shRNA. Two weeks later, the hearts were subjected to I/R, and electrocardiography was performed to assess malignant arrhythmias. Tissues from peri-infarct areas of the left ventricle were collected for molecular biological measurement.

Results: We found that PIASy was upregulated by I/R, with increased SUMO2/3 modification of Cav-3, reduced membrane $\text{Na}_v1.5$ density, and increased ventricular arrhythmia frequency. These effects were significantly reversed by PIASy silencing. In addition, PIASy silencing enhanced Cav-3 binding to $\text{Na}_v1.5$ and prevented I/R-induced $\text{Na}_v1.5$ re-localization. Using *in vitro* models of HEK293T cells and isolated adult rat cardiomyocytes exposed to hypoxia/reoxygenation (H/R), this research further confirmed that PIASy promoted Cav-3 modification by SUMO2/3 and $\text{Na}_v1.5$ /Cav-3 dissociation after H/R. Mutation of the SUMO Consensus Sites Lysine in Cav-3 (K38R or K144R) alters the membrane expression levels of $\text{Na}_v1.5$ and Cav-3 before and after H/R in HEK293T cells.

Conclusions: I/R-induced cardiac PIASy activation contributes to Cav-3 SUMOylation by SUMO2/3 and dysregulated $\text{Na}_v1.5$ - related ventricular arrhythmias. Cardiac-targeted PIASy gene silencing mediates deSUMOylation of Cav-3 and prevents I/R-induced $\text{Na}_v1.5$ down-regulation and ventricular arrhythmias in rats, identifying PIASy as a potential therapeutic target for relevant life-threatening arrhythmias in patients with ischemic heart diseases.

Bullet Points

- a. PIASy was upregulated by ischemia/reperfusion (I/R), with increased SUMO2/3 modification of Cav-3, reduced membrane $\text{Na}_v1.5$ density, and increased ventricular arrhythmia frequency.
- b. PIASy silencing ameliorated I/R-induced ventricular arrhythmias.
- c. PIASy silencing enhanced Cav-3 binding to $\text{Na}_v1.5$ and prevented I/R-induced $\text{Na}_v1.5$ re-localization.
- d. PIASy promoted Cav-3 modification by SUMO2/3 and $\text{Na}_v1.5$ /Cav-3 dissociation after I/R.
- e. Mutation of the SUMO Consensus Sites Lysine in Cav-3 alters the membrane expression levels of $\text{Na}_v1.5$ and Cav-3.

Introduction

Cardiac dysrhythmias are common causes of morbidity and mortality in patients with heart diseases. Ion channel alterations play pivotal roles in the development of cardiac arrhythmias. $\text{Na}_v1.5$, encoded by the *SCN5A* gene, is the α -subunit of cardiac Na^+ channel¹ and determines heart excitability and conduction². Dysregulated Na^+ channel expression has been reported in several cardiac pathophysiological conditions such as myocardial infarction, heart failure, and other heart diseases, leading to life-threatening arrhythmias³. Dysfunctional Na^+ channel has also been shown in *SCN5A* gene mutations linked to Brugada syndrome, long QT syndrome (LQTS), cardiac conduction defects and atrial fibrillation⁴. Under normal circumstances, Na^+ channel activity is finely regulated by complex molecular mechanisms. Accumulating evidence indicates that multitudinous protein complexes, including ankyrin-G⁵, caveolin-3 (Cav-3), E3 ubiquitin ligase Nedd4⁶, MOG1⁷, syntrophin⁸ and dystrophin⁹, can interact with $\text{Na}_v1.5$ and contribute to its functional changes¹⁰.

As one of the multiple $\text{Na}_v1.5$ interacting proteins, Cav-3, encoded by the *CAV3* gene, may regulate the expression and function of $\text{Na}_v1.5$ and is a major subtype of caveolins, which include caveolin-1 α , -1 β , -2 α , -2 β , -2 γ and Cav-3¹¹. Recent findings reveal that several cardiac ion channels, such as $\text{Na}_v1.5$, might be integrated into specific macromolecular signaling complexes for precise regulation¹². Cav-3 is specifically expressed in cardiomyocytes and regulates $\text{Na}_v1.5$ in caveolae by inhibiting nNOS-dependent S-nitrosylation of $\text{Na}_v1.5$ ¹³. Cav-3 mutation has been implicated in type 9 long QT syndrome and sudden infant death syndrome (SIDS), with increased late sodium current (I_{NaL})¹⁴. However, the molecular mechanisms by which Cav-3 interacts and regulates $\text{Na}_v1.5$ activity remain largely unknown.

SUMOylation is a reversible post-transcriptional modification of proteins by small ubiquitin like modifier (SUMO) proteins, which dynamically mediate functional changes in SUMO-target proteins¹⁵. The biochemical processes of SUMOylation are mediated subsequently by the E1, E2 and E3 enzymes, leading to altered protein-protein interactions, subcellular localization, or degradation of target proteins¹⁶. It was shown that Cav-3 SUMOylation may affect the expression and desensitization of β -adrenergic receptor ($\beta_2\text{AR}$)¹⁷.

The mammalian protein inhibitor of activated STAT (PIAS) protein represents a SUMO E3 ligase containing four members, including PIAS1, PIAS2, PIAS3, and PIASy¹⁸. As an important SUMO E3 ligase, PIASy is involved in the regulation of several metabolism-related proteins, such as peroxisome proliferator-activated receptor (PPAR), AMPK and SIRT1^{19,20}. Cav-3 is also a target protein of PIASy, both in heterogeneous transfected cells and in cardiomyocytes¹⁷. In a previous study, we found decreased membrane $\text{Na}_v1.5$ density upon cardiac ischemia/reperfusion (I/R), which related to cardiac arrhythmias²¹. Although dysfunctional $\text{Na}_v1.5$ under I/R leads to lethal cardiac arrhythmias, it remains unclear whether activated SUMOylation of Cav-3 (an important interacting protein of $\text{Na}_v1.5$) by PIASy plays a role in altering $\text{Na}_v1.5$ abundance after I/R.

Therefore, in the present study, AAV9-transferred PIASy shRNA were used both in *in vivo* models of myocardial I/R in rats and *in vitro* models of hypoxia/reoxygenation (H/R) in the isolated adult rat cardiomyocytes as well as HEK293 cells transfected with SUMO machinery, to test the hypotheses that enhanced PIASy activity-mediated Cav-3 SUMOylation after myocardial I/R contributes to plasma membrane Nav1.5 downregulation and ventricular arrhythmias.

Methods

Animals and cell line

All animal experiments were performed in strict accordance with the guidelines for the Care and Use of Laboratory Animals formulated by the National Institutes of Health (NIH, Bethesda, MD). The protocols were approved by the Animal Care and Use Committee of Tongji Medical College of Huazhong University of Science and Technology (permission number: SCXK (E) 2016-0057).

Eight to ten week-old male Sprague-Dawley rats, weighing 200 - 300 g (Wuhan University Laboratory Animal Center, Wuhan, China) were used in this study. The human HEK293 cell line (American Type Culture Collection) was cultured in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum and 100 mg•ml⁻¹ sodium pyruvate, in a humidified atmosphere containing 5% CO₂ and 95% air at 37°C. Adult rat cardiomyocytes were isolated by the Langendorff method and cultured as described previously²².

Construction and *in vivo* cardiac transfection of AAV9-ZsGreen-shRNA

AAV9-ZsGreen vectors carrying PIASy-shRNA or scramble-shRNA were commercially synthesized from Shenzheng BioWit Technologies Co., Ltd and its titer were 1.0×10¹²vg/ml. Briefly, three shRNAs targeting PIASy were designed and used to build the respective plasmids. After enzyme digestion and sequencing, the plasmids were transfected into H9c2 cells to pack with AAV9 harboring ZsGreen. The shRNA with the best interfering efficiency was selected based on Q-PCR results. Purification and titer determination of the AAV9 shRNA vector were performed following standard protocols.

Cardiac delivery of the AAV9 vectors *in vivo* was performed by intraventricular injection in 8-week-old male Sprague-Dawley rats according to a previous description²³. The rats were anesthetized with pentobarbital sodium (70 mg/kg, iv.) and fixed in the supine position. An Insulin syringe was inserted through the thoracic wall into the left ventricular chamber, which was confirmed by blood withdrawal. Then, AAV9 suspension (50μL, 1.3×10¹¹ vg) was slowly injected.

Grouping

Eighty rats were randomly assigned to four groups (n=20 per group), including Scramble-shRNA (AAV9 ZsGreen scramble shRNA transfer with sham operation), Scramble-shRNA + I/R (AAV9 ZsGreen scramble shRNA transfer with I/R), PIASy-shRNA (AAV9 ZsGreen PIASy shRNA transfer with sham operation) and PIASy-shRNA + I/R (AAV9 ZsGreen PIASy shRNA transfer with I/R) groups. Rats were subjected to I/R 14 days after intraventricular injection of AAV9 shRNA.

***In vivo* I/R and *in vitro* H/R models**

An *in vivo* myocardial I/R model was established by surgical ligation of the left anterior descending coronary artery (LAD) as described previously²¹. Rats in the Scramble-shRNA and PIASy-shRNA groups underwent the same procedure except for LAD ligation. Two weeks after systemic delivery of AAV9 vectors, the animals were subjected to a 45 min of ischemia followed by 2h reperfusion. Fresh ventricular tissue samples in the peri-infarct region (area of approximately 3 mm surrounding the infarction induced by LAD ligation) were obtained, snap frozen in liquid nitrogen and stored at -80°C.

An *in vitro* simulated I/R model was established by exposing transfected HEK293 cells to hypoxia (serum-free DMEM in an incubator containing a humidified atmosphere with 1% air + 5% CO₂ + 94% N₂) for 3h followed by 2h of reoxygenation (DMEM with serum in 5% CO₂ + 95% air), as well as isolated adult rat cardiomyocytes to hypoxia for 40 min followed by 30 min of reoxygenation (H/R).

Plasmids and transfection

The plasmid pTracer-SV40 containing the WT human Na_v1.5 gene was a kind gift from Dr. Thomas ZIMMER at Friedrich Schiller University Jena. Flag-tagged Cav-3 (Human caveolin 3) was purchased from Origene Technologies. The Flag-hPIASy, HA-SUMO1, HA-SUMO2/3, ubc9, scramble pCMV6-entry plasmids were obtained from Addgene. Cav-3 mutations at the K38 and K149 positions (Flag-tagged) were generated with the QuickChange II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions, and verified by DNA sequencing. Plasmids, including the SUMO machinery (SUMO2/3, SUMO1, ubc9, and PIASy), Cav-3 (Wt, K38R and K149R mutants), and Na_v1.5, were transfected into cells with Attractene from Qiagen, following the manufacturer's instructions. HEK 293 cells transfected with various plasmids were exposed to hypoxia or hypoxia/reoxygenation to detect Cav-3 binding to SUMO2/3 and Na_v1.5, respectively.

Immunofluorescence and histology

Two weeks after transfer of the AAV9 carrying reporter gene (ZsGreen) into the rat myocardium, six-micron cryosections were cut from rat heart, lung and liver tissues, and observed directly for green fluorescence protein expression by fluorescence microscopy (Olympus BX-51 Microscope). Transfected HEK293 cells and isolated adult rat cardiomyocytes exposed to H/R, as well as fresh frozen ventricular

sections from the four animal groups were fixed for 10 minutes in 4% formaldehyde (Beyotime Biotechnology, Germany) and stained with primary antibodies targeting PIASy, Cav-3, Na_v1.5 and SUMO2/3, respectively, followed by incubation with secondary antibodies. Nuclei were stained with 4'6-diamidino-2-phenylindole (DAPI, Beyotime Biotechnology) during the secondary antibody incubation step. Sections were mounted with Gelvatol for microscopy. Fresh ventricular tissues were processed by paraffin embedding, and 5-μm thick sections were stained with hematoxylin and eosin (H&E). An Olympus fluorescence microscope was used for imaging.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from ventricular tissue samples using TRIzol Reagent (Invitrogen) and treated with DNase I to remove genomic DNA. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on a Bio-Rad thermocycler with a SYBR green kit (Invitrogen) following the manufacturer's instructions. The primers used for amplification were: Na_v1.5, forward 5'-CCTTCACTGCCATCTACAC-3' and reverse 5'-GCCTGAAATGACCGATAT-3'; CAVEOLIN-3, forward 5'-GACATTGTGAAGGTGGATTT-3' and reverse 5'-GTAGACAGCAGGCGGTAG-3'; GAPDH, forward 5'-AAGGGCTCATGACCACAGTC-3' and reverse 5'-GGATGCAGGGATGATGTTCT-3' (Wuhan BioBuffer Biotechnology, Co. Ltd., Wuhan, China).

Co-immunoprecipitation and Western blot

The following primary antibodies were used for Western blot and/or co-immunoprecipitation (Co-IP): anti-SUMO1 (ab11672), anti-SUMO2/3 (ab3754), anti-Caveolin-3 (rabbit polyclonal, ab2912), and anti-Sodium Potassium ATPase antibody (ab198366), purchased from Abcam; anti-phosphor-Tyrosine (#9411), anti-phospho-Threonine Antibody (#9381), anti-GFP (#2555), and anti-β-actin (#4970), from Cell Signaling Technology; anti-Caveolin-3 (mouse polyclonal, sc-55518) and anti-PIASy (sc-166706, sc-50348), obtained from Santa Cruz Biotechnology; anti-Na_v1.5 (#ASC-005), from Alomone Labs; anti-myc-tag (No. 66004-1-Ig) and anti-HA-tag (No. 66006-1-Ig), from Proteintech. Horseradish peroxidase (HRP) conjugated anti-mouse IgG (cs7076) and anti-rabbit IgG (cs7074) secondary antibodies were from Cell Signaling Technology; anti-mouse AlexaFluor488 (ab150113) and anti-rabbit AlexaFluor647 (ab150115) secondary antibodies were from Abcam. N-Ethylmaleimide (NEM) was purchased from Sigma-Aldrich.

Forty-eight hours after transfection, HEK293 cells were washed with PBS. Cells and tissue samples (stored at -80°C) were lysed for 30 minutes on ice in RIPA lysis buffer (Beyotime, Shanghai, China) containing 0.1 mM PMSF and a protease inhibitor cocktail (Roche). Equal amounts of protein in cell or tissue lysates were separated by 10% SDS-PAGE and electro-transferred onto PVDF membranes. Then, the membranes were blocked with 5% BSA (bovine serum albumin) for 1 hour at room temperature, and sequentially incubated with respective primary antibodies overnight and HRP-conjugated secondary antibodies (1:3000) for 1 hour. The blots were evaluated by ECL (enhanced chemiluminescence), with β-actin (A2228; 1:5000 dilution; Sigma-Aldrich) as a loading control. Cytosolic and membrane fractions

were prepared with Mem-PER Plus Membrane Protein Extraction Kit (Pierce Protein Biology) according to the manufacturers' instructions.

For immunoprecipitation, 50µg of clarified cell and tissue lysate samples were immunoprecipitated with 1µg of anti-Cav-3 or anti-Na_v1.5 overnight, and then incubated with 15µL protein G/A-agarose (Santa Cruz, CA) for another 4h at 4°C. Agarose beads were sedimented and washed 5 times with cell lysis buffer, and bound proteins were released with 30µl of 2×SDS-loading buffer. The immunoprecipitated proteins were further analyzed by Western blot as described above.

For the detection of SUMOylation, 20 mmol/L NEM was added to the lysis buffer for protein extraction. Importantly, the samples were not boiled for SUMOylation determination. Individual bands were quantified by densitometry with the ImageJ software (version 5, NIH).

Electrocardiography (ECG)

ECG was performed continuously during the experiments. Surface ECG parameters were analyzed under stable baseline conditions at least 5 minutes after anesthesia induction and before jugular vein preparation. P duration, PR interval, QRS duration, and corrected QT interval (QTc) were measured on standard limb lead II. ECG recordings were evaluated with the LabChart software (version 7.3, AD Instruments Pty Ltd, Australia). The durations of ventricular arrhythmias, and episodes of ventricular tachycardia (VT), sustained VT and ventricular fibrillation (VF) within every consecutive 5-min block after ligation were determined. VT is 3 or more consecutive ventricular premature beats; sustained VT is an episode of VT lasting 10 sec or more. We used Curist-Walker scoring systems to grade the severity of arrhythmias occurring in rats under the experimental conditions of myocardial I/R²⁴.

Statistical Analysis

The results were presented as mean ± SEM and analyzed by Student's *t*-test and one-way analysis of variance (one-way ANOVA) followed by the Newman–Keuls *post hoc* test. For non-normally distributed parameters, such as episodes of ventricular arrhythmias, durations of sustained VT and VF, the Kruskal Wallis test was used. For ECG wave changes, two-way ANOVA was employed for analysis. Percent survival was analyzed with the Kaplan-Meier method. The GraphPad Prism 6.0 software package (version 5 for Windows, San Diego, CA) was used for all statistical analyses. Statistical significance was set at $p < 0.05$.

Results

Experimental model of AAV9-mediated PIASy silencing in the rat heart

To achieve direct AAV9-mediated PIASy shRNA transfer to the adult rat heart, scramble shRNA and PIASy shRNA were packed into AAV9 vectors. According to previous reports, AAV9 capsids were directly injected into the left ventricular chamber. After injection into the left ventricle, Evans blue was evenly distributed to the whole ventricular myocardium (right panel in Fig. 1A) compared with the normal heart (left panel in Fig. 1A), which confirmed the efficiency of the injection. Two weeks after intraventricular transfer of the vectors, 6 μ m short-axis sections of the apical and anterior left ventricles as well as lung and liver tissue sections were prepared to assess ZsGreen expression by fluorescence microscopy. As shown in Fig. 1B, ZsGreen was predominantly expressed in the heart, especially in the mid-ventricular region, in both scramble shRNA and PIASy shRNA-treated animals. In contrast, very low ZsGreen expression was found in the liver and the lung. Meanwhile, PIASy shRNA mediated PIASy silencing resulted in significantly decreased mRNA levels ($p < 0.05$) (Fig. 1C) and down-regulated protein expression ($p < 0.05$) (Fig. 1D) compared with the scramble shRNA group. These findings indicated that AAV9 could yield a robust expression of AAV9-mediated PIASy shRNA, preferably in cardiomyocytes, mediating cardiac-specific knockdown of the target protein. To exclude the potential toxic effect of AAV9-capsids to the animals, the survival rate and histological changes were examined. A slightly lower survival rate (16/20 rats) was observed in the PIASy-shRNA group compared with 18/20 rats in the Scramble-shRNA group ($p > 0.05$), 15 days after the administration of AAV9 vectors (Fig. 1E). Hematoxylin-eosin (H&E) stained sections of the anterior left ventricle were prepared. No significant structural alterations, e.g. cardiomyocyte swelling and necrosis, myocardial filament disruption, interstitial inflammatory cell infiltration, and replacement fibrosis, were found in both groups, which implied a limited toxicity of AAV9 capsids on the myocardium (Fig. 1F).

PIASy silencing with AAV9 PIASy shRNA improves I/R-induced Na_v1.5 down-regulation both in the membrane and cytosolic fractions

It has been reported that hypoxia could induce global SUMO modification and PIASy upregulation^{17,25}. Since PIASy is a specific E3 ligase for Cav-3, we hypothesized that PIASy might promote SUMO conjugation to Cav-3 during I/R. As an important protein interacting with Cav-3, Na_v1.5 was further assessed after I/R-induced SUMOylation of Cav-3. We first evaluated the effect of H/R on protein expression levels of PIASy, Na_v1.5, Cav-3 and SUMO2/3 in isolated rat cardiomyocytes.

Immunofluorescence showed enhanced staining of the PIASy protein in cardiomyocytes exposed to 40 min hypoxia followed by 30 min reoxygenation, which was accompanied by markedly diminished Na_v1.5 levels, predominantly in the membrane region (Fig. 2A). Furthermore, declined Cav-3 and increased SUMO2/3 signals were also observed in H/R-treated individual cardiomyocytes compared with normal cells (Fig. 2A). To explore the *in vivo* role of PIASy-mediated Cav-3 SUMOylation in Na_v1.5 expression during I/R, we next silenced PIASy gene expression in the myocardium by AAV9 shRNA vector transfection, 15 days before I/R. The RNA extracted from the myocardium in each group was analyzed

for mRNA levels of PIASy, Na_v1.5 and Cav-3. No significant changes of Na_v1.5 and Cav-3 mRNA expression levels were observed in the Scramble-shRNA and PIASy-shRNA groups, with or without I/R ($p>0.05$). PIASy mRNA levels were significantly higher ($p<0.01$) after I/R in both Scramble-shRNA and PIASy-shRNA groups, corroborating previous findings. Although AAV9 PIASy shRNA actually decreased the levels of PIASy mRNA in both PIASy-shRNA and PIASy-shRNA + I/R groups ($p<0.05$, vs. Scramble-shRNA and Scramble-shRNA + I/R, respectively), PIASy mRNA level increase in the PIASy-shRNA + I/R group indicated an incomplete silencing (Fig. 2B). Further examination was performed by Western blot on lysates from left ventricular peri-infarct tissue samples. As shown in Fig. 2C, the changes of PIASy protein expression were consistent with mRNA data, especially in whole cell and cytoplasmic specimens. Meanwhile, I/R induced a significant decrease of Na_v1.5 protein expression in both Scramble-shRNA and PIASy-shRNA groups, in whole cell, membrane and cytoplasmic fractions ($p<0.05$). However, this decrease was partially reversed by cardiac-targeted interference of PIASy shRNA ($p<0.05$). Though Cav-3 can be modified by PIASy-mediated SUMO conjugation, Cav-3 mRNA and protein expression levels showed no significant changes in this model ($p>0.05$). Since Na_v1.5 itself was not SUMO-modified but Cav-3-regulated, further investigation is required to identify the role of the functional interaction between Cav-3 (particular SUMO-modified Cav-3) and Na_v1.5, but no alteration of Cav-3 amounts, in I/R-induced Na_v1.5 dysfunction.

PIASy-induced Cav-3 SUMOylation by SUMO2/3 affects the interaction between Cav-3 and Na_v1.5 both *in vitro* and *in vivo*

It is well reported that Cav-3 can be sumoylated by SUMO1 or SUMO2/3 in heterologously transfected cells¹⁷. To determine which SUMO molecule is functionally involved in Cav-3 SUMO modification in the rat myocardium, Cav-3 was precipitated from cell lysates using anti-Cav-3 monoclonal antibody, followed by immunoblotting with antibodies specific to SUMO2/3 and SUMO1, respectively. Co-IP results showed distinct SUMO2/3-modified Cav-3 bands around 50 kDa. In contrast, no visible changes of SUMO1 immunoreactive bands were found in the Cav-3 precipitated protein. Interestingly, I/R resulted in increased levels of SUMO2/3-modified Cav-3, which can be partially abrogated by PIASy shRNA interference (Fig. 3A). To further verify the role of PIASy in modulating Cav-3 SUMOylation *in vitro*, cultured HEK 293 cells were transfected with plasmids of the SUMO machinery (SUMO2/3, ubc9, and incremental doses of PIASy), along with Cav-3 and Na_v1.5 plasmids. Co-IP revealed that SUMO2/3-modified Cav-3 increased with PIASy dose in precipitated Cav-3, 48h after plasmid transfection. In addition, hypoxia caused a more pronounced SUMOylation of the Cav-3 protein compared with H/R stimulation in co-transfected HEK cells at a stable dose of PIASy (Fig. 3B). It is known that Cav-3 belongs to a scaffold protein involved in the functions and cellular translocation of several channel proteins such as Na_v1.5 and Kv1.5, and may regulate their functions. To assess whether the molecular interaction between Cav-3 and Na_v1.5 contributes to I/R-induced dysfunction of Na_v1.5, reciprocal Co-IP was performed on lysates from both

ventricular tissue specimens and transfected HEK 293 cells. As expected, overt physical binding between Cav-3 and Na_v1.5 was observed both *in vivo* and *in vitro* (Fig. 3C). In the rat myocardium, I/R resulted in increased SUMO conjugation to Cav-3 as well as enhanced dissociation of Cav-3 from Na_v1.5, which could be partially reversed by PIASy shRNA (Fig. 3D). Immunofluorescence (double staining with Cav-3 and Na_v1.5) in the myocardium further revealed that I/R disturbed the interaction between Cav-3 and Na_v1.5, especially in the intercalated disc and the lateral membrane. Meanwhile, PIASy shRNA restored Na_v1.5 expression in both the intercalated disc and the lateral membrane (Fig. 3E). These results suggested that PIASy-mediated SUMO2/3 modification of Cav-3 could cause abnormal interaction between Cav-3 and Na_v1.5, consequently affecting the localization and abundance of Na_v1.5 during I/R.

Mutation of the SUMO Consensus Sites Lysine in Cav-3 (K38R or K144R) alters the membrane expression levels of Na_v1.5 and Cav-3 both under normal conditions and after H/R

Analysis of the human Cav-3 sequence revealed several sequences with predicted SUMOylation sites of canonical $\Psi K_X D/E$. One of them was centered on Lys-38 and lies between the N-terminus and the caveolin scaffolding domain (CSD). The other one, centered on Lys-144, was located in the C-terminal tail beyond the sixth transmembrane fragment (Fig. 4A). Sequence comparison across several vertebrate species revealed that both sites were evolutionally conserved (Fig. 4A). To further confirm the functional role of sumoylated Cav-3 in the Cav-3/Na_v1.5 interaction, we mutated both lysine residues of Cav-3 to arginine (KR mutation), and assessed membrane and cytoplasmic levels of Na_v1.5 in these SUMOylation-deficient Cav-3 mutants. When the plasmid harboring the K38R mutation was co-transfected with SUMO2/3, ubc9, PIASy and Na_v1.5 into HEK 293 cells, membrane Na_v1.5 protein levels were significantly increased compared with the wild type Cav-3 group under normal conditions. However, only slightly increased Na_v1.5 levels in the membrane were found for the K144R mutant-transfected 293 cells (Fig. 4B). Consistent with Western blot findings, immunofluorescence showed stronger signals of Na_v1.5 (red) in K38R and K144R mutant cells than wild type counterparts (Fig. 4C). We next examined whether such SUMO mutations affect H/R-induced aberrant Na_v1.5/Cav-3 interaction as well as Na_v1.5 translocation by exposing K38R- and K144R-transfected 293T cells to H/R. While H/R induced significantly decreased membrane Na_v1.5 and Cav-3 levels in wild type Cav-3 cells, the K38R mutation only prevented Na_v1.5 loss in the membrane after H/R, with no visible effect on Cav-3 expression. Interestingly, K144R mutation restored both Na_v1.5 and Cav-3 in the membrane after H/R conditioning (Fig. 4D). The alterations of Na_v1.5 and Cav-3 in the cytosolic fraction were mild during H/R, and a modest increase of Na_v1.5 in K144R-transfected cells after H/R was obtained, indicating a functional role for SUMO-modified Cav-3 in its interaction with Na_v1.5 during H/R (Fig. 4E).

PIASy-mediated Cav-3 SUMOylation may be associated with Na_v1.5 phosphorylation

To explore the exact mechanisms of Na_v1.5 dislocation and downregulation by I/R-induced aberrant interaction between sumoylated Cav-3 and Na_v1.5, Na_v1.5 phosphorylation was assessed in rats treated with scramble shRNA and PIASy shRNA, respectively. Co-IP using the precipitated Na_v1.5 from the rat heart revealed that although no phosphorylated protein bands were detected around the Na_v1.5 molecule (~250 kDa), a significant phosphor band was found around 70-90 kDa, presumably from Na_v1.5 fragments (Fig. 5A-B). The phosphorylation of Na_v1.5 fragments was enhanced by I/R in both scramble shRNA and PIASy shRNA groups. After PIASy shRNA induced suppression of Cav-3 SUMO, the increased phosphorylation of Na_v1.5 partially reversed (Fig. 5A-B).

PIASy shRNA improves I/R-induced prolongation of QTc and QRS in rats

Hypoxia-induced Na_v1.5 dysregulation contributes to abnormal cardiac electrical conduction and subsequent fetal arrhythmias. ECG was performed in scramble shRNA and PIASy shRNA treated rats, with or without I/R insult. PIASy silencing caused no detectable alterations in P duration, PR interval, QRS duration and QTc compared with the scramble shRNA group ($p > 0.05$) (Fig. 6A). Furthermore, PIASy shRNA shortened I/R-induced prolongation of QTc and QRS duration ($p < 0.05$, vs. scramble shRNA) (Fig. 6A-B).

PIASy shRNA interfering reduces lethal ventricular arrhythmias in I/R injured rats

Consistent with our hypothesis, PIASy shRNA transfection into the rat heart resulted in reduced I/R-induced fatal arrhythmias, reflected by a modest decrease in the duration of ventricular fibrillation ($p < 0.05$, vs. scramble shRNA) and significantly declined arrhythmia score ($p < 0.01$, vs. scramble shRNA) (Fig. 7B-D). The anti-arrhythmic effect of PIASy silencing was also evidenced by decreased episodes of VT, sustained VT and VF, especially 5 to 10 minutes after ischemia ($p < 0.05$, vs. scramble shRNA) (Fig. 7E-G).

Discussion

The main finding of the present study is that PIASy-mediated Cav-3 SUMOylation by SUMO2/3 could control the magnitude of Cav-3/Na_v1.5 interaction, which affects Na_v1.5 abundance on the cardiomyocyte membrane, subsequently modulating functional cardiac conduction and lethal ventricular arrhythmias. In addition, cardiac-targeted PIASy gene silencing mediates deSUMOylation of Cav-3 and

prevents I/R-induced $\text{Na}_v1.5$ down-regulation and ventricular arrhythmias. Further, mutation of the SUMO Consensus Sites Lysine in Cav-3 (K38R or K144R) alters the membrane expression levels of $\text{Na}_v1.5$ and Cav-3 before and after H/R in HEK293T cells.

The current data showed that PIASy expression was increased both by I/R in the rat heart and H/R in isolated rat cardiomyocytes, and associated with reduced $\text{Na}_v1.5$ membrane density. Elevated PIASy was evidenced not only on the cardiomyocyte membrane but also in the cytoplasm. PIASy is considered to mainly localize near the nucleus, but could also be found in the cytoplasm²⁵. It is well known that as a SUMO E3 ligase, PIASy mediates the SUMOylation of various proteins, further regulating the activity, translocation and autophagy of conjugated proteins²⁵. As one of the major cardiac proteins specifically localized to caveolae, Cav-3 may interact with many cardiac ion channels, including $\text{Na}_v1.5$ and $\text{K}_v1.5$ ¹². Since no SUMO position has been found in $\text{Na}_v1.5$, which has not been reported to be SUMOylated, we hypothesized that SUMO modification of Cav-3 may alter its interaction with $\text{Na}_v1.5$. As expected, our co-IP data revealed that Cav-3 was physically conjugated with SUMO2/3, but not with SUMO1 in myocardial cells from rat left ventricle. Furthermore, *in vitro* assay for SUMO in HEK293 cells co-transfected with Cav-3 and the SUMOylation machinery also confirmed the possibility of SUMO2/3 modified-Cav-3. Interestingly, I/R, H/R and incremental dose of PIASy correspondingly increased the levels of SUMO2/3 modified-Cav-3. These results demonstrated that Cav-3 is the target of covalent SUMO conjugation mediated by PIASy. We also observed substantial binding between Cav-3 and $\text{Na}_v1.5$ *in vivo* and *in vitro*. I/R may lessen the binding between Cav-3 and $\text{Na}_v1.5$. Therefore, the present findings indicated that upregulated PIASy is associated with enhanced Cav-3 SUMOylation by SUMO2/3 and decreased interaction between $\text{Na}_v1.5$ and Cav-3 upon I/R.

To further assess the role of PIASy-mediated Cav-3 SUMOylation in Cav-3/ $\text{Na}_v1.5$ interaction and plasma membrane $\text{Na}_v1.5$ amounts, AAV9-mediated shRNA cardiac transfection was used to interfere with PIASy gene expression in the current study. AAV9 is a safe and useful vector in gene therapy, and can mediate efficient cardiac-targeted interference even by intravenous delivery^{26,27}. Consistent with previous findings²⁷, we noted that intraventricular injection of the AAV9 vector could markedly reduce target gene expression in adult rat heart as evidenced by reduced PIASy mRNA and protein levels of in left ventricular tissue. In the AAV9 PIASy shRNA- group, PIASy protein expression was significantly reduced under normal conditions and after I/R, accompanied by decreased SUMO2/3-modified Cav-3 and Cav-3/ $\text{Na}_v1.5$ dissociation, with increased $\text{Na}_v1.5$ after I/R. In addition to PIASy knockdown by AAV9 shRNA interference *in vivo* for reducing SUMOylation, the SUMOylation-deficient mutants of Cav-3 (Cav-3 K38R and K144R) were used in HEK293 cells heterologously transfected with $\text{Na}_v1.5$ and the SUMOylation machinery. Cav-3 has conserved SUMO consensus sites, suggesting that they are potential targets for SUMOylation. Among these, Lys-38 was shown to be the preferred SUMOylation site through poly-SUMO3 chains, with PIASy necessary for its SUMOylation. Mutations of Lys-38 and Lys-144, respectively, partially reversed H/R-induced decrease of $\text{Na}_v1.5$, especially on the cardiomyocyte membrane, while increasing $\text{Na}_v1.5$ protein amounts in the membrane compared with wild type Cav-3 under normal conditions. These

findings suggested that mutating the SUMO sites of Cav-3 affects Na_v1.5/Cav-3 interaction and Na_v1.5 translocation, increases Cav-3 SUMOylation by PIASy upon I/R, contributes to Cav-3 dissociation from Na_v1.5, resulting in altered plasma membrane Na_v1.5 density.

As an important caveolin, Cav-3 plays a pivotal role in cardioprotection by interacting with several ion channels and signaling molecules through its scaffold domains²⁸. Our previous study showed that Cav-3 is essential for propofol induced cardiac protection against I/R injury²⁹. Accumulating evidence indicates that Cav-3 may regulate Na_v1.5 activity by binding to the latter³⁰. The physical binding of Cav-3 and Na_v1.5 was also supported by Co-IP data in both rat heart tissue samples and heterologously transfected cells in this study. The functional role of Cav-3 in Na_v1.5 activity is further confirmed by the evidence that mutations in specific sites of Cav3 are associated with dysregulated Na_v1.5 activity and severe arrhythmias such as Long-QT syndrome³¹. How Cav-3 modification affects its interaction with Na_v1.5 remains elusive. Since protein SUMOylation emerged as an important protein modification strategy for functional regulation in diverse cellular processes, including protein localization, stability and stress responses, more E3 SUMO ligases such as the PIAS family of proteins, NSE2 and EGR2, have been identified³². As shown above, PIASy was important in terms of its ability to regulate Cav-3 SUMOylation in response to I/R stimulation. Similar roles for PIASy have been reported in regulating specific molecules, including VHL³³, NEMO³⁴, p53³⁵ and Tip60³⁶, in response to different cellular environments.

Although we found that sumoylated Cav-3 altered the amounts of Na_v1.5 in cardiomyocytes, the underlying mechanism remains unclear. Decreased Na_v1.5 expression induced by I/R was evidenced not only in the plasma membrane fraction, but also in the cytosolic fraction. These results suggested an involvement of both localization and degradation of Na_v1.5. Recent findings show that Na_v1.5 phosphorylation in specific sites regulates its intracellular translocation and activity³⁷. Meanwhile, PKA-dependent phosphorylation on S526 or S529 in ID 1-2 promotes Na_v1.5 trafficking to the plasma membrane³⁸, while PKC-dependent phosphorylation in S1503 decreases its membrane levels³⁹. Increased Na_v1.5 phosphorylation after I/R was found in this study, and was linked to reduced membrane Na_v1.5 levels. Silencing of PIASy *in vivo* blunted I/R-induced Na_v1.5 phosphorylation and restored membrane Na_v1.5 abundance. These data indicated that activating Na_v1.5 phosphorylation following its dissociation with SUMO-regulated Cav-3 may play a role in its trafficking away from the membrane. Na_v1.5 is ubiquitinated by UBR3/6 and subsequently degraded via the ubiquitin-protease system⁴⁰. Although we observed that enhanced Cav-3 SUMO was associated with decreased Na_v1.5 binding to Cav-3 as well as Na_v1.5 downregulation, no active ubiquitination of Na_v1.5 was detected in I/R-treated rat heart (data not shown). Whether other posttranscriptional events, such as dysfunctional trafficking and endocytosis, may contribute to decreased membrane Na_v1.5 expression during I/R remains undefined. Notably, the exact mechanisms by which PIASy promotes Na_v1.5 dissociation from Cav-3 and facilitates Na_v1.5 translocation during I/R require further investigation.

As the main sodium channel subunit for developing cardiomyocyte action potential, $\text{Na}_v1.5$ plays a pivotal role in cardiac conduction. Abnormal expression and distribution of $\text{Na}_v1.5$ may cause deficient Na^+ current and conduction, which are arrhythmogenic⁴¹. Reduced $\text{Na}_v1.5$ protein expression after I/R, primarily on the cardiomyocyte membrane, results in prolonged QRS complex due to deficient conduction and lethal ventricular arrhythmias, as shown in our previous study²¹. In agreement with other reports, the present data showed that PIASy silencing prevents I/R-induced loss of membrane $\text{Na}_v1.5$, QRS duration increase, arrhythmia score elevation, VF duration, and episodes of sustained VT and VF. It should be noted that enhanced late sodium current (I_{NaL}) induced by I/R is another mechanism underlying fatal arrhythmias. I_{NaL} is generated by delayed inactivated or reopened cardiac sodium channels, and leads to prolonged QTc⁴². Inhibition of I_{NaL} by ranolazine reverses I/R-induced prolongation of QTc and effectively ameliorates ventricular arrhythmias⁴³. In this study, PIASy shRNA silencing showed similar effects as ranolazine, indicating a possibility that PIASy may affect both I_{NaL} and $\text{Na}_v1.5$ expression. Further investigation is needed to determine how PIASy regulates I_{NaL} and $\text{Na}_v1.5$ function in pathological environments such as I/R, heart failure and et al.

Conclusions

In summary, the present study revealed a novel link between PIASy related Cav-3 SUMOylation and $\text{Na}_v1.5$ translocation and stability, and found a new mechanism underlying I/R-induced $\text{Na}_v1.5$ downregulation and fetal ventricular arrhythmias (Fig. 8). Silencing of PIASy by shRNA prevents Cav-3 dissociation from $\text{Na}_v1.5$ as well as membrane $\text{Na}_v1.5$ level reduction after I/R, identifying PIASy as a potential therapeutic target for relevant life-threatening arrhythmias in patients with ischemic heart diseases.

Abbreviations

$\text{Nav}1.5$: a member of the voltage-dependent family of Na channels; PIASy: the mammalian protein inhibitor of activated STAT-y; Cav-3: Caveolin-3; I_{NaL} : late sodium current; SUMO: Small ubiquitin-related modifier; AAV9: adeno-associated virus subtype 9; VT: ventricular tachycardia; VF: ventricular fibrillation; H/R: hypoxia / reoxygenation; I/R: ischemia/reperfusion.

Declarations

Ethics approval and consent to participate

All animal experiments were approved by the Institutional Animal Use and Care Committee at Tongji Medical College, Huazhong University of Science & Technology (permission number: SCXK (E) 2016-0057).

Consent for publication

Not applicable.

Availability of data and materials

The data and materials used to support the findings of this study are available from the corresponding authors upon request.

Competing interests

The authors declare no conflict of interest.

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Authors' contributions

CH, XW, TW and WM conceived and designed the experiments; CH, XW, JL, LH, CX, JW, TY and AZ performed the experiments; CH, XW, JL, LH, CX, JW, TY, AZ, TW and WM analyzed the data; CH, XW, SY, SY, HX, ZX, TW and WM contributed to discussion and wrote the paper. All authors read and approved the final paper.

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Figures

Figure 1

Efficiency and safety of AAV9-mediated PIASy silencing in the rat heart. (A) Distribution of Evens blue (2% v/w, 50µl) in the rat heart at 10 minutes after intraventricular injection. (B) Green fluorescent protein (ZsGreen) expression mediated by the AAV9 vector in the liver, lung and heart samples 15 days after intraventricular injection of AAV9-ZsGreen-shRNA (50µL, 1.3×10^{11} vg). (C) (D) AAV9 PIASy shRNA-mediated PIASy silencing in the rat heart. RNA and protein samples from left ventricle specimens were obtained for RT-PCR and Western blot analysis, respectively, 15 days after administration of AAV9-ZsGreen-shRNA (n=5). GAPDH was used as an internal control. Values are mean $\pm$ SEM, * p < 0.05 vs. Scramble-shRNA, determined by Student's *t*-test. (E) Effect of AAV9 PIASy shRNA on the survival of rats during the 15 days following treatment with the AAV9 vector (n=20, p > 0.05, Kaplan-Meier analysis). (F) Histological analysis after hematoxylin-eosin staining of rat tissue samples from left ventricle free wall 15 days after intraventricular injection of AAV9-ZsGreen-shRNA (200×).

Figure 2

Effects of ischemia/reperfusion (I/R) and PIASy shRNA on PIASy, Na_v1.5 and Cav-3 expression levels. (A) Hypoxia/reoxygenation (H/R)-induced changes of PIASy, Na_v1.5, Cav-3 and SUMO2/3 levels in isolated cardiomyocytes from left ventricle of adult SD rats. PIASy (red) and SUMO2/3 (green) were detected at high levels by immunofluorescence, while Na_v1.5 (green) and Cav-3 (red) showed decreased localization especially on the membrane (400×). (B) Ischemia/reperfusion (I/R) and PIASy shRNA mediated mRNA level alteration of PIASy, Na_v1.5 and Cav-3 in rat left ventricle tissue samples, assessed by RT-PCR. (C) Protein expression levels of PIASy, Na_v1.5 and Cav-3 in whole cardiomyocytes, and in the membrane and cytoplasmic fractions after I/R in the scramble shRNA and PIASy shRNA groups. The left panel represents western blot bands, while the right panel represents densitometric analysis. Values are mean ± SEM, n=10. * p < 0.05, ** p < 0.01 vs. Scramble-shRNA, # p < 0.05, ## p < 0.01 vs. PIASy-shRNA + I/R, one-way ANOVA.

Figure 3

Effects of PIASy silencing on Cav-3 binding to SUMO2/3 and Na_v1.5, and Na_v1.5 distribution. (A) SUMO modified Cav-3 in the rat heart. Rat myocardial lysates were collected and subjected to immunoprecipitation (IP) with anti-Cav-3 antibody, followed by immunoblotting with anti-SUMO2/3 (left panel) or anti-SUMO1 antibody (right panel) after I/R injury. (B) Cav-3 binding to SUMO2/3 in transfected cells. HEK293 cells were co-transfected with Cav-3, Na_v1.5, ubc9, SUMO2/3 and increasing amounts of PIASy (left panel) or identical amounts of PIASy with hypoxia or H/R (right panel). (C) Physical binding of Cav-3 and Na_v1.5 in the rat heart, and in Cav-3 and SUMO machinery co-transfected HEK293 cells. Normal left ventricle and HEK293 cells after 48h transfection were obtained for immunoprecipitation with anti-Cav-3 antibody followed by Western blot with anti-Na_v1.5 antibody. (D) Effect of PIASy shRNA on binding between Cav-3 and Na_v1.5 by Co-IP in the I/R rat heart. (E) Cav-3 and Na_v1.5 co-localization in the rat myocardium. Immunofluorescent staining of the peri-infarct zone of rat left ventricle showing the localizations and expression levels of Na_v1.5 (red) and Cav-3 (green), mainly on the lateral membrane (arrows) and intercalated disc (arrowheads) of cardiomyocytes. Co-localization was shown in the merged image, along with the cell nuclei counterstained by 4',6-diamidino-2-phenylindole (DAPI). Scale bar=40μm.

Figure 4

Effects of Cav-3 mutations at SUMO consensus sites on Na_v1.5 abundance in transfected HEK293 cells. (A) Schematic diagram of Cav-3 SUMO consensus sites. Cav-3 has a SUMO consensus motif (ΨKxD/E) near the caveolin scaffold domain (CSD) at the N-terminus. This SUMO consensus site (K38) and the

other SUMO site (K144) at the C-terminus are highly conserved in different species. (B) (C) Changes of Na_v1.5 abundance in transfected HEK293 cells between the wild type and mutants. HEK293 cells were co-transfected with the SUMO machinery, including HA-SUMO2/3, ubc9 and Flag-hPIASy, Na_v1.5 and Flag-tagged Cav-3 (wild-type, K38R mutant or K144R mutant). Membrane proteins extracted with a commercial kit were assessed by Western blot, 48h after transfection. Immunofluorescent staining was completed with anti-Na_v1.5 antibody and DAPI (red, Na_v1.5; blue, nucleus, 400×). (D) (E) Effects of Cav-3 mutations on Na_v1.5 abundance after H/R. HEK293 cells transfected with the SUMO machinery, Na_v1.5 and Cav-3 (wild-type, K38R mutant or K144R mutant) exposed to 3h hypoxia followed by 2h reoxygenation. Membrane and cytosolic proteins were prepared for immunoblot with anti-Na_v1.5 and anti-flag (Cav-3) antibodies, respectively. Na⁺/K⁺ ATPase and β-actin were used as internal controls for membrane and cytosolic proteins, respectively.

Figure 5

Effect of PIASy silencing on Na_v1.5 phosphorylation in I/R-model rats. I/R rats' myocardial lysates were collected and subjected to immunoprecipitation with anti-Na_v1.5 antibody, followed by immunoblot with anti-phospho-tyrosine and anti-phospho-threonine antibodies, respectively.

Figure 6

Effects of PIASy gene silencing on P duration, PR interval, QRS duration and QTc during I/R. (A) Changes of P duration, PR interval, QRS duration and QTc during ischemia and reperfusion. (B) Representative changes of QRS wave and QT interval (upper panel) in electrocardiography (ECG) and statistical analyses of QRS duration and QTc (bottom panel) at 20 min after ischemia and 1h after reperfusion. QTc, corrected QT interval; isch, ischemia; rep, reperfusion. Values are mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ vs. Scramble-shRNA, # $p < 0.05$ vs. ## $p < 0.01$ vs. PIASy-shRNA + I/R, n=10, two-way ANOVA.

Figure 7

AVV9-mediated PIASy gene silencing decreases lethal arrhythmias during myocardial I/R. (A) Schematic diagram of VT and VF during I/R. Duration of sustained VT (B) and VF (C) after I/R. (D) Arrhythmia score in I/R-injured rats with PIASy shRNA or scramble shRNA treatment. Episodes of VT (E), sustained VT (F) and VF (G) within every consecutive 5-min block after ligation of left anterior descending coronary artery, respectively. VT, ventricular tachycardia, consecutive ventricular premature beats ≥ 3 ; sustained VT, an episode of VT ≥ 10 sec; VF, ventricular fibrillation. Values are mean $\pm$ SEM. ** $p < 0.01$ vs. Scramble-shRNA, # $p < 0.05$ vs. ## $p < 0.01$ vs. PIASy-shRNA + I/R, n=10. Kruskal-Wallis test in B, C, E-G; one-way ANOVA in D.

Figure 8

Schematic of proposed signaling involved PIASy-mediated Cav-3 SUMOylation effects on reperfusion arrhythmias. PIASy-mediated Cav-3 SUMOylation by SUMO2/3 controls the magnitude of Cav-3/Na_v1.5 interaction, which affects Na_v1.5 abundance on the cardiomyocyte membrane, subsequently modulating lethal reperfusion arrhythmias.