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S-nitrosylation of TRIM72 at cysteine 144 is critical for protection against oxidation-induced protein degradation and cell death

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Abstract

Oxidative stress and membrane damage following myocardial ischemia/reperfusion injury are important contributors to cardiomyocyte death and the loss of myocardial function. Our previous study identified cysteine 144 (C144) of tripartite motif-containing protein 72 (TRIM72) as a potential site for S-nitrosylation (SNO). TRIM72 is a cardioprotective membrane repair protein that can be both activated and targeted for degradation by different oxidative modifications. Consistent with the potential regulation of TRIM72 by various oxidative modifications, we found that SNO levels increased at C144 of TRIM72 with ischemic preconditioning. Therefore, to investigate the role of C144 in the regulation of TRIM72 function, we mutated C144 of TRIM72 to a serine residue (TRIM72_{C144S}), and expressed either TRIM72_{WT} or TRIM72_{C144S} in HEK-293 cells, which lack endogenous TRIM72, in order to examine the effect of this mutation on the functional stability of TRIM72 and on cell survival. We hypothesized that SNO of TRIM72 stabilizes the protein, thus allowing for membrane repair and enhanced cell survival. Upon treatment with hydrogen peroxide (H₂O₂), we found that TRIM72_{WT} levels were decreased, but not TRIM72_{C144S} and this correlated with increased H₂O₂-induced cell death in TRIM72_{WT} cells. Additionally, we found that treatment with the cardioprotective S-nitrosylating agent S-nitrosoglutathione (GSNO), was able to preserve TRIM72_{WT} protein levels and enhance TRIM72_{WT}-mediated cell survival, but had no effect on TRIM72_{C144S} levels. Consistent with our hypothesis, GSNO was also found to increase SNO levels and inhibit H₂O₂-induced irreversible oxidation for TRIM72_{WT} without affecting TRIM72_{C144S}. In further support of our hypothesis, GSNO blocked the ischemia/reperfusion-induced decrease in TRIM72 levels and reduced infarct size in a Langendorff-perfused heart model. The results of these studies have important implications for cardioprotection and suggest that SNO of TRIM72 at C144 prevents the

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DISCLOSURES

None.

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oxidation-induced degradation of TRIM72 following oxidative insult, therefore enhancing cardiomyocyte survival.

Keywords

tripartite motif-containing protein 72; mitsugumin 53; S-nitrosylation; oxidation; cardioprotection; cell death

1. INTRODUCTION

Repair of damaged cellular membranes is an essential process for preserving cardiomyocyte viability following oxidative injury, as occurs with ischemia-reperfusion (I/R) where membrane rupture has been shown to play a key role in cell death [1, 2]. Tripartite motif-containing protein 72 (TRIM72) or mitsugumin-53 (MG53), is a membrane repair protein that is only expressed in cardiac and skeletal muscle [3]. Membrane damage leads to the activation of TRIM72, and this has been shown to occur during I/R injury [4, 5] or with exposure to oxidants, including hydrogen peroxide (H₂O₂) [6]. Following activation, TRIM72 translocates to the site of membrane damage and acts as a scaffold to facilitate membrane repair [3, 7, 8]. TRIM72 activation is mediated, in part, via oxidation and disulfide bridge formation at cysteine 242 [3, 9], which leads to the oligomerization of TRIM72 and the stabilization of the repair complex [3, 7, 8]. We have also shown that TRIM72 can be S-nitrosylated (SNO) at cysteine 144 (C144) [10], but the functional consequences of this reversible modification have not been determined.

Myocardial I/R injury is accompanied by an increase in damaging oxidants that can lead to the loss or inhibition of many important cardiac proteins, such as SERCA2a [11] and myosin light chain [12, 13]. Recent studies have shown that TRIM72 is an essential component of both ischemic preconditioning (IPC) and ischemic post-conditioning (PostC) [5, 6], the former of which we have shown to protect the heart, in part, by increasing protein SNO and inhibiting detrimental cysteine oxidation [10, 14–17]. I/R injury (and/or oxidative stress) has been shown to decrease TRIM72 protein levels and this correlates with cell death [6]. Consistent with the down-regulation of TRIM72, the genetic ablation of TRIM72 has also been shown to increase cellular damage and increase the release of lactate dehydrogenase following myocardial ischemic injury [6]. Interestingly, cardioprotective interventions such as IPC [5, 6], have been shown to block the I/R-induced decrease in TRIM72 levels, suggesting a redox-dependent mechanism for TRIM72 regulation.

In the current study, we tested the hypothesis that irreversible oxidation of TRIM72 at C144 leads to protein degradation, while SNO at C144 protects against oxidation, thereby stabilizing TRIM72 and enhancing cell survival. Consistent with this hypothesis, we demonstrate that SNO-TRIM72 levels are enhanced with IPC in the myocardium, and that SNO provides protection against oxidant-induced TRIM72 degradation, thus preserving cell viability. We previously identified C144 as a site of SNO for TRIM72 [10], and mutation of this site to a serine residue (TRIM72_{C144S}) prevented oxidation, resulting in the preservation of TRIM72 protein levels and the enhancement of cell survival following oxidative insult. Additionally, we found that pre-treatment of TRIM72 with the cardioprotective nitrosylating agent S-nitrosoglutathione (GSNO) increased SNO of TRIM72_{WT}, but not TRIM72_{C144S}, and also preserved TRIM72_{WT} levels and cell viability following oxidative insult. In further support of our hypothesis, GSNO protected against the I/R-induced degradation of TRIM72 and reduced infarct size in a Langendorff-perfused heart model. Therefore, we propose that cardioprotection induces SNO of TRIM72 at C144, and as a result, TRIM72 protein levels are stabilized and cardiomyocyte survival is enhanced following oxidative injury.

2. MATERIALS AND METHODS

2.1 Perfusion protocols, infarct size determination, and whole heart homogenate preparation

Male and female C57BL/6J mice (12–15 weeks) were obtained from Jackson Laboratories (Bar Harbor, ME). Hearts were Langendorff-perfused in the dark as previously described [10, 14–17], and randomly subjected to a perfusion protocol. For TRIM72 SNO experiments, hearts were subjected to control perfusion (60 minutes perfusion) or an IPC protocol (20 minutes equilibration, 4 cycles of 5 minutes ischemia/5 minutes reperfusion). For TRIM72 degradation experiments, hearts were subjected to control perfusion (20 minutes perfusion), an I/R protocol (20 minutes equilibration, 30 minutes ischemia, 180 minutes reperfusion), or a GSNO-I/R protocol (20 minutes equilibration, 30 minutes GSNO [100 μ mol/L; Sigma], 30 minutes ischemia, 180 minutes reperfusion). Hearts were snap frozen in liquid nitrogen immediately following the treatment protocol. Hearts were then powdered on liquid nitrogen with a mortar and pestle, and homogenized as previously described [10, 14–17]. Protein concentration was determined using the Bradford protein assay. Infarct size was assessed in a separate set of hearts as previously described [15, 17]. At the conclusion of each perfusion protocol, hearts were perfused/incubated with 1% (w/v) 2,3,5-triphenyltetrazolium chloride, followed by fixation in 10% (w/v) formaldehyde. Infarct size was expressed as a percentage of the total cross sectional area of the ventricles. This investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication No. 85–23, revised 2011) and was approved by the Institutional Laboratory Animal Care and Use Committee.

2.2 S-nitrosylation-resin assisted capture (SNO-RAC)

SNO-RAC was used to examine protein SNO as previously described [10, 14] with the exception that an LTQ Orbitrap Elite mass spectrometer (Thermo, San Jose, CA) was used for a targeted MS/MS search for the MQLQEACMR peptide of TRIM72 (this peptide corresponds to the cysteine 144 SNO site of TRIM72). The mass spectrometer automatic gain target was set to 5×10^4 for SIM scans and 1×10^4 for MS/MS scans, both in the Orbitrap analyzer. Precursor ions were detected at a resolution of 120,000 FWHM and the SIM window was defined between 40 amu over the mass range of 535–575 m/z, and the precursor retention time set to a window of ± 5 minutes. Each RAW file was then analyzed using Xcalibur software (Thermo) by focusing on the precursor with a 5 ppm window for extracted ion chromatograms, and the precursor area was used for quantitative measurements.

2.3 Biotin iodoacetamide (BIAM) labeling and enrichment

SNO or oxidation of TRIM72 were assessed separately in whole heart homogenates and cell lysates using a modified biotin switch assay as previously described [10, 14, 18, 19]. Cell lysates were prepared in radio-immunoprecipitation assay buffer (RIPA, Thermo) containing protease and phosphatase inhibitor cocktail (Roche, Indianapolis, IN). For SNO labeling, 500 μ g of protein was added to HEN buffer (250 mmol/L HEPES, 1 mmol/L EDTA, 0.1 mol/L Neocuproine) and incubated with 2% sodium dodecyl sulfate (SDS) and 25 mmol/L N-ethylmaleimide (NEM, Sigma) for 20 minutes at 50°C; NEM was removed via acetone precipitation. Protein was resuspended in HEN with 1% SDS and incubated with 1 mmol/L ascorbate and 2 mmol/L freshly-prepared biotin polyethyleneoxide iodoacetamide (Sigma) for 2 hours at room temperature. For oxidized protein labeling, 500 μ g of protein was added to HEN Buffer and incubated with 2% SDS, 1 mmol/L ascorbate and 25 mmol/L NEM for 20 minutes at 50°C; NEM was removed via acetone precipitation. Protein was resuspended in HEN buffer with 1% SDS and treated with 100 μ mol/L tris(2-carboxyethyl)phosphine hydrochloride (TCEP, Thermo) for 45 minutes at room temperature; TCEP was removed via

acetone precipitation. Protein was resuspended in HEN with 1% SDS and incubated with 2 mmol/L freshly-prepared biotin polyethyleneoxide iodoacetamide for two hours at room temperature. BIAM-labeled proteins were pulled down with EZview Red Streptavidin Affinity Gel (Sigma) overnight at 4°C. Captured proteins were washed with HEN Buffer and then eluted in NuPage sample loading buffer (Life Technologies, Carlsbad, CA) containing 10 mol/L urea.

2.4 Western Blotting

Samples were run on a 4–12% gel and transferred onto a nitrocellulose membrane. Membranes were blocked with 5% milk in Tris-buffered saline with 0.1% Tween-20 and subsequently incubated with primary antibodies against TRIM72 (1:1000; Acris, San Diego, CA), GAPDH (1:5000; Santa Cruz, Santa Cruz, CA), or β -actin (1:1000, Abcam, Cambridge, MA) overnight at 4°C. Membranes were then probed with the corresponding secondary antibodies for 1 hour and visualized by electrogenerated chemiluminescence (GE Healthcare, Piscataway, NJ).

2.5 Transfection of TRIM72

Green fluorescent protein (GFP)-tagged human TRIM72 was purchased from Origene (Rockville, MD), and mutation of C144 to a serine residue was performed by Mutagenex Inc. (Piscataway, NJ). TRIM72_{WT} or TRIM72_{C144S} or a GFP control vector (pMax-GFP, Lonza, Walkersville, MD) was transfected into HEK-293 cells using FugeneHD (Roche) according to the manufacturer's instruction. Cells were incubated with the vector/transfection mixture for 48 hours in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum and 1% penicillin/streptomycin (Life Technologies). Culture media was replaced prior to experiments.

2.6 TRIM72 membrane translocation

Glass-bottom cell culture dishes (MatTek, Ashland, MA) were prepared with sterile 0.1% gelatin in phosphate buffered saline at least 30 minutes prior to cell plating. HEK-293 cells were transfected with GFP-tagged TRIM72_{WT} or TRIM72_{C144S} as described, and maintained in culture medium during imaging. Cells were treated with 0.001% saponin [6], followed by 200 μ mol/L H₂O₂, and imaged for 5 minutes at 60 $\times$ magnification using a Zeiss LSM510 Confocal Microscope.

2.7 TRIM72 degradation

HEK-293 cells expressing either TRIM72_{WT} or TRIM72_{C144S} were treated with 200 μ mol/L H₂O₂ for 3 or 6 hours and then harvested in RIPA buffer containing complete protease and phosphatase inhibitor cocktail. In some experiments, the proteasomal inhibitor MG132 (10 μ mol/L; Sigma) was co-administered at the time of H₂O₂ addition or GSNO (1 mmol/L) was administered one hour prior to the addition of H₂O₂ in fresh media.

2.8 Live-dead Assay

Glass-bottom cell culture dishes were prepared with sterile 0.1% gelatin in phosphate buffered saline at least 30 minutes prior to cell plating. HEK-293 cells were transfected with GFP-tagged TRIM72_{WT}, TRIM72_{C144S} or a GFP control vector as described above. Cells were treated with medium alone or medium containing 1 mmol/L GSNO for one hour, followed by treatment with medium alone or medium containing 200 μ mol/L H₂O₂ for 24 hours. Cells were then treated with 2 μ mol/L ethidium homodimer-1 (Life Technologies) for 30 minutes. GFP and ethidium staining were assessed using a Nikon E800 Wide-field Fluorescence Microscope at 20 $\times$ magnification and cell death was assessed as the

percentage of ethidium-stained cells to total cells. Cell number was counted using the ImageJ Analyze Particles function.

2.9 Statistical analysis

All data are presented as mean $\pm$ SEM. Statistical significance ($P < 0.05$) was determined between groups using an ANOVA followed by a Bonferroni post-hoc test for multiple groups or a Student's *t*-test for two groups.

3. RESULTS

3.1 Identification of the SNO site for TRIM72

We previously found TRIM72 to be endogenously *S*-nitrosylated at cysteine 144 (C144) in whole heart homogenates using SNO-RAC [10], and following treatment with the cardioprotective agent GSNO, we observed an increase in SNO at the same C144 residue compared to baseline. We also detected SNO-TRIM72 at additional cysteine residues beyond C144 (including C242), but C144 showed the most robust change in SNO upon GSNO treatment, potentially indicating a role for SNO in the regulation of TRIM72 function. In the current study, we confirmed our findings using a similar approach and a representative peptide spectrum for the C144 SNO site of TRIM72 is shown in Figure 1. Interestingly, C144 is highly conserved among mammalian species, and this may signify an important role in the regulation of TRIM72.

3.2 SNO-TRIM72 increases with cardioprotection

TRIM72 has been shown to play a critical role in both IPC and PostC in the myocardium [5, 6]. In previous studies, we have demonstrated that IPC can increase SNO protein levels in the myocardium [14–17], but we did not detect SNO-TRIM72. Therefore, we utilized a targeted approach to specifically examine SNO-TRIM72 in the setting of cardioprotection. Langendorff-perfused mouse hearts were subjected to IPC and SNO-TRIM72 levels were assessed using SNO-RAC in tandem with mass spectrometry, as we performed in our previous study [14]. However, in the current study, we used a targeted mass spectrometry search to examine the specific peptide that contains the C144 SNO site of TRIM72 (MQLQEACMR). As shown in Figure 2a, IPC-treated hearts showed a 7-fold increase in SNO at the C144 residue of TRIM72 compared to control hearts. We confirmed this IPC-induced increase in SNO-TRIM72 levels using a modified biotin switch [10, 18, 19], and although the fold-change in SNO was not as high compared to our mass spectrometry-based method, we still observed a significant increase in SNO-TRIM72 levels with IPC compared to control (Figure 2b). The difference in fold-change between the targeted mass spectrometry-based approach and the biotin switch technique likely stems from methodological sensitivity. Our targeted mass spectrometry-based approach allowed us to focus exclusively on SNO-TRIM72 at the C144 residue, therefore enhancing the signal-to-noise ratio by increasing sensitivity and reducing background noise. Conversely, the biotin switch technique detects all SNO sites for a given protein, thus making it difficult to resolve changes at specific cysteine residues. We next performed a series of experiments to examine the potential biological ramifications SNO of TRIM72 at the C144 site.

3.3 Mutation of C144 does not block the recruitment of TRIM72 to sites of membrane damage

TRIM72 initiates membrane repair by translocation to sites of membrane damage and acting as a repair scaffold, but the role of SNO at C144 in regulating TRIM72 function has not been investigated. Therefore, we mutated C144 of TRIM72 to a serine residue (TRIM72_{C144S}) to prevent SNO at this site. In order to determine if the C144 mutation

altered the ability of TRIM72 to translocate to the plasma membrane, we first examined the membrane recruitment of TRIM72_{WT} and TRIM72_{C144S} in response to oxidative damage. To avoid interference with endogenous TRIM72, we performed these studies in non-muscle cells (HEK-293 cells) that lack TRIM72. Membrane localization was assessed in HEK-293 cells expressing either TRIM72_{WT} or TRIM72_{C144S} via membrane permeabilization with saponin (0.001%) [6], followed by oxidation with H₂O₂ (200 μM). TRIM72_{WT} and TRIM72_{C144S} were both found to be distributed diffusely throughout the cytosol under baseline conditions (Figure 3). However, following membrane disruption with saponin/H₂O₂, TRIM72_{WT} and TRIM72_{C144S} were both recruited to the membrane.

3.4 SNO protects C144 of TRIM72 from irreversible oxidation

TRIM72_{WT} or TRIM72_{C144S} was expressed in HEK-293 cells, and the oxidation state of TRIM72 was assessed using a modified biotin switch [10, 18, 19]. Following treatment of cells with H₂O₂ (200 μM), increased oxidation was detected for TRIM72_{WT}, but not for TRIM72_{C144S}. In addition, pre-treatment with GSNO prevented the H₂O₂-induced increase in TRIM72 oxidation in cells expressing TRIM72_{WT}, but was without effect in cells expressing TRIM72_{C144S} (Figure 4A). These results indicate that C144 of TRIM72 is a critical target of irreversible oxidation, and by occupying the C144 residue, SNO can effectively protect against H₂O₂-induced oxidation.

We and others have shown that SNO can protect proteins from the damaging effects of irreversible oxidation by shielding critical cysteine residues [14, 20, 21]. To further demonstrate the reciprocal relationship between SNO and oxidation, TRIM72_{WT} and TRIM72_{C144S} were assessed for SNO levels following treatment with GSNO, H₂O₂ or the combination of both. GSNO treatment increased SNO-TRIM72 levels in cells expressing TRIM72_{WT}, and these GSNO-induced SNO-TRIM72 levels were maintained even after treatment with H₂O₂ (Figure 4B). However, as expected, treatment of TRIM72_{C144S} expressing cells with either GSNO or H₂O₂ did not alter SNO-TRIM72 levels (Figure 4B), thus providing further evidence that SNO can protect C144 from the damaging effects of H₂O₂-induced oxidation. These results also implicate C144 as a primary target for SNO and an important residue in the regulation of TRIM72 oxidation.

3.5 Irreversible oxidation induces the degradation of TRIM72

Decreased TRIM72 levels following hypoxia or treatment with H₂O₂ have been demonstrated in previous studies, and this is known to correlate with cell survival [6]. Therefore, we tested the hypothesis that oxidation of TRIM72 may lead to the degradation of TRIM72 and SNO, by reducing oxidation, may block the degradation of TRIM72. We first examined the effect of oxidation on TRIM72 protein levels. HEK-293 cells expressing either TRIM72_{WT} or TRIM72_{C144S} were treated with H₂O₂ (200 μM) for 3 or 6 hours and TRIM72 protein levels were quantified. After 6 hours, TRIM72_{WT} protein levels in cells treated with H₂O₂ were significantly lower than that of untreated controls (Figure 5A), and this effect could be prevented by co-administration of the proteasomal inhibitor MG132 (Figure 5B). In contrast, TRIM72_{C144S} protein levels were not changed by H₂O₂ treatment or MG132. These results provide a potential mechanism for the reduction in TRIM72 degradation that has been reported with IPC [6]. These findings also correlate well with our previous study, which shows that IPC blocks the I/R-induced increase in TRIM72 oxidation at C144 (Control: 1.0±0.7 vs. I/R: 1.7±0.9 vs. IPC-I/R: 1.2±0.8) [14].

Since we established that SNO can protect C144 of TRIM72 from irreversible oxidation, we next examined whether TRIM72_{WT} degradation is altered by SNO. HEK-293 cells expressing either TRIM72_{WT} or TRIM72_{C144S} were treated with GSNO prior to the administration of H₂O₂. Interestingly, pre-treatment with GSNO prevented the H₂O₂-

induced degradation of TRIM72_{WT}, but was without effect on TRIM72_{C144S} protein levels (Figure 5B). This is consistent with our hypothesis that the irreversible oxidation of TRIM72 at C144 leads to proteasomal degradation.

3.6 SNO of TRIM72 protects from oxidation-induced cell death

TRIM72 is an important contributor to cell survival following oxidative insult [3, 4, 6], so we next examined the role of SNO-TRIM72 in cell death. HEK-293 cells expressing either TRIM72_{WT} or TRIM72_{C144S} were assessed for cell viability following 3 or 24 hours of H₂O₂ treatment. After 3 hours, a time point at which TRIM72_{WT} was not significantly degraded, cells expressing either TRIM72_{WT} or TRIM72_{C144S} showed no significant loss of viability following H₂O₂ treatment, and this result is consistent with a protective effect for TRIM72. Conversely, cells expressing a GFP control vector (there is no TRIM72 in HEK-293 cells since it is a muscle-specific protein) showed approximately 20% cell death, and this effect could not be rescued by GSNO pre-treatment (Figure 6A). However, after 24 hours of H₂O₂ treatment, cells expressing TRIM72_{WT} showed a 40% decrease in viability compared to a minimal loss of viability in non-treated cells expressing TRIM72_{WT} (Figure 6B). Cells expressing a GFP control vector alone also showed a 40% decrease in viability after 24 hours. However, cells expressing TRIM72_{C144S} showed significantly less H₂O₂-induced cell death compared to TRIM72_{WT} or GFP-expressing cells. Consistent with the preservation of TRIM72_{WT} levels following GSNO pre-treatment, cells expressing TRIM72_{WT}, but not GFP alone, were rescued from H₂O₂-induced cell death and showed viability levels similar to those of TRIM72_{C144S} expressing cells.

3.7 TRIM72 degradation and cell death in the heart

Myocardial I/R injury has been shown to reduce TRIM72 expression levels [6]. Therefore, we further tested our hypothesis that SNO protects against I/R-induced TRIM72 degradation and cell death using Langendorff-perfused mouse hearts, which endogenously express TRIM72 in cardiomyocytes, and GSNO as a preconditioning agent. In support of this model, we have previously shown that treatment of Langendorff-perfused mouse hearts with GSNO protects against I/R-induced injury by lessening post-ischemic contractile dysfunction and reducing infarct size [15]. We have also previously shown that GSNO treatment leads to the S-nitrosylation of TRIM72 at C144 in the myocardium [10]. Further, we have demonstrated that ischemia-reperfusion injury leads to an increase in TRIM72 oxidation at C144 in Langendorff-perfused mouse hearts, and that this increase in oxidation is blocked by IPC (Control: 1.0±0.7 vs. I/R: 1.7±0.9 vs. IPC-I/R: 1.2±0.8) [14]. After 30 minutes of ischemia followed by three hours of reperfusion, TRIM72 expression levels were reduced by more than 80% compared to perfusion control hearts (Figure 7A). However, hearts perfused with GSNO prior to ischemia showed significantly higher TRIM72 expression levels following I/R injury compared to hearts subjected to I/R injury alone. Consistent with the preservation of TRIM72 levels, GSNO also provided a significant infarct-sparing effect compared to I/R alone (Figure 7B). These results suggest that SNO protects TRIM72 against oxidation-induced degradation in the myocardium, thus preserving cardiomyocyte viability.

4. DISCUSSION

Membrane repair is vital for maintaining cardiomyocyte viability in response to an oxidative insult, such as I/R injury. TRIM72 plays a critical role in the membrane repair process in cardiomyocytes [3–6], but oxidative stress has been shown to decrease TRIM72 protein levels and this correlates negatively with cell survival [6]. Protein SNO, however, is known to protect proteins from degradation, in part, by reducing the irreversible oxidation of critical cysteine residues [14, 20, 21], which can lead to ubiquitin-independent protein degradation by the 20S proteasome [22]. We previously quantified the level of irreversible oxidation in

hearts following I/R injury and determined that increased levels of SNO elicited via IPC prevented I/R-induced oxidation at the same cysteine residue [14]. Interestingly, IPC and PostC have been shown to increase protein SNO [10, 14–17] and preserve TRIM72 levels [6], and we have shown that TRIM72 can be *S*-nitrosylated at C144 [10]. The combination of these findings led to the hypothesis that SNO protects TRIM72 from oxidation-induced degradation and subsequent cell death by shielding C144 from irreversible oxidation (see Figure 8). In accordance with this hypothesis, we found herein that SNO or mutation of the C144 residue protected TRIM72 from H₂O₂-induced oxidation and degradation, and protected against H₂O₂-induced cell death. Further, we found that GSNO blocked I/R-induced TRIM72 degradation and reduced infarct size in a Langendorff-perfused heart model. These results suggest that oxidation and degradation of TRIM72, as occurs during I/R injury [6, 14], may lead to a decrease in cardiomyocyte viability.

4.1 Redox-dependent TRIM72 stability

In the current study, we confirmed that TRIM72 can be *S*-nitrosylated at C144 at baseline (Figures 1 & 2), and SNO is increased with IPC (Figure 2). C144 of TRIM72 is highly conserved among mammalian species, and this highlights the potential importance of this residue in regulating TRIM72. Mutation of C144 did not block the translocation of TRIM72 to the plasma membrane (Figure 3), but the stability of this mutant protein was enhanced in response to oxidative stress in HEK-293 cells. We found that the GSNO-induced SNO of TRIM72 prevented H₂O₂-induced oxidation, while the TRIM72 mutant could not be *S*-nitrosylated or oxidized by GSNO or H₂O₂ treatment, respectively (Figure 4). Interestingly, the C242 residue of TRIM72 has been shown to play a key role in the activation and dimerization of TRIM72 through the formation of a disulfide bridge [3, 9], and SNO is thought to play a role in disulfide bridge formation [23]. This likely explains the maintenance of basal SNO in cells expressing the TRIM72 C144S mutant. Further, we showed that H₂O₂-induced oxidative stress led to the degradation of TRIM72, but this effect could be blocked with GSNO pre-treatment (to induce SNO) or via mutation of C144 (Figure 5). We also showed a direct correlation between oxidant-induced TRIM72 degradation and decreased cell viability in response to H₂O₂ challenge (Figure 6). However, the effect of oxidant stress on cell viability could be alleviated with GSNO pre-treatment or via mutation of C144. In addition, GSNO-induced protein SNO was effective at reducing TRIM72 degradation and infarct size following I/R injury in a Langendorff-perfused heart model (Figure 7). These results indicate that C144 of TRIM72 plays a critical role in the redox-dependent regulation of TRIM72 stability and cellular viability (Figure 8).

4.2 SNO-TRIM72 and cardioprotection

Increased protein SNO, as occurs with IPC, GSNO perfusion, or the loss of GSNO-reductase, has been shown to correlate with myocardial protection against I/R injury [10, 14–17, 24]. Additionally, TRIM72 has been identified as an essential component of cardioprotection, and IPC and PostC have both been shown to reduce the I/R injury-induced degradation of TRIM72 [5, 6], which has been shown to lead to a reduction in cellular viability. Consistent with these studies, we showed that IPC increased the levels of SNO-TRIM72 in the myocardium (Figure 2), thus providing a potential mechanism for the IPC/PostC-induced preservation of TRIM72 levels. In addition, we demonstrated that the cardioprotective agent GSNO [15], which we have also shown to increase SNO-TRIM72 levels in the myocardium [10], blocked the I/R-induced degradation of TRIM72 in Langendorff-perfused hearts which endogenously express TRIM72 (Figure 7). Although TRIM72 levels were decreased by ~50% in GSNO treated hearts, this level is significantly higher than non-GSNO treated hearts and the level of TRIM72 is critical for cellular survival, as Cao *et al.* recently demonstrated that TRIM72 overexpression protects neonatal cardiomyocytes from hypoxia and oxidative stress-induced cell death, while the loss of

TRIM72 decreases cellular viability [6]. Thus, the preservation of TRIM72 levels likely contributes to the infarct-sparing effects of GSNO (Figure 7). Further, we demonstrated that I/R injury increased the levels of irreversible oxidation of TRIM72 at C144, but this increase was blocked by IPC, likely via SNO [14]. Conversely, oxidation of TRIM72 at C242 was increased with I/R injury, regardless of whether hearts were preconditioned, suggesting disulfide bridge formation indicative of TRIM72 dimerization/activation. These results suggest that SNO-mediated cardioprotection is modulated, in part, by the preservation of TRIM72 levels and the maintenance of cardiomyocyte viability.

4.3 Conclusions

TRIM72 is a membrane repair protein that plays an essential role in protecting the heart against I/R injury. In the current study, we found that IPC increased SNO-TRIM72 levels in the myocardium, and mutation of C144 of TRIM72 was able to prevent oxidant-induced TRIM72 degradation and the loss of cell viability. In addition, this protection could be mimicked by treatment with the *S*-nitrosylating agent GSNO, which enhanced SNO-TRIM72 levels and prevented irreversible oxidation elicited by oxidant challenge. We also showed that GSNO could block I/R-induced TRIM72 degradation and reduce infarct size in a perfused heart model. The role of TRIM72 C144 in cardioprotection has not been explored previously, but our results suggest that the SNO of TRIM72 at C144, as occurs with IPC, protects against oxidant-induced cell death and this mechanism may be critically important in the setting of cardioprotection.

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ABBREVIATIONS

BIAM	Biotin iodoacetamide
DMEM	Dulbecco's modified eagle medium
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
GSNO	<i>S</i> -nitrosoglutathione
HEK-293	<i>Homo sapiens</i> embryonic kidney
H₂O₂	Hydrogen peroxide
IPC	Ischemic preconditioning
I/R	Ischemia-reperfusion
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
MG53	Misugumin 53
MS	Mass spectrometry
PostC	Ischemic post-conditioning
RIPA	Radio-immunoprecipitation assay buffer
SERCA2a	Sarcoplasmic reticulum Ca ²⁺ -ATPase 2a

SNO	S-nitrosylation
SNO-RAC	S-nitrosylation-resin assisted capture
TRIM72	Tripartite motif-containing protein 72

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HIGHLIGHTS

- TRIM72 is SNO at C144 with myocardial ischemic preconditioning.
- Oxidation of TRIM72 resulted in protein loss via proteosomal degradation.
- Degradation of TRIM72 correlated with increased oxidant-induced cell death.
- TRIM72 C144 SNO or mutation blocked oxidation-induced degradation and cell death.
- SNO reduced I/R-dependent TRIM72 degradation and infarct size in a perfused heart.

Figure 1. MS/MS spectra for the C144 SNO site of TRIM72

Representative MS/MS spectra showing fragmentation of the MQLQEACMR peptide. The peptide sequence above the representative spectrum shows theoretical *b* ion identifications (red, N terminal fragments) and theoretical *y* ion identifications (blue, C terminal fragments). Peaks in the spectrum that are marked red correspond to matched *b* ions and peaks that are marked blue correspond to matched *y* ions. The number paired with each ion identification (i.e., *b*2, *y*4, etc.) indicates the number of amino acids present on N terminal fragments for *b* ions and C terminal fragments for *y* ions.

Figure 2. Cardioprotection results in SNO of TRIM72

SNO-TRIM72 levels were assessed in whole heart homogenates using (A) SNO-RAC and (B) a modified biotin switch assay (+/- IPC). (A) Intensity of SNO-TRIM72 at C144 as determined by a targeted mass spectrometry search for the MQLQEACMR peptide. (B) Representative western blot, together with the densitometry of SNO-TRIM72 normalized to total TRIM72 input. The upper blot shows SNO-TRIM72 pulled-down by BIAM labeling, the center blot shows total TRIM72, the lower blot shows GAPDH (* $p < 0.05$; $n=3$).

Figure 3. TRIM72_{WT} and TRIM72_{C144S} translocate to the plasma membrane in the presence of cell damage and oxidant stress

HEK293 cells expressing GFP-tagged TRIM72_{WT} or TRIM_{C144S} were treated with saponin (0.001%) to induce membrane damage and H₂O₂ (200 μmol/L). Cells were imaged for 5 minutes at 60× magnification using a Zeiss LSM510 Confocal Microscope.

Figure 4. Cysteine 144 of TRIM72 is a target of irreversible oxidation

A) Irreversible oxidation of TRIM72 was assessed using a modified biotin switch assay (+/- 60 minute pre-treatment with GSNO [1 mmol/L] followed by +/- 30 minute treatment with H₂O₂ [200 μmol/L]). A representative western blot is shown together with the densitometry of oxidized TRIM72 normalized to total TRIM72 input. The upper blot shows oxidized TRIM72 pulled-down by BIAM labeling, the lower blot shows total TRIM72 (*p< 0.05; n=3). B) SNO-TRIM72 was assessed using a modified biotin switch assay (+/- 60 minute pre-treatment with GSNO [1 mmol/L] followed by +/- 30 minute treatment with H₂O₂ [200 μmol/L]). A representative western blot is shown together with the densitometry of SNO-TRIM72 normalized to total TRIM72 input. The upper blot shows SNO-TRIM72 pulled-down by BIAM labeling, the lower blot shows total TRIM72 (*p< 0.05; n=3).

Figure 5. Oxidant stress promotes the degradation of TRIM72_{WT}

A) HEK-293 cells were transfected with plasmids encoding for either TRIM72_{WT} or TRIM72_{C144S} prior to treatment with H₂O₂ (200 μmol/L) for 3 or 6 hours. A representative western blot is shown together with the densitometry of TRIM72 normalized to GAPDH. The upper blot shows total TRIM72, the lower blot shows GAPDH (*p< 0.05; n=4). B) HEK-293 cells expressing TRIM72_{WT} or TRIM72_{C144S} were pre-treated with GSNO (1 mmol/L) or treated with MG132 (10 μmol/L) concurrently with 200 μmol/L H₂O₂ and assessed for TRIM72 protein levels. A representative western blot is shown together with the densitometry of TRIM72 normalized to GAPDH. The upper blot shows total TRIM72, the lower blot shows GAPDH; H₂O₂ treatment is indicated by hashed bars (*p< 0.05 H₂O₂ vs. non-H₂O₂ condition control; n=3).

Figure 6. Mutation or SNO of C144 inhibits oxidant-induced cell death

HEK-293 cells expressing either GFP-tagged TRIM72_{WT} or TRIM72_{C144S}, or GFP alone were treated for (A) 3 hours or (B) 24 hours with H₂O₂ (200 μ mol/L) with or without GSNO pre-treatment (1 mmol/L, 1 hour). Cell viability was assayed by fluorescent imaging of ethidium bromide staining normalized to cell number (*p < 0.05; n=3).

Figure 7. SNO protects against I/R-induced TRIM72 degradation and cell death in the myocardium

Langendorff-perfused mouse hearts were subjected to I/R injury with or without GSNO pre-treatment (100 μ mol/L, 30 minutes). A) A representative western blot is shown, together with the densitometry of TRIM72 normalized to β -actin. The upper blot shows TRIM72, the lower blot shows β -actin (* p < 0.05 vs. control, GSNO+I/R; ** p < 0.05 vs. control, I/R; n =3). B) Infarct size, measured after two hours of reperfusion via 2,3,5-triphenyltetrazolium chloride staining (* p < 0.05 vs. I/R; n =3).

Figure 8. SNO-induced protection of TRIM72

(LEFT PANEL) SNO of cysteine 144 blocks the cysteine residue from further oxidation, thus preventing protein degradation and enhancing cardiomyocyte survival. (CENTER PANEL) Similarly, mutation of cysteine 144 of TRIM72 prevents oxidation from occurring at this site and mimics the blocked SNO state. (RIGHT PANEL) In the absence of SNO or mutation at cysteine 144, treatment with H_2O_2 induces the irreversible oxidation of TRIM72, which results in TRIM72 degradation and the subsequent loss of cardiomyocyte viability.