Thioredoxin uses a GSH-independent route to de-glutathionylate endothelial nitric oxide synthase and protect against myocardial infarction

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Running Title: Thioredoxin protects coronary endothelial dysfunction

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ABSTRACT

Reversible glutathionylation plays a critical role in protecting protein function in conditions of oxidative stress generally, and for endothelial nitric oxide synthase (eNOS) specifically. Glutathione-dependent glutaredoxin-mediated deglutathionylation of eNOS has been shown to confer protection in a model of heart damage termed ischemia-reperfusion injury, motivating further study of eNOS deglutathionylation in general. In this report we present evidence for an alternative mechanism of deglutathionylation. In this pathway, thioredoxin (Trx), a small cellular redox protein, is shown to rescue eNOS from glutathionylation during ischemia-reperfusion in a GSH-independent manner. By comparing mice with global overexpression of Trx and mice with cardiomyocyte-specific overexpression of Trx, we demonstrate that vascular Trx-mediated deglutathionylation of eNOS protects against ischemia-reperfusion-mediated myocardial infarction. Trx deficiency in endothelial cells promoted eNOS glutathionylation and reduced its enzymatic activity, whereas increased levels of Trx led to deglutathionylated eNOS. Thioredoxin-mediated deglutathionylation of eNOS in the coronary artery in vivo protected against reperfusion injury, even in the presence of normal levels of GSH. We further show that Trx directly interacts with eNOS, and confirmed that Cys691 and Cys910 are the glutathionylated sites, as mutation of these cysteines partially rescued the decrease in eNOS activity whereas mutation of a distal site, Cys384, did not. Collectively, this study shows for the first time that Trx is a potent deglutathionylating protein in vivo and in vitro that can deglutathionylate proteins in the presence of high levels of GSSG in conditions of oxidative stress.

INTRODUCTION

Occlusion of coronary artery due to an atherosclerotic plaque interrupts the flow of...
blood to the cardiac tissue, depriving it of nutrients and oxygen, resulting in the progressive death of affected tissue termed as ischemia. When the occlusion is removed either by surgical or other interventions, ensuring the reflow of blood to the affected heart tissue, the process of reperfusion of ischemic tissue is established. Paradoxically, this reperfusion causes more damage to the affected heart tissue, which is known as reperfusion injury (1). The luminal surface of coronary artery and their branches are lined with endothelial cells which are adversely impacted by ischemia-reperfusion resulting in loss or decreased production vascular relaxing factors such as nitric oxide (NO). This dysfunction of the endothelium is known as endothelial dysfunction. Endothelial dysfunction is a key mechanism in the pathogenesis of myocardial infarction (MI) due to ischemia/reperfusion (I/R) of the heart (2,3). Several studies have provided unequivocal evidence that increased production of vascular superoxide anion (O$_2^-$) is a major cause of endothelial dysfunction (2-4). The sources of O$_2^-$ in endothelial cells (ECs) include the mitochondrial electron transport chain, NADPH oxidases (Noxs), activation of xanthine oxidase (XO), and dysfunctional eNOS (1,5,6). Recent studies have shown that pretreatment with NO donors or drugs that increase NO synthesis prior to ischemia protect the myocardium against I/R injury (7-9) supporting a major role of eNOS dysfunction in the pathogenesis of myocardial injury. Although both eNOS and neuronal NOS (nNOS) are present in cardiomyocytes, endothelial cells within the heart express 4 times higher level of eNOS compared to myocytes (10). Further, 60% of total non-myocyte cell population of heart is composed of endothelial cells (11), suggesting a crucial role of endothelial eNOS in the regulation of myocyte function. Consistent with this notion, NO in the heart affects the onset of ventricular relaxation that allows for a precise optimization of pump function (12), demonstrating the availability of endothelial NO is critical for the heart function. Additionally, oxidative stress in I/R results in a dysfunctional eNOS that produce O$_2^-$ by transferring electrons to molecular oxygen instead of L-arginine, resulting in uncoupling of eNOS-L-arginine pathway of production of NO. This uncoupling of eNOS further increases oxidative load and accentuates vascular dysfunction that sets the stage for various cardiovascular diseases such as atherosclerosis, hypertension or myocardial infarction (13-15). It remains unclear whether maintaining the normal oxidation-reduction status (redox) of ECs during reperfusion of occluded coronary artery in vivo would prevent eNOS dysfunction, and consequent protection against ischemia-reperfusion mediated myocardial infarction in vivo.

Besides uncoupling, eNOS is inactivated by conjugation of oxidized glutathione (GSSG) to cysteine moieties, referred to as “glutathionylation” in the conditions of oxidative stress. Recently, it has been shown that increased concentration of GSSG due to the inhibition of glutathione reductase (GR) causes S-glutathionylation of eNOS at cysteine 689 and 908 residues of the reductase domain using cell culture studies (16,17). Further, S-glutathionylated eNOS has been shown to unouple eNOS leading to the production of O$_2^-$. eNOS S-glutathionylation is reversed by glutaredoxin (Grx1), a cytosolic oxidoreductase. Grx1 is an enzyme that catalyzes the transfer of electrons from glutathione (GSH) to glutathionylated proteins (PrS-SG). Utilization of GSH by Grx1 generates GSSG that is converted back to GSH via GR using NADPH (18). Grx1 efficiently reduces protein-mixed-disulfide bonds by
deglutathionylation in the presence of reduced glutathione (GSH) (18-20). It has been shown that the efficiency of eNOS deglutathionylation by Grx1 is primarily influenced by the level of GSH (20).

In addition to GSH-Grx1 system there is ubiquitous presence of another powerful Trx redox system that is involved in the regulation of several important cellular functions. Trx is a 12-kDa small cytosolic redox protein that is primarily involved as an electron donor for ribonucleotide reductase (RNR), a rate-limiting enzyme in DNA replication (21). Trx also regenerates oxidatively inactivated proteins with reducing equivalents supplied by NADPH via thioredoxin reductase (TrxR) (22-24). Although potentially important, the role of Trx in deglutathionylation of mammalian proteins remains unknown. Since, glutathionylation of eNOS in hypoxia-reoxygenation is an underlying mechanism of endothelial dysfunction (25), we hypothesized that high levels of Trx would deglutathionylate eNOS in I/R due to its disulfide reductase activity. In this report we show that I/R of left anterior descending coronary artery (LAD) induced significant glutathionylation of eNOS concomitant with increase in area-at-risk (AAR) in the heart. In contrast, coronary arteries from Trx-Tg mice with high levels of Trx failed to show significant glutathionylation of eNOS in I/R due to its disulfide reductase activity. In this report we show that I/R of left anterior descending coronary artery (LAD) induced significant glutathionylation of eNOS concomitant with increase in area-at-risk (AAR) in the heart. In contrast, coronary arteries from Trx-Tg mice with high levels of Trx failed to show significant glutathionylation of eNOS in I/R due to its disulfide reductase activity. In this report we show that I/R of left anterior descending coronary artery (LAD) induced significant glutathionylation of eNOS concomitant with increase in area-at-risk (AAR) in the heart. In contrast, coronary arteries from Trx-Tg mice with high levels of Trx failed to show significant glutathionylation of eNOS in I/R due to its disulfide reductase activity.
Trx-Tg or αMHC-Trx-Tg are similar, yet αMHC-Trx-Tg mice showed significant MI due to I/R, demonstrating a significant role cardiac endothelial Trx in protection against I/R injury, since other non-myocyte cells such as fibroblasts or inflammatory cells constitute a very low percentage of cells in the heart and these cells do not express eNOS. We also found increased apoptosis in the heart sections in NT or αMHC-Trx littermates, but the TUNEL positive nuclei were significantly lower in the Trx-Tg heart sections (Fig 1H and I). These data show that high levels of functional hTrx expressed in ECs in the heart could be significant in protection against I/R-related MI. Taken together our data show that high level of endothelial Trx in the heart is critical for the protection of heart against I/R-mediated MI.

High levels of Trx maintains coronary endothelial function during I/R:
Since endothelial dysfunction due to I/R is a major contributing factor to cardiomyocyte death and resultant MI (26), we analyzed the effect of high levels of Trx in coronary endothelial dysfunction in I/R. As shown in Fig 2A, I/R significantly blunted endothelium-dependent acetylcholine (Ach)-mediated relaxations in left coronary arteries (LCAs) derived from NT mice that underwent I/R compared to sham-operated LCAs. Endothelium-dependent ACh-induced relaxation was preserved in LCAs from Trx-Tg mice after I/R (Fig 2B). Since NO is the major vasodilator we determined the effect of Trx on eNOS function in I/R. As shown in Fig 2C, basal NO inhibition by L-NAME did not result in an enhanced contraction in LCAs derived from NT mice that underwent I/R. This is in contrast to LCAs derived from Trx-Tg I/R mice, which showed a significant increase in basal tone after the addition of L-NAME, indicating increased basal NO release (Fig. 2D). To determine if vascular Trx is specifically required for improved endothelial function in I/R, LCA from αMHC-Trx-Tg mice was subjected to I/R. As shown in Fig 2E, LCA from these mice failed to show protection against endothelial dysfunction in I/R, demonstrating that vascular Trx is specifically required for endothelial function in I/R.

Hypoxia-Reoxygenation (H/R) causes S-glutathionylation and decreased activity of eNOS:
Recent studies have demonstrated that H/R impairs eNOS function resulting in decreased levels of NO release in bovine aortic endothelial cells (BAEC) (25). Since eNOS is inactivated via S-glutathionylation (16), we examined whether NO production in human coronary artery endothelial cells (HCAEC) via eNOS is impaired in H/R due to S-glutathionylation. Exposure of HCAECs to H/R caused S-glutathionylation of eNOS in a sustained manner as detected by immunoblotting following immunoprecipitation of total cellular lysate with eNOS antibody (Fig. 3A). Treatment with 2-mercaptoethanol released the GSH from eNOS-SG indicating that S-glutathionylation of eNOS was indeed induced due to H/R (Fig. 3B). We also confirmed the glutathionylation of eNOS in HCAEC using immunofluorescence that shows colocalization of eNOS and PrS-SG (Fig 3C). To determine whether the enzymatic activity of eNOS is compromised following exposure of HCAECs to H/R, we measured the eNOS activity in HCAEC exposed to H/R. As shown in Fig. 3D & E, eNOS enzymatic activity was decreased in a time-dependent manner, and after 8h of H/R eNOS activity was reduced to less than 1%. Taken together, these data establish that HCAEC in H/R fail to maintain adequate NO production due to S-glutathionylation of eNOS that diminished its enzymatic activity.
Depletion of Trx in HCAECs causes eNOS S-glutathionylation and reduces its activity:
We have previously shown that Trx undergoes significant oxidation in hypoxia resulting in its inactivity in cancer cells (27). Therefore, we speculated that unavailability of functional Trx might promote glutathionylation. To test this hypothesis we depleted Trx in HCAEC and determined its effect on eNOS glutathionylation and activity. As shown in Fig 4A, Trx was effectively downregulated due to siRNA treatment of HCAECs. This depletion of endogenous Trx promoted significant increase in eNOS glutathionylation (Fig 4B and C). We further observed that down regulation of Trx in HCAEC resulted in differential distribution of eNOS (Fig 4C). Therefore, we sought to determine whether Trx depletion promoted the translocation of glutathionylated eNOS to a specific organelle within the cell. Since eNOS distribution pattern in Trx-depleted cells coincided with lysosome distribution, and lysosomal protein degradation is a major mechanism of elimination of dysfunctional proteins, we determined whether eNOS undergoes lysosomal degradation under Trx-deficient conditions. As shown in Fig 4D, in Trx depleted cells exposed to H/R eNOS was localized within lysosomal-associated membrane protein 1 (LAMP1) vesicle. Since lysosomal degradation of eNOS could result in the loss of specific epitope reactive to the antibody and hence, decreased eNOS fluorescence, we treated cells with lysosomal proton pump inhibitor lansoprazole and determined the localization of eNOS. As shown in Fig 4E, there was not much difference in the eNOS intensity in lansoprazole treated cells, suggesting that the eNOS may remain in the lysosome for a prolonged time without loss of reacting epitope. Thus, lysosomal localization of glutathionylated eNOS suggests that eNOS may be undergoing lysosomal degradation in response to H/R. Further, eNOS glutathionylation due to Trx depletion decreased its enzymatic activity (Fig. 4F). Additionally, treatment with L-NNA , an eNOS inhibitor, abolished the production of [3H]-Citruline indicating the specificity of the reaction for eNOS activity (Fig 4G). These data show for the first time that Trx is a critical regulator of glutathionylation even in the presence of an intact GSH-Grx system. Further, these results indicate that Trx is required to prevent glutathionylation and maintain eNOS function.

Overexpression of Trx protects against eNOS S-glutathionylation in HCAEC:
Since depletion of Trx induces glutathionylation of eNOS we speculated that high levels of Trx would protect against H/R-induced glutathionylation. HCAECs were transduced with adenovirus to overexpress Trx (Fig. 5A) and exposed to H/R. As shown in Fig. 5B, H/R induced eNOS glutathionylation in Ad-LacZ expressing cells, but not in HCAECs overexpressing Trx. Additionally, immunofluorescence staining showed that the co-localization of eNOS and PrS-SG was increased after H/R in LacZ expressing cells, but not in Trx overexpressed cells as detected by staining for PrS-SG (Fig. 5C). To further confirm the glutathionylation of eNOS we implemented the proximity ligation assay (PLA) (28). As shown in Fig 5D, exposure of HCAEC to H/R induced increased PLA signal for eNOS and PrS-SG staining suggesting significant increase in eNOS glutathionylation. However, when PLA was performed in cells with increased expression of Trx (Ad-Trx infection) the PLA signal was significantly decreased, suggesting either protection against S-glutathionylation or deglutathionylation of eNOS by Trx in H/R (Fig 5D and E).
Overexpression of Trx restores eNOS activity and NO release:
We analyzed the effect of Trx on eNOS enzymatic activity and NO release. Trx overexpression protected against H/R-induced loss in eNOS enzymatic activity (Fig 6A). In addition, overexpression of Trx restored eNOS ability to catalyze conversion of L-arginine to L-citrulline (Fig 6B). Specificity of the reaction is shown by treatment of cells with NOS inhibitor, L-NNA that completely prevented the conversion of L-arginine to citruline. We further analyzed the effect of Trx on NO release using the NO sensitive probe DAF-FM. As shown in Fig. 6C, H/R decreased ACh-induced NO release, which was restored by overexpression of Trx in response to 10 \( \mu \text{M} \) Ach. Collectively, these data establish that high levels of Trx prevent eNOS glutathionylation and consequently maintain eNOS function in H/R. Since it is well established that the Grx1-GSH system is the predominant deglutathionylating system in cells (29), we next determined whether high levels of Trx could protect eNOS function in the presence of increased levels of GSSG.

Trx prevents glutathionylation of eNOS in presence of high levels of GSSG:
Inhibition of GR affects the redox balance of cells and causes glutathionylation of eNOS due to increased intracellular concentration of GSSG (16). We determined whether Trx overexpression would protect eNOS from S-glutathionylation in the presence of increased accumulation of GSSG due to inhibition of GR by 1,3-Bis (2-chloroethyl)-1-nitrosourea (BCNU). As shown in Fig 7A, treatment of cells with BCNU increased S-glutathionylation of eNOS, but overexpression of Trx protected eNOS from S-glutathionylation. We further tested whether depletion of GR by RNAi would promote eNOS glutathionylation. As shown in Fig 7B, GR was effectively downregulated by GR siRNA and this decrease in GR promoted eNOS glutathionylation. However, eNOS glutathionylation due to depletion of GR was abolished in the presence of high levels of Trx (Fig 7C). Additionally, high levels of Trx restored eNOS enzymatic activity in the face of depletion of GR (Fig 7D). Grx1 is the major deglutathionylation enzyme and uses reducing equivalents from GSH (20). To further delineate the role of Grx1, we depleted Grx1 (Fig 7E) and determined eNOS glutathionylation. As shown in Fig 6F depletion of Grx1 induced eNOS glutathionylation. However, in the presence of a Trx reducing system (Trx+TrxR1+NADPH) glutathionylation of eNOS was rescued even in the absence of Grx1 (Fig 7F). These data demonstrate that Trx is an efficient deglutathionylating agent and functionally Grx1-independent. In addition, downregulation of thioredoxin reductase 1(TrxR1) induced eNOS glutathionylation further demonstrating that Trx-redox cycle is an important regulator of eNOS function (Fig 7G and H). Taken together, our results demonstrate that Trx-dependent deglutathionylation of eNOS occurs in the absence of GSH, providing compelling evidence that Trx is a powerful deglutathionylating protein, and can function as an independent mechanism to protect eNOS against oxidant-mediated inactivation.

Accumulation of GSSG has been shown to cause defects in the endothelium-dependent vascular relaxation (16). To test the efficacy of Trx in the preservation of endothelial function in the presence of high levels of GSSG, we inhibited GR activity by BCNU (Fig 7I) in myograph-mounted LCA, which significantly blunted the endothelium-dependent ACh-mediated relaxations (Fig. 7I). We next addressed whether elevated Trx
levels would protect against the BCNU-induced impairment in ACh-mediated relaxation. Although, BCNU resulted in a significant rightward shift in the concentration-response curves to ACh, maximal relaxation in response to ACh did not reach statistical significance for BCNU-treated compared to control LCAs from Trx-Tg mice (Fig 7J). Addition of DTT fully restored ACh-mediated relaxations similar to control LCAs from Trx-Tg mice, suggesting that fully reduced Trx exerts maximal relaxing effect.

**Trx inhibits H/R or I/R- induced eNOS dysfunction:**

To test whether Trx protects eNOS from oxidative modification and thereby retains it in coupled state, we examined the effect of H/R on \( \text{O}_2^- \) production by DHE staining. As shown in Fig. 8A and B, exposure of HCAECs with Ad-LacZ to H/R resulted in increased production of \( \text{O}_2^- \) that was inhibited by NOS inhibitor L-NAME. This data demonstrates that the uncoupled eNOS contributes to \( \text{O}_2^- \) production in H/R. Importantly, H/R-induced \( \text{O}_2^- \) production was diminished due to Trx overexpression. We further confirmed the generation of \( \text{O}_2^- \) using EPR spin-trapping technique. As shown in Fig 8C and D, coronary arteries from NT mice subjected to I/R demonstrated significant \( \text{O}_2^- \) production compared to coronary arteries from Trx-Tg mice in I/R or coronary arteries from NT mice treated with L-NAME for I/R. In additional experiments, we also determined NO release in coronary arteries from I/R subjected NT and Trx-Tg mice using EPR-spin-trapping. As shown in Fig 8E and F, coronary arteries from Trx-Tg mice generated higher levels of NO compared to NT mice in I/R.

Cardiomyocytes constitute the major volume of myocardial tissue. Besides, eNOS is also expressed in cardiomyocytes albeit to a lesser extent than the endothelium. Therefore, we determined the relative contribution of eNOS-mediated \( \text{O}_2^- \) in response to H/R in cardiomyocytes. As shown in Fig. 8G and H, cardiomyocytes isolated from NT, Trx-Tg or \( \alpha\text{MHC-Trx-Tg} \) without any treatment did not show any detectable level of superoxide. However, H/R induced significant generation of \( \text{O}_2^- \), which was not blocked by L-NAME, demonstrating that the \( \text{O}_2^- \) released form myocytes originate from other sources such as the mitochondria or NADPH oxidase, but not from eNOS. However, \( \text{O}_2^- \) released from myocytes isolated form Trx-Tg or \( \alpha\text{MHC-Trx-Tg} \) mice was significantly lower compared to NT mice in H/R, but was significantly higher compared to Trx-Tg or \( \alpha\text{MHC-Trx-Tg} \) myocytes in normoxia. These data suggest that although high levels of Trx was able to bring down the level of \( \text{O}_2^- \) in cardiomyocytes, the level did not decrease to baseline level with still a significant amount of \( \text{O}_2^- \) released from these myocytes. Based on these results, we speculate that cardiomyocytes are not a significant source for eNOS-dependent \( \text{O}_2^- \) production.

**Trx directly interacts with eNOS and prevents S-glutathionylation in vivo:**

Next, we determined how Trx de-glutathionylate eNOS in vivo. To differentiate the mechanism of Trx-mediated protection of eNOS from S-glutathionylation, the HCAEC lysates were incubated with GSSG and/or recombinant hTrx and its impact on glutathionylation was evaluated. As shown in Fig 9A addition of GSSG increased eNOS glutathionylation in the lysate of HCAEC. However, the glutathionylation was restored to the control level when both GSSG and hTrx were added to lysates. Further, hTrx interacted with eNOS and could be immunoblotted from eNOS immunoprecipitated (IP) samples,
demonstrating that eNOS and hTrx interact \textit{in vitro} (Fig 9B), and this interaction was increased in H/R (Fig 9C). We also studied the interaction of hTrx with eNOS using a PLA assay. As demonstrated in Fig 9D (right panel), H/R induced significant number of PLA signals compared to normoxic HCAECs (Fig 9E). To determine if we could rescue the loss of eNOS activity due to mutation of cysteine residues that undergo glutathionylation, we mutated the previously reported(16,19) glutathionylation susceptible cysteines (384, 691 and 910) to serine in bovine eNOS construct (Fig 9F&G). The mutant eNOS enzymatic analysis revealed that C691S and C910S mutations partially rescued the H/R mediated decrease in eNOS activity and Trx overexpression completely restored the activity in either of the eNOS mutants. We believe that Trx deglutathionylate either cysteine 691 or 910 to restore eNOS activity during H/R. No rescue of activity in C384S-beNOS mutant indicates that this cysteine is not susceptible to glutathionylation during H/R. To determine whether eNOS is glutathionylated \textit{in vivo}, we implemented tissue PLA using anti-eNOS and anti-PrS-SG antibodies as described in the methods. As shown in Fig 9H (right panels), there was no PLA signal in the arteries within a heart section of NT mice that under went sham surgery although isolectin staining show the presence of endothelial cells (Fig 9H). However, significant number of endothelial cells underwent eNOS glutathionylation \textit{in vivo} in the coronary artery branches in response to I/R (Fig 9H, top lower panels and Fig 9I). In contrast, the PLA signals of eNOS-SG and isolectin were significantly lower in the endothelium of coronary arteries from Trx-Tg mice that underwent I/R (Fig 9H, middle panels and I), but the PLA signal of eNOS-SG and isolectin did not change in coronary arteries of αMHC-Trx-Tg mice that underwent I/R (Fig 9H, bottom panels and I). Collectively, these studies show that Trx deglutathionylate eNOS \textit{in vivo} and \textit{in vitro} in response to I/R or H/R.

\textbf{DISCUSSION}

We have shown that endothelial cells in coronary arteries undergo extensive S-glutathionylation of eNOS following I/R and consequently show impaired endothelial function. Using Trx-Tg and αMHC-Trx-Tg mice we have shown that functional Trx in vascular endothelium is critically required for deglutathionylation of eNOS and maintenance of endothelial function following I/R. Further, we demonstrated that I/R impairs endothelium-dependent relaxation of coronary arteries that is protected in Trx-Tg mice, but not in αMHC-Trx-Tg mice. Thus, vascular Trx plays a critical role in the protection against I/R-mediated endothelial dysfunction. Since eNOS was glutathionylated following I/R and consequently its activity was decreased significantly in NT and αMHC-Trx-Tg mice but not in Trx-Tg mice coronary arteries, deglutathionylation by Trx constitutes a major protective mechanism in endothelial dysfunction in I/R by Trx. Further evidence is provided by the fact that Trx-Tg mice are protected from BCNU-induced impairment in vascular relaxation, demonstrating that Trx could independently deglutathionylate eNOS even when the GSSG/GSH ratio is quite high, as BCNU treatment increases GSSG due to inhibition of GR. Finally we show that Trx directly interacts with eNOS to deglutathionylate it in I/R. This is the first report demonstrating that Trx is an efficient deglutathionylating protein even in the presence of normal levels of GSH and Grx1 in a cellular model and \textit{in vivo} in a ischemia-reperfusion model of oxidative stress.
Trx has been shown to be upregulated in oxidative stress conditions such as ischemia-reperfusion injury (30), hyperoxia (31) and also in hypoxia (27). However, the mechanisms associated with protection of myocardial ischemia-reperfusion injury due to high levels of Trx has remained unclear. We used both, global Trx overexpressing mice and mice that specifically overexpress Trx in the cardiomyocyte to determine the relative contribution of Trx in the protection against I/R. Although myocytes from αMHC-Trx-Tg and Trx-Tg showed almost equal expression of Trx, the αMHC-Trx-Tg mice underwent significant myocardial injury in I/R with resultant increase in MI. The only major difference between these mice is that endothelial cells from Trx-Tg mice heart express significant levels of Trx. Since endothelial cells constitute 60% of total cell type of the heart (26), and endothelial cells have been shown to release survival factors in I/R that promote cardiomyocyte survival (32,33), we speculated a significant contribution of endothelial Trx for survival of myocytes in I/R. Myocyte eNOS constitute about 25% or total eNOS of the heart (12). Therefore, endothelial eNOS is critically required for myocyte function in addition to myocyte eNOS. We may mention here that although non-myocyte cells such as fibroblasts and other cells might have higher levels of Trx in Trx-Tg compared to αMHC-Trx-Tg we believe that higher Trx levels in the endothelial cells play a major role in the protection against MI due to deglutathionylation of eNOS, as fibroblasts or other inflammatory cells do not express eNOS. A dysfunctional eNOS due to glutathionylation is expected to cause endothelial dysfunction in I/R that impairs myocardial perfusion and resultant myocyte death. Our study shows that deglutathionylation of eNOS by high levels of vascular Trx is a critical endothelial mechanism by which myocyte survive during I/R with consequent decrease in MI. Cardiomyocytes isolated from Trx-Tg or αMHC-Trx-Tg mice had similar levels of O$_2^-$ generation in H/R, which was lower than the NT mice, but was significantly higher than myocytes in normoxia and this O$_2^-$ generation was not dependent on eNOS as L-NAME did not decrease their level. Thus, the source of O$_2^-$ in cardiomyocyte could be other than eNOS, such as mitochondria or NADPH oxidase. Further, the decrease in O$_2^-$ in both myocytes were equal, yet the αMHC-Trx-Tg mice showed significant myocardial damage and MI. We believe that dysfunctional eNOS due to glutathionylation contributed significantly to the myocardial injury of αMHC-Trx-Tg mice, whereas Trx-Tg mice were protected due to deglutathionylation of eNOS resulting in a functional eNOS during I/R due to high levels of vascular Trx in addition to myocardial Trx. It is important to mention here that Trx is not a O$_2^-$ scavenger, but could scavenge hydroxyl radicals and singlet oxygen in a redox-independent manner (34). However, it rescues oxidatively inactivated proteins due to its disulfide reductase properties via NADPH and redox cycles using NADPH (35). Therefore, it is plausible that an impairment in the mitochondrial redox-sensitive enzymes such asaconitase or NADH dehydrogenase (36) could occur due excessive O$_2^-$ generation due to I/R. High levels of Trx in the myocytes could restore the activities of these enzymes and thus could bring down the levels of O$_2^-$ in I/R in myocytes. We believe that the 50% decrease in the production of O$_2^-$ in the myocytes from Trx-Tg or αMHC-Trx-Tg mice could account for the mitochondrial production of O$_2^-$ . However, deglutathionylation of eNOS by Trx could provide much needed NO for the proper functioning of myocytes as endothelial NO is needed for a variety of myocyte function...
such as cardiomyocyte contractile function and survival in I/R (12,37-39).

Our data show that significant deglutathionylation of eNOS occurs with high levels of Trx in the absence Grx1. Since Trx was able to decrease eNOS glutathionylation in the presence of increasing intracellular GSSG (by BCNU treatment), it is likely that high levels of Trx deglutathionylate eNOS in the presence of GSSG. Supporting this notion, we have shown that Trx directly interacts with eNOS and deglutathionylate it in an in vitro system. These studies point to the fact that deglutathionylation by Trx is an independent additional mechanism, but not a compensatory mechanism in the absence of Grx1. This was further supported by the fact that Grx1 depletion mediated glutathionylation of eNOS was rescued by increased levels of Trx. Further in vivo evidence is provided by Grx1 knockout mice, which were not susceptible to I/R injury of the heart, suggesting that Grx1 is not essential for deglutathionylation in I/R (40). Thus, our studies establish for the first time that Trx is a potent deglutathionylating protein, which could rescue eNOS deglutathionylation during I/R.

Grx1 is the major deglutathionylating enzyme with selective preference for PrS-SG(19). The catalytic efficiency of Grx1 is much higher for PrS-SG compared to Trx (41). For example, the $K_{cat}/K_{m}$ is 5000-fold higher for PrS-SG compared to Trx(41).

In conclusion, our data establish that Trx prevents eNOS S-glutathionylation during I/R, thereby preventing uncoupling of eNOS resulting in improved NO release, and decreased oxidative load, consequently maintaining coronary artery perfusion and endothelial function following I/R (Fig. 9J).

Materials and Methods:
Animals and Cells
Wildtype C57BL6 strain (WT) was purchased from Charles River Laboratory. Transgenic mice with overexpression of hTrx (Trx-Tg) were bred and maintained in the animal facility of Texas Tech University Health Sciences Center (TTUHSC) and have been described previously(43,44). αMHC-
Trx-Tg mice are a generous gift from Dr. Junichi Sadoshima, UMDNJ, New Jersey and has been described (45). Both males and females were used in this study. All mice strains used in this study are from C57BL/6 background and of 12-16 weeks of age. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the TTUHSC and were consistent with the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health. Human coronary artery endothelial cells (HCAEC) were purchased from Clonetics and propagated in endothelial basal medium (EBM) supplemented with additives (Bulletkit, Clonetics). Mouse coronary artery endothelial cells (MCAEC) and ECG media were purchased from Cell Biologics, Chicago, IL.

**Antibodies and chemicals**

The antibodies and chemicals were obtained from following vendors: Anti-eNOS from BD biosciences (San Jose, CA); anti-Trx, anti-NOS3 (C-20) agarose conjugate (AC), anti-GR, anti-TrxR1 antibodies were obtained from Santa Cruz Biotech. (Dallas, TX); anti-GSH from Virogen (Watertown, MA), Anti-Grx from GeneTex (Irvine, CA), Anti-LAMP1 from DSHB (Iowa City, IA), Anti α-actinin, anti-GAPDH, anti β-actin, Nicotinamide-adenine dinucleotide phosphate reduced form (NADPH) sodium, N-ethylmaleimide (NEM), BCNU, reduced glutathione (GSH), and oxidized glutathione (GSSG) were purchased from Sigma-Aldrich (St. Louis, MO). NOS activity assay kit was purchased from Cayman chemicals (Ann Arbor, Michigan). Secondary anti-rabbit and anti-mouse IgG-HRP antibodies were purchased from thermoscientific (Waltham, MA). 4-amino-5-methylamino-2’, 7’ -difluorofluorescein diacetate (DAF-FM), dihydroethidium (DHE) and fluorescent conjugated secondary antibodies were purchased from life technologies (Grand Island, NY). All other chemicals were purchased from Sigma Chemical Co. unless otherwise stated.

**Isolation of adult mouse cardiomyocytes**

Adult cardiomyocytes were isolated from 12-16 weeks old mice as described by O’Connell et al. and Louch with minor modifications (46,47). In brief, mice were anesthetized with 2% isoflurane and 100% O$_2$ (1.0 L/min) and intraperitoneally injected with 50 IU of heparin. Thoracic cavity was opened and hearts were dissected at aortic arch, immersed in ice-cold isolation buffer containing (in mM) NaCl 120.4, KCl 14.7, KH$_2$PO$_4$ 0.6, Na$_2$HPO$_4$ 0.6, MgSO$_4$-7H$_2$O 1.2, Na-HEPES 10, NaHCO$_3$ 4.6, taurine 30, butanedione monoxime (BDM) 10, glucose 5.5. The aorta was cannulated just above the coronary artery branches under dissection microscope. Hearts were retrograde perfused through the aorta using a Langendorff perfusion apparatus with isolation buffer (3ml/min) for 5 min, then switched to Digestion buffer containing (in mM) NaCl 120.4, KCl 14.7, KH$_2$PO$_4$ 0.6, Na$_2$HPO$_4$ 0.6, MgSO$_4$-7H$_2$O 1.2, Na-HEPES 10, NaHCO$_3$ 4.6, taurine 30, butanedione monoxime (BDM) 10, glucose 5.5. The aorta was cannulated just above the coronary artery branches under dissection microscope. Hearts were retrograde perfused through the aorta using a Langendorff perfusion apparatus with isolation buffer (3ml/min) for 5 min, then switched to Digestion buffer containing Liberase TH from Roche; 0.3mg/mL, trypsin and 100 μM CaCl$_2$ in isolation buffer (4mL/min) for 15 min. Hearts were placed in a 6 cm Petri dish containing 3 ml digestion buffer, the atria were removed, the ventricles were cut into 4-5 pieces and cell suspension was made by gentle dispersion with transfer pipettes. The cells were filtered through 200 µm strainer and stop solution (0.5% FBS in isolation buffer) was added. The cell suspension was allowed to settle for 10 min, the supernatant containing damaged myocytes and non-myocytes were removed and pellet was dissolved in stop buffer. The CaCl$_2$ was added in sequentially, washed, resuspended in cardiomyocyte culture medium (minimum essential medium with insulin, transferrin, selenium, glutamine and...
antibiotics) and plated in laminin coated dishes.

**Endothelial cell isolation:**
Endothelial cells from mouse hearts were isolated as described by Jin et al (48). In brief, mouse were anesthetized with ketamine (100mg/Kg) and xylazine (10mg/Kg), after opening the thoracic cavity hearts were perfused, dissected and placed in HBSS solution. Hearts were minced to small pieces and digested in 0.2% collagenase II from Worthington (Lakewood, NJ) containing HBSS for 45 min. Then the mixture was triturated by passing through the 16G cannula and filtered with 70 µm strainer. After centrifugation the pellet was dissolved in DMEM and endothelial cells were selected by incubating with anti-PECAM (BD bioscience) coated sheep anti-rat Dynabeads (Invitrogen) for 30 min, washed and trypsinized to detach cells from Dynabeads. Finally the cells were suspened in EBM-2 medium and plated in gelatin coated plates

**Adenovirus Production**
AdenoX system was obtained from Stratagene Corp. (La Jolla, CA), and LacZ or Trx cDNA was cloned into pAdenoX vector as described previously(49). Recombinant virus was allowed to infect HEK293 cells for generation of viral particles.

**RNA Interference**
Small interfering RNAs were obtained for Nontargeting siRNA control, Trx, TrxR1, Glutathione reductase (GR) and Glutaredoxin1 from Dharmacon Inc. (Arvada, CO) and transfected to HCAEC as described earlier (49).

**Site directed mutagenesis of eNOS**
Bovine eNOS construct(50) was obtained from Addgene. Cysteine 384, 691 and 910 were mutated to serine by Cyagen Biosciences (Santa Clara, CA).

**Cell culture and hypoxia-reoxygenation (H/R):**
HCAECs, and mouse coronary artery endothelial cells (MCAEC) in complete medium and isolated cardiomyocytes were flushed with a 95% N2/5% CO2 gas mixture while in Billups-Rothenberg modular chambers to create hypoxic environment. The oxygen level was kept below 1% by measuring with an oxygen electrode. Chambers were kept inside the incubator at 37°C for indicated periods of time and followed by 1h of reoxygenation in normoxic condition.

**Myocardial ischemia and reperfusion**
Trx-Tg, αMHC-Trx-Tg or non-transgenic littermates (NT) were anaesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). After an equilibration period of 10 min, left thoracotomy was performed in the fourth intercostal space and the pericardium was opened to expose the heart. A 8-0 silk suture was passed around the left anterior coronary artery (LAD) at a point two thirds of the way between its origin near the pulmonary conus and the cardiac apex. Coronary artery occlusion was achieved by ligating the left descending coronary artery using a slipknot. Following ischemia, the slipknot was released and the myocardium was reperfused. Sham mice underwent the same procedure without the slipknot tied.

**Determination of infarct size:**
Myocardial infarct size was determined as described previously (51). Briefly, after reperfusion, animals were sacrificed and aorta was cannulated, perfused with saline to remove blood. 0.25 mL of 1.5 % Evans blue was perfused after religating the coronary artery to demarcate remote myocardium (blue) and area at risk (AAR). 1.0 mm heart
sections were taken and stained with 1.0 % triphenyltetrazolium chloride (TTC) for 15 min at 37°C. After TTC staining PFA fixed heart sections were imaged with Nikon camera. TTC stained and unstained area (infarct) at AAR was quantified.

**eNOS activity assay:** eNOS activity was assayed using Cayman’s NOS activity assay kit (Cat. No. 781001) following manufacturer’s instruction.

**Detection of nitric oxide and superoxide anion:** HCAECs were cultured on glass coverslips, which were placed in the wells of a four-well plate. For detection of NO after H/R, cells were loaded with DAF-FM (10 µM) then treated with acetylcholine (ACh, 10 µM) in the presence or absence of eNOS inhibitor L-NAME. For O$_2$\textsuperscript{-} detection, H/R-exposed cells were treated with 5.0 µM of dihydroethydium (DHE) in the presence or absence of L-NAME, fixed and counter stained with Hoechst 33342.

**Immunoprecipitation and immunoblotting**
HCAECs were lysed in 25 mM Tris-HCl (pH 7.4) buffer containing 1% NP-40, 150 mM NaCl, and 20 mM NEM and protease inhibitors. The cell lysate was then incubated with the bead-conjugated eNOS antibody overnight at 4°C under constant rotation. eNOS was then eluted from the bead–antibody–eNOS complex using the loading buffer without reducing agent and the supernatant was separated by SDS–PAGE.

**Isolation and mounting of left coronary artery segments:** The heart was removed and placed in cold Krebs-Ringer buffer (KRB) and the left coronary artery below the ligation point was carefully dissected and mounted in a wire-myograph (model 620M; Danish Myotechnology, Aarhus, Denmark) for the recording of isometric force development.

**Contractile responses:** After a 30 min washout period, cumulative concentration-response curves (CRCs) were performed to serotonin (5-HT; 0.001 – 30 µmol/L).

**Relaxation responses:** During contraction with a single concentration of 5-HT (30 µmol/L), relaxing responses to ACh (0.001 – 10 µmol/L) were recorded with or without inhibitors.

**Immunofluorescence and PLA:**
PLA and immunofluorescence staining were performed as described in our recently published report (28).

**TUNEL assay:** TUNEL assay was performed by In Situ Cell Death Detection kit from Roche Applied Science, Indianapolis, MN (Cat. No. 11684795910) following supplier protocol.

**In situ PLA:** Deparaffinized heart sections were permeabilized with 0.1% Triton X-100 for 10 min at RT, blocked with 5% donkey serum and 3% BSA in PBS for 1 hour at RT and incubated with primary antibodies in 50% Da Vinci Green antibody diluent (Abcam, Cambridge, MA). PLA was performed following supplier’s instruction using Duolink Anti-Rabbit PLUS and anti-mouse MINUS PLA probes and Duolink green detection reagent (Duolink, Sigma, St. Louis, MO).

**Electron paramagnetic spectrometry (EPR) for detection of O$_2$\textsuperscript{-} and NO:**
Superoxide production by coronary artery or isolated cardiomyocytes was measured by EPR spectrometry using spin trap BMPO. Isolated coronary arteries (1.5 - 3 mm) were
incubated in 50 µL of Krebs-HEPES buffer containing 25 mM BMPO. Superoxide generated by coronary arterial sections was detected as BMPO-OOH adduct using Bruker EMX X-band spectrometer at room temperature as described in the online methods. NO formation by isolated LCA was detected by EPR spectroscopy using NO spin trap Fe²⁺-(N-methyl-D-glucamine dithiocarbamate)₂ (Fe-MGD). LCA was longitudinally opened and washed with MEM. LCA was incubated in 50 µL of MEM containing 10 µM ACh, 0.1 mM sodium ascorbate and 2 mM Fe-MGD. NO generated by LCA was detected as paramagnetic NO-Fe²⁺-MGD₂ adduct using Bruker EMX X-band spectroscope at room temperature.

Statistical Analysis
All mice numbers are mentioned in the figure legends. A minimum of n=3 was used in this study. The experiments were performed in triplicate and repeated for a minimum of 2 times. All cell culture studies are performed in triplicate and are repeated at least twice. Data were statistically analyzed by analysis of variance (ANOVA) for multiple means with Tukey’s post-hoc analysis. Student's t-test was used to compare 2 means. Prism software (Version 6.0) was used for all statistical analysis.

Disclosures: None

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Figure legends:

Figure 1. Trx-Tg mice are protected from I/R-induced myocardial infarction. (A) NT, Trx-Tg, and αMHC-Trx-Tg mice were subjected to 30 min ischemia and 1h reperfusion and TTC staining was performed as described in the methods. TTC stains viable tissue brick red and necrotic tissue as white; (B) Infarct area in relation to area-at-risk (AAR). *p <0.05 versus NT or αMHC-Trx-Tg; (C) Percent protection relative to NT. *p <0.05 versus Trx-Tg; (D) Isolated cardiomyocytes from Trx-Tg and αMHC-Trx-Tg were lysed and analyzed for Trx and α-actinin by western blotting; (E) Quantitation of Fig 1D; (F) Cardiomyocytes and endothelial cells were isolated from Trx-Tg mice, lysed and equal amount of protein from cell lysates were analyzed for Trx, α-actinin and β-actin by western blotting using their specific antibodies; (G) Quantitation of Fig 1F *p<0.05 versus cardiomyocytes; (H) Apoptosis was evaluated by TUNEL assay of heart sections from NT, Trx-Tg and αMHC-Trx-Tg mice that were subjected to I/R surgery. Images were obtained from infarct zone from endocardial border to the center via a 10X objective using Zeiss AxioObserver Z2 microscope. Green: apoptotic cells; red: cardiomyocytes (α-actinin); blue: nucleus (DAPI). (I) Percent TUNEL positive nuclei. *p <0.05 versus NT or αMHC-Trx-Tg.

Figure 2. Trx-Tg mice are protected from I/R-induced impairment of coronary artery relaxation. (A). Endothelium-dependent ACh-mediated vasorelaxing responses in LCA derived from sham and I/R-operated NT mice, (B) Trx-Tg mice. LCA relaxation is plotted as the percentage decrease in serotonin (1 μM)-induced contraction against agonist concentration of ACh on a logarithmic scale. Results are shown as mean ± SEM (n=4-9). (C) Contractile responses to cumulative concentrations of serotonin (5-HT; 0.001 – 3 μM) in LCA from NT with I/R, (D) Trx-Tg mice that underwent I/R in either the absence or presence of L-NAME (100 μM). Coronary artery contraction is plotted as the percentage of depolarization (60 μM KCl in KRB)-induced contraction, which is set as 100% against agonist concentration of 5-HT on a logarithmic scale. Results are shown as mean±SEM (n=4-9). (E) Endothelium-dependent ACh-mediated vasorelaxing responses in LCA derived from sham and I/R in αMHC-Trx-Tg. *p <0.05 versus control or sham.

Figure 3. H/R results in eNOS S-glutathionylation and reduced enzyme activity. (A) HCAECs were exposed to H/R, lysed and immunoprecipitated using anti-eNOS antibody and the immunoprecipitates were analyzed by Western blotting using anti-PrS-SG or anti-eNOS antibodies. (B) Immunoprecipitates described in panel A
was boiled with or without β-mercaptoethanol and analyzed by Western blotting using anti-PrS-SG or anti-eNOS antibodies. (C) HCAEC were exposed to H/R for indicated time periods, fixed, permeabilized and immunostained with anti-eNOS and anti-PrS-SG antibodies. (D) HCAECs were exposed to H/R for indicated time periods, lysed and the cell lysates were analyzed for eNOS enzymatic activity as described in “Methods” (E) Thin layer chromatography (TLC) of eNOS enzyme assay products from each groups were spotted onto silica-gel TLC plates and developed.

**Figure 4. Trx deficiency promotes eNOS S-glutathionylation and reduces its enzymatic activity.**
(A) HCAECs were transfected with indicated concentrations of NT or Trx siRNA and after 36 h cell lysates were collected. The lysates were analyzed for level of Trx and β-actin by Western blotting. (B) Cell lysates described in panel A were immunoprecipitated using anti-eNOS antibodies and analyzed by Western blotting using anti-PrS-SG and anti-eNOS antibodies. (C) NT or Trx siRNA (100 nM) transfected HCAEC were immunostained with anti-eNOS and anti-PrS-SG antibodies. (D) HCAEC were transfected with NT/Trx siRNA (100 nM), exposed to H/R and immunostained for eNOS and lysosomal-associated membrane protein 1 (LAMP1). (E) Experiment described in panel ‘D’ was performed in presence of proton-pump inhibitor, Lansoprazole (25 µM). (F) NT or Trx siRNA transfected HCAEC lysates were analyzed for eNOS enzymatic activity *p < 0.05 and (G) The specificity of production of 3H-Citruline was analyzed in presence or absence of L-NAA, an inhibitor of eNOS by thin layer chromatography (TLC).

**Figure 5. Trx overexpression prevents H/R-mediated eNOS glutathionylation.**
(A) HCAECs were infected with Ad-LacZ or Ad-Trx and cell lysates were analyzed by Western blotting using anti-Trx and anti-β-actin antibodies. (B) Ad-LacZ or Ad-Trx infected HCAEC were exposed to hypoxia (8h) followed by reoxygenation (1h), lysed and eNOS glutathionylation was analyzed by immunoprecipitation and Western blotting. (C) Ad-LacZ or Ad-Trx infected H/R treated HCAEC were analyzed for colocalization of eNOS and Prs-SG by immunofluorescence staining. (D) Ad-LacZ or Ad-Trx infected HCAEC were exposed to H/R and eNOS glutathionylation was examined by PLA as previously published (28). (E) Proximity signals (green foci) were counted and plotted as bar graph. *p<0.05 versus Ad-LacZ and **p<0.05 versus Ad-LacZ+H/R.

**Figure 6. Overexpression of Trx prevents H/R-mediated decrease in eNOS enzymatic activity and NO release.** (A) Ad-LacZ or Ad-Trx infected HCAEC were exposed to H/R and eNOS enzymatic activity was assayed. *p<0.05 versus Ad-LacZ and **p<0.05 versus Ad-LacZ+H/R (B) eNOS Enzyme assay products were analyzed by TLC in the presence or absence of L-NAA, a specific inhibitor of eNOS. (C) Ad-LacZ or Ad-Trx infected HCAEC were exposed to H/R, washed and loaded with DAF-FM, stimulated with ACh (10 µM) in presence or absence of 100 µM L-NAME and images were acquired via 20X objective.

**Figure 7. Trx prevents eNOS S-glutathionylation and impairment of endothelium-dependent relaxation in coronary arteries in response to GR inhibition.** (A) Ad-LacZ or Ad-Trx infected HCAEC were treated with 100 µM of
BCNU, lysed and cell lysates were analyzed for eNOS S-glutathionylation by immunoprecipitation and western blotting. (B) HCAEC were transfected with NT or GR siRNA then infected with Ad-LacZ or Ad-Trx for 36 h. Cell lysate was analyzed for GR and β-actin; (C) eNOS glutathionylation detected by immunoprecipitation and western blotting; (D) enzymatic activity of eNOS, *p<0.05 versus NT siRNA/Ad-LacZ and **p<0.05 versus GR siRNA/Ad-LacZ. (E) HCAECs were transfected with NT or Grx siRNA and after 48 h, cell extracts were prepared and analyzed for Grx and β-actin levels by Western blotting. (F) Equal amount of protein samples from NT or Grx siRNA group were incubated with/without Trx system components (Trx: 10 μg, NADPH: 200 μM, and rat TrxR: 0.1 μM) for 15 min, the reaction was stopped with 10 mM of NEM and analyzed for eNOS-SG and Trx interaction by immunoprecipitation and Western blotting. (G) HCAECs were transfected with NT or TrxR1siRNA and after 36 hours, cell extracts were prepared and analyzed for level of TrxR1 and β-actin by Western blotting; (H) eNOS was immunoprecipitated from NT or TrxR1 siRNA treated HCAEC lysates and samples were analyzed by western blotting with anti-PrS-SG and anti-eNOS antibodies. (I) Endothelium-dependent ACh-mediated vasorelaxing responses in control, BCNU (10 μmol/L) or BCNU + DTT (100 μmol/L; 20 min)-treated LAD from NT, and (J) Trx-Tg mice. Coronary artery relaxation is plotted as the percentage decrease in serotonin (1 μmol/L)-induced contraction against agonist concentration of ACh on a logarithmic scale. Results are shown as means ± SEM (n=4-5); * p < 0.05 versus control.

Figure 8. Overexpression of Trx prevents H/R or I/R mediated eNOS dysfunction and superoxide anion production. (A) Ad-LacZ or Ad-Trx treated HCAECs were exposed to H/R, washed and treated with O$_2^-$ indicator dihydroethidium (DHE) in the presence or absence of L-NAME (100 μM) and then images were acquired by Zeiss microscope (20X); (B) DHE-fluorescence signals (mean gray values) were plotted as bar graph. *p<0.05 versus Ad-LacZ and **p<0.05 versus Ad-LacZ+H/R (C) O$_2^-$ production in coronary arteries from sham or I/R-treated NT or Trx-Tg mice was measured by EPR spectrometry using spin trap BMPO as described in the methods. (D) The height of peaks indicate the magnitude of O$_2^-$ generation and were calculated and expressed as arbitrary unit. *p<0.05 versus NT and **p<0.05 versus NT I/R (E) LCAs from NT or Trx-Tg mice were isolated after sham or I/R surgery. NO formation by isolated LCA was detected by EPR spectroscopy using NO spin trap Fe-MGD as described in the methods in the presence or absence of L-NAME. (F) The height of peaks indicates the magnitude of NO generation and were calculated and expressed as arbitrary unit. *p<0.05 versus NT and **p<0.05 versus NT I/R; (G) O$_2^-•$ production was measured via EPR spectrometry of isolated cardiomyocytes from adult (12-16 weeks) NT, Trx-Tg and αMHC Trx-Tg mice after exposing them to 30 min hypoxia followed by 60 min reoxygenation, *p<0.05 vs respective normoxia control, **p<0.05 vs Trx-Tg or αMHC-Trx-Tg with L-NAME.

Figure 9. Trx directly interacts with eNOS to prevent glutathionylation ex vivo and in vivo. (A) HCAEC extract was incubated with 2.0 mM of oxidized glutathione (GSSG) in the presence and absence of recombinant Trx and the reaction was quenched with 10 mM of NEM. Cell lysate was analyzed for eNOS S-glutathionylation by immunoprecipitation.
and western blotting or (B) immunoprecipitated with anti-eNOS antibody analyzed for Trx and eNOS by Western blotting. (C) HCAECs were exposed to H/R and cell lysates were immunoprecipitated with anti-eNOS antibody and analyzed for Trx and eNOS by Western blotting. (D) PLA was performed in HCAEC using anti-Trx and anti-eNOS antibodies as described in the methods. (E) Green foci-proximity signals of Trx and eNOS were counted and plotted as a bar graph. *, Significantly higher in H/R exposed HCAEC (P < 0.05, Student’s t test) (F) Mouse coronary artery endothelial cells were transfected with pcDNA3, pcDNA3-beNOS, pcDNA3-beNOS(C384S), pcDNA3-beNOS(C691S) and pcDNA3-beNOS (C910S) constructs and then transduced with Ad-LacZ or Ad-Trx. 36 h after transfections cells were exposed to 8h hypoxia and 1h reoxygenation, cell lysates were prepared and analyzed for eNOS enzymatic activity as described earlier *p<0.05 versus Ad-LacZ normoxia and **p<0.05 versus Ad-LacZ H/R. (G) eNOS expression in cell lysates were determined by Western blotting, (H) Sham and I/R heart from NT, Trx Tg and aMHC-Trx-Tg mice were sectioned below the LAD ligation point and analyzed for eNOS S-glutathionylation by PLA. (I) Green foci of proximity signals (PrS-SG and eNOS) around the coronary artery endothelial cells were imaged and quantitated (n=5) * p<0.05 versus NT sham, ** p< 0.05 versus NT I/R or a-MHC-Trx-Tg I/R. (J) Schematic diagram showing Trx-mediated protection of eNOS from I/R-induced glutathionylation and preservation of vascular function.
Figure 1

A

B

C

D

E

F

G

H

I

NT

Trx-Tg

αMHC-Trx-Tg

Infarct / AAR

% protection

Trx-Tg

αMHC-Trx-Tg

Trx

α-actinin

Trx levels (arbitrary units)

CM

EC

Trx

α-actinin

β-actin

NT

Trx-Tg

αMHC-Trx-Tg

% TUNEL positive nuclei

NT

Trx-Tg

αMHC-Trx-Tg
Figure 2

A

B

C

D

E

Relaxation (%) vs. - LOG [ACh] in M

Relaxation (%) vs. - LOG [ACh] in M

Contraction (%) vs. - LOG [5-HT] in M

Contraction (%) vs. - LOG [5-HT] in M

Relaxation (%) vs. - LOG [ACh] in M

Sham

I/R

Control

L-NAME

Control

L-NAME

Sham

I/R

Sham

I/R

* * *

* *

* * *
**Figure 3**

A. 

| Condition       | IP: eNOS | PrS-SG | eNOS |
|-----------------|----------|--------|------|
| Normoxia        |          |        |      |
| Hypoxia(h)+1h RO|          |        |      |
| 2               | ![Image](https://example.com/image1) | ![Image](https://example.com/image2) | ![Image](https://example.com/image3) |
| 4               | ![Image](https://example.com/image4) | ![Image](https://example.com/image5) | ![Image](https://example.com/image6) |
| 8               | ![Image](https://example.com/image7) | ![Image](https://example.com/image8) | ![Image](https://example.com/image9) |

B. 

| Condition       | IP: eNOS | IB: PrS-SG | IB: eNOS |
|-----------------|----------|------------|----------|
| Normoxia        | ![Image](https://example.com/image10) | ![Image](https://example.com/image11) | ![Image](https://example.com/image12) |
| H/R             | ![Image](https://example.com/image13) | ![Image](https://example.com/image14) | ![Image](https://example.com/image15) |
| H/R             | ![Image](https://example.com/image16) | ![Image](https://example.com/image17) | ![Image](https://example.com/image18) |

Boiled: ![Image](https://example.com/image19) Boiled+L-ME: ![Image](https://example.com/image20)

C. 

| Condition       | eNOS | PrS-SG | Merge |
|-----------------|------|--------|-------|
| Normoxia        | ![Image](https://example.com/image21) | ![Image](https://example.com/image22) | ![Image](https://example.com/image23) |
| 2h Hypoxia 1h RO| ![Image](https://example.com/image24) | ![Image](https://example.com/image25) | ![Image](https://example.com/image26) |
| 4h Hypoxia 1h RO| ![Image](https://example.com/image27) | ![Image](https://example.com/image28) | ![Image](https://example.com/image29) |
| 8h Hypoxia 1h RO| ![Image](https://example.com/image30) | ![Image](https://example.com/image31) | ![Image](https://example.com/image32) |

D. 

![Image](https://example.com/image33) 

**eNOS activity (units/mg)**

Control: ![Image](https://example.com/image34) Normoxia: ![Image](https://example.com/image35) H/R: ![Image](https://example.com/image36)

Hypoxia (h)+1 h RO: ![Image](https://example.com/image37) 2, 4, 8

E. 

![Image](https://example.com/image38) 

**[H]-Citrine**

Hypoxia (h)+1 h RO: ![Image](https://example.com/image39) 2, 4, 8 L-NNNA: ![Image](https://example.com/image40) Blank: ![Image](https://example.com/image41)

**[H]-Arginine**
Figure 4

A

\[ \begin{array}{cccc}
  & + & - & + & - \\
10 \text{ nM} & + & - & + & - \\
25 \text{ nM} & - & + & - & + \\
100 \text{ nM} & + & - & + & - \\
\end{array} \]

- NT siRNA
- Trx siRNA
- Trx
- β-actin

B

\begin{array}{cccc}
  & 10 \text{ nM} & 25 \text{ nM} & 100 \text{ nM} \\
\end{array}

- NT siRNA
- Trx siRNA
- IB: PrS-SG
- IB: eNOS

C

\begin{array}{cccc}
\text{eNOS} & \text{PrS-SG} & \text{Merge} & \text{Zoomed} \\
\hline
\text{NT siRNA} & & & \\
\text{Trx siRNA} & & & \\
\end{array}

D

\begin{array}{cccc}
\text{Normoxia} & \text{H/R} & \text{Normoxia} & \text{H/R} \\
\hline
\text{eNOS} & & & \\
\text{LAMP1} & & & \\
\text{Merge} & & & \\
\text{Zoomed} & & & \\
\end{array}
Figure 8

A

|          | Ad-LacZ | Ad-Trx |
|----------|---------|--------|
|          | Normoxia | H/R | Normoxia | H/R |
| Control  | ![control image] | ![control image] | ![control image] | ![control image] |
| L-NAME   | ![L-NAME image] | ![L-NAME image] | ![L-NAME image] | ![L-NAME image] |

B

|          | Normoxia | H/R |
|----------|----------|-----|
| Ad-LacZ  | +        | -   | +     | -   |
| Ad-Trx   | -        | +   | -     | +   |
| L-NAME   | +        | +   | -     | +   |

Mean Gray Value (arbitrary Unit)

C

![Graph C](image)

D

![Graph D](image)

E

![Graph E](image)
Thioredoxin uses a GSH-independent route to deglutathionylate endothelial nitric oxide synthase and protect against myocardial infarction
Jaganathan Subramani, Venkatesh Kundumani-Sridharan, Robert H.P. Hilgers, Cade Owens and Kumuda C. Das

J. Biol. Chem. published online September 1, 2016

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