Nitrative Inactivation of Thioredoxin-1 and Its Role in Postischemic Myocardial Apoptosis

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Background—Intracellular proteins involved in oxidative stress and apoptosis are nitrated in diseased tissues but not in normal tissues; definitive evidence to support a causative link between a specific protein that is nitratively modified with tissue injury in a specific disease is limited, however. The aims of the present study were to determine whether thioredoxin (Trx), a novel antioxidant and antiapoptotic molecule, is susceptible to nitrative inactivation and to establish a causative link between Trx nitration and postischemic myocardial apoptosis.

Methods and Results—In vitro exposure of human Trx-1 to 3-morpholinosydnonimine resulted in significant Trx-1 nitration and almost abolished Trx-1 activity. 3-morpholinosydnonimine–induced nitrative Trx-1 inactivation was completely blocked by MnTE-2-PyP5+/H11001 (a superoxide dismutase mimetic) and markedly attenuated by PTIO (a nitric oxide scavenger). Administration of either reduced or oxidized Trx-1 in vivo attenuated myocardial ischemia/reperfusion injury (50% reduction in apoptosis and infarct size, \( P < 0.01 \)). However, administration of nitrated Trx-1 failed to exert a cardioprotective effect. In cardiac tissues obtained from ischemic/reperfused heart, significant Trx-1 nitration was detected, Trx activity was markedly inhibited, Trx-1/ASK1 (apoptosis signal-regulating kinase-1) complex formation was abolished, and apoptosis signal-regulating kinase-1 activity was increased. Treatment with either FP15 (a peroxynitrite decomposition catalyst) or MnTE-2-PyP5+/H11022 10 minutes before reperfusion blocked nitrative Trx inactivation, attenuated apoptosis signal-regulating kinase-1 activation, and reduced postischemic myocardial apoptosis.

Conclusions—These results strongly suggest that nitrative inactivation of Trx plays a proapoptotic role under those pathological conditions in which production of reactive nitrogen species is increased and that antinitrating treatment may have therapeutic value in those diseases, such as myocardial ischemia/reperfusion, in which pathological apoptosis is increased. (Circulation. 2006;114:1395-1402.)

Key Words: apoptosis ■ thioredoxin ■ ischemia ■ nitric oxide ■ reperfusion

Apoptosis plays a critical pathogenic role in many cardiovascular diseases, including myocardial ischemia/reperfusion (MI/R) and heart failure. Under physiological conditions, apoptosis is tightly controlled by a balance between proapoptotic and antiapoptotic molecules. Because a majority of the signaling molecules involved in apoptosis are proteins, the normal balance between these forces can be destroyed not only at the gene level (transcriptional regulation) but also at the protein level (posttranslational modification). Substantial evidence exists that posttranslational protein phosphorylation/dephosphorylation plays a critical role in regulating apoptotic signaling. Interventions that modulate protein phosphorylation/dephosphorylation (eg, kinase inhibitors or activators) have been proposed in the treatment of cardiovascular diseases.

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Thioredoxin (Trx) is a small protein expressed in all living cells. Clinical and experimental results have demonstrated that Trx is markedly upregulated in cancer tissues, and molecules that inhibit Trx promote apoptosis and reduce cancer development. On the other hand, genetic inhibition of endogenous Trx in the heart increases oxidative stress, and acute inhibition of Trx abolishes preconditioning-induced cardiac protection. Moreover, Trx-deficient cells show an accumulation of intracellular reactive oxygen species (ROS) and mitochondria-dependent apoptosis, and genetic ablation of Trx in mice causes massively increased apoptosis in embryos on embryonic day 10.5 (E10.5), leading to embryonic lethality. Conversely, overexpression of Trx confers resistance to ROS-induced cell...
Methods
In Vitro Nitration of Trx-1
Human Trx-1 (the cytosolic form of Trx, Sigma Chemical Co, St Louis, Mo) was subjected to in vitro nitration with a modified procedure recently described by Guo and colleagues for MnSOD nitration. In brief, purified human Trx-1 (dissolved in 0.1 μmol/L phosphate buffer, pH 7.4, final concentration 50 μmol/L) was incubated with 3-morpholinosydnonimine (SIN-1; final concentration 100 μmol/L, Cayman Chemical, Ann Arbor, Mich) at 37°C for 30 minutes in the presence and absence of MnTe-2-PyP31 (500 μmol/L), a cell-permeable superoxide dismutase (SOD) mimic (kindly provided by Dr Batinic-Haberle, Duke University, Durham, NC). Unreacted SIN-1 was removed by ultrafiltration over membranes with a 5-kDa cutoff. Samples were electrophoretically size-fractionated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride–plus membrane, and nitrated Trx-1 was detected with anti-nitrotyrosine antibody.

Statistical Analysis
All values in the text and figures are presented as mean±SEM of n independent experiments. All data (except Western blot density) were subjected to ANOVA followed by Bonferroni correction for post hoc t test. Western blot densities were analyzed with the Kruskal-Wallis test followed by Dunn post hoc test. Probabilities of 0.05 or less were considered to be statistically significant.

Results
Trx-1 Is Susceptible to Nitrative Modification and Its Activity Irreversibly Inhibited
Protein nitration has been shown to be a selective posttranslational modification process, and it has been shown that not all tyrosine-containing proteins are susceptible to nitration. Previous molecular and structural studies have demonstrated that nitrative modification of a number of cytoprotective molecules, including superoxide dismutase (SOD) and prostacyclin synthase, results in their enzymatic inactivation. However, definitive evidence that supports a causative link between nitrative inactivation of a specific protein with tissue injury in a specific disease is currently lacking. Therefore, the aims of the present study were (1) to determine whether Trx is susceptible to nitrative modification in vitro and in vivo; (2) to investigate the functional consequence of Trx nitration; and (3) to establish a causative link between Trx nitration and postischemic myocardial apoptosis.
the single-tyrosine–containing human Trx-1 is susceptible to nitrative modification, purified human Trx-1 was incubated with SIN-1, a molecule that simultaneously generates nitric oxide and superoxide, thus functioning as a peroxynitrite donor, and Trx-1 nitration was determined by Western blot with a monoclonal antibody against nitrotyrosine. As illustrated in Figure 1A, exposure of Trx-1 to SIN-1 (lane 1) resulted in significant Trx-1 nitration, which was completely blocked when a superoxide scavenger was added at 5 times higher concentration than SIN-1 (lane 2). Moreover, SIN-1–induced Trx-1 nitration was completely abolished when tyrosine-49 was replaced with phenylalanine (lane 4). These results indicate that although human Trx-1 with 100 μmol/L SIN-1 resulted in significant Trx nitration, which was completely blocked when a superoxide scavenger was added at 5 times higher concentration than SIN-1.

Evidence of nitrative inactivation of Trx-1 in vitro. A, Representative Western blots showing that in vitro incubation of human Trx-1 with SIN-1 resulted in significant Trx nitration, which is completely inhibited by an SOD mimetic. M indicates molecular marker; lane 1, Trx-1 + SIN-1; lane 2, Trx-1 + MnTE-2-PyP5⁺ + SIN-1; lane 3, wild-type Trx-1 + SIN-1; and lane 4, Trx-Y49F + SIN-1. B, SIN-1 inhibited Trx-1 activity in a peroxynitrite-dependent manner. n=6 to 8 samples/group. **P<0.001 vs vehicle. C, Concentration-dependent inhibition of Trx-1 activity by SIN-1.

Figure 1. Evidence of nitrative inactivation of Trx-1 in vitro. A, Representative Western blots showing that in vitro incubation of human Trx-1 with SIN-1 resulted in significant Trx nitration, which is completely inhibited by an SOD mimetic. M indicates molecular marker; lane 1, Trx-1 + SIN-1; lane 2, Trx-1 + MnTE-2-PyP5⁺ + SIN-1; lane 3, wild-type Trx-1 + SIN-1; and lane 4, Trx-Y49F + SIN-1. B, SIN-1 inhibited Trx-1 activity in a peroxynitrite-dependent manner. n=6 to 8 samples/group. **P<0.001 vs vehicle. C, Concentration-dependent inhibition of Trx-1 activity by SIN-1.

et al27 demonstrating that although glyceraldehyde phosphate dehydrogenase can be S-nitrosylated by SIN-1, this reaction requires much a higher concentration of SIN-1 (800 μmol/L) and a much longer incubation time (40 minutes) than that induced by a pure NO donor (200 μmol/L and 2.5 minutes). These results demonstrated that peroxynitrite is a strong nitrating agent but a weak nitrosylation molecule.

Having demonstrated that Trx-1 is susceptible to nitration, we further determined whether the activity of Trx-1 is altered by this posttranslational modification. As summarized in Figure 1B, treatment with SIN-1 almost completely inactivated Trx-1 (P<0.001). Coincubation with MnTE-2-PyP5⁺ (to remove SIN-1–released O₂⁻) completely and coincubation with nitric oxide scavenger PTIO (to remove SIN-1–released NO; AG Scientific, San Diego, Calif) markedly blocked SIN-1–induced inactivation of Trx-1. Because Trx-1 was incubated with dithiothreitol (DTT) after SIN-1 exposure, this SIN-1 induced Trx-1 inactivation cannot be explained by oxidative Trx-1 inhibition. Moreover, preincubation of H₂O₂-treated Trx-1 with DTT reducing buffer resulted in complete recovery of Trx-1 activity (data not shown).

Nitrative Inactivation of Trx-1 Blocks Its Antiapoptotic and Cardioprotective Effects

We22 and others28 have recently demonstrated that administration of recombinant human Trx-1 (shTrx-1) to animals subjected to MI/R or to autoimmune myocarditis exerts significant cardioprotective effects. Having demonstrated that nitrative modification resulted in its inactivation in vitro, we further investigated whether Trx-1 nitration may also abolish its antiapoptotic and cardioprotective effect in vivo. Consistent with our previous finding, treatment with reduced Trx-1 exerted significant cardioprotective effects, as evidenced by decreased TUNEL-positive nuclei (Figure 2), attenuated DNA ladder formation (Figure 3A), reduced caspase-3 activity (Figure 3B), and decreased infarct size (Figure 4). However, exposure of Trx-1 to SIN-1 before its administration (N-Trx-1) completely blocked its cardioprotective effect (Figures 2 through 4). To obtain more evidence that SIN-1 inhibits Trx-1 activity by nitration modification, 2 additional experiments were performed. First, a mutant form of Trx-1 (Trx-Y49F; tyrosine replaced with phenylalanine) was preincubated with phosphate-buffered saline or SIN-1 for 30 minutes and administered (2 mg/kg IP) 10 minutes before reperfusion. Their effect on postischemic myocardial apoptosis was determined by caspase-3 activity assay. As illustrated in Figure 3, administration of Trx-Y49F significantly reduced cardiomyocyte apoptosis. Most importantly, exposure of this nitrative-resistant mutant form of Trx-1 to SIN-1 before its in vivo administration failed to abolish its cardioprotective effect. Second, Trx-1 was preincubated with H₂O₂ (100 μmol/L) for 30 minutes, and this oxidized form of Trx-1 (2 mg/kg) was administered 10 minutes before reperfusion. Our experimental results demonstrated that administration of this oxidized Trx significantly reduced myocardial infarct size (Figure 4), which indicates that exogenously administered oxidized Trx can be
reduced in vivo and exerts cardioprotective effects. Collectively, these in vivo experimental results provide strong evidence that tyrosine nitrination is the primary cause of peroxynitrite-induced Trx inactivation.

Trx-1 Is Nitrated and Inactivated in Ischemic/Reperfused Cardiac Tissue

Many investigators have recently used a proteomic technique and demonstrated that a variety of proteins are nitrated in diseased tissue. This approach is most appropriate for generating a hypothesis that can be further investigated. However, nitrative modification of many proteins may have no biological significance, and the selection of a targeting protein can be a challenge. In the present study, we took a different approach and first determined whether nitrative modification of Trx-1 may alter its biological function. After demonstrating that Trx-1 is susceptible to nitrative modification in vitro and that its biological functions are inhibited after nitrination, we further investigated whether

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**Figure 2.** Nitration of Trx-1 (N-Trx-1) abolished its antiapoptotic effect in vivo (TUNEL staining). Using a 20× objective, the tissue slide was digitally photographed. Total nuclei (DAPI staining, blue) and TUNEL-positive nuclei (green) were counted by an IP Lab (Rockville, Md) image analysis software program with a custom-made script. The index of apoptosis (number of positively stained myocytes/total number of myocytes ×100%). 80 fields/heart ×5 to 6 hearts/group) was automatically calculated. Assays were performed in a blinded manner. A, Representative photomicrographs of in situ detection of DNA fragments. B, Summary of percent TUNEL-positive myocytes. n=5 to 6 hearts/group. **P<0.01 vs MI/R + Vehicle.

**Figure 3.** Nitration of Trx-1 abolished its antiapoptotic effect in vivo. Left, Representative photograph of electrophoretic analysis of internucleosomal DNA extracted from sham-operated control hearts (lane 1) or mouse hearts exposed to ischemia/reperfusion that received vehicle (lane 2), Trx-1 (lane 3), or SIN-1–pretreated Trx-1 (lane 4). M indicates DNA size markers. Right, Effect of Trx-1 or nitrated Trx-1 on ischemia/reperfusion-induced caspase-3 activation. N-Trx-1 indicates Sin-1–pretreated wild-type Trx-1; N-Trx-Y49F, SIN-1–pretreated mutant form of Trx-1 (Y49F). n=9 to 12 mice/group. **P<0.01 vs vehicle.

**Figure 4.** Nitration of Trx-1 (N-Trx-1), but not oxidation (O-Trx-1), resulted in a complete loss of its infarct reduction effect when administered in vivo. Top, Representative photographs of heart sections. Black-stained portion indicates nonischemic, normal region; red-stained portion, ischemic/reperfused but not infarcted region; and negative-stained portion, ischemic/reperfused infarcted region. Bottom, Summary of myocardial infarct size expressed as a percent of total ischemic-reperfused area (area-at-risk, AAR). n=12 mice/group. **P<0.01 vs vehicle-treated ischemic/reperfused hearts.
Trx-1 is nitratively modified and its activity inhibited in vivo after MI/R. A group of mice was subjected to 30 minutes of myocardial ischemia followed by 3 hours of reperfusion (MI/R). Trx-1 nitration was determined by anti-Trx-1 immunoprecipitation followed by antinitrotyrosine immunoblotting. As illustrated in Figure 5A, nitrated Trx-1 was not detected in cardiac samples from sham MI/R animals. In contrast, 30 minutes of ischemia followed by 3 hours of reperfusion resulted in significant Trx-1 nitration. Consistent with the nitrative Trx-1 modification by ischemia/reperfusion, Trx activity in ischemic/reperfused cardiac tissue was significantly reduced compared with that of tissues isolated from sham MI/R animals (Figure 5B).

**Figure 5.** Trx-1 is nitratively modified and its activity inhibited by in vivo MI/R. A, Representative Western blots. Lane 1 indicates sham MI/R; Lane 2, MI/R+Vehicle; Lane 3, MI/R+FP15; and Lane 4, MI/R+SODM. B, Trx activity in ischemic/reperfused cardiac tissue. SODM indicates a cell-permeable SOD mimic (MnTE-2-PyP5). n=6 to 8 mice/group. **P<0.01 vs Sham MI/R, +++P<0.01 vs MI/R+Vehicle.

**Downstream Signaling Mechanisms by Which Trx-1 Nitration May Induce Cardiomyocyte Apoptosis**

Recent in vitro studies have demonstrated that binding/inhibition of Trx-1 with ASK1 is the primary mechanism by which Trx-1 exerts its antiapoptotic effect. To determine whether Trx-1 nitration may alter the Trx-1/ASK1 interaction, thus activating the downstream proapoptotic kinase, 2 additional observations were made. As illustrated in Figure 6, Trx-1 is physically associated with ASK-1 (anti-Trx-1 immunoprecipitation and anti-ASK1 immunoblotting) in cardiac tissues from sham MI/R animals. However, this protein/protein interaction was virtually abolished in ischemic/reperfused cardiac tissue. Consequently, a >12-fold increase in p38 MAPK activity, a downstream molecule by which ASK1 exerts its proapoptotic effect, was observed in the ischemic/reperfused heart (Figure 7A and 7B).

**Blockade of Trx Nitration Protected the Heart From Ischemia/Reperfusion Injury**

Considerable evidence exists that peroxynitrite is the most pathologically relevant nitrating agent in vivo. To obtain more evidence to support our hypothesis that Trx-1 nitration is causatively related to postischemic myocardial apoptosis, mice were treated with either FP-15 (5 mg/kg, 10 minutes before reperfusion; Inotek Pharmaceuticals Corp, Beverly, Mass), a novel peroxynitrite decomposition catalyst, or MnTE-2-PyP5 (25 μg/kg), a cell-permeable SOD mimic. Impressively, treatment with FP-15 or MnTE-2-PyP5 shortly before reperfusion blocked Trx-1 nitration (Figure 5A), preserved Trx activity (Figure 5B), maintained Trx-1 binding activity with ASK1 (Figure 6), attenuated p38 MAPK activation (Figure 7A and 7B), and reduced postischemic myocardial apoptosis (Figure 7C). These results provided strong evidence that peroxynitrite-induced Trx nitration plays a causative role in postischemic cardiomyocyte apoptosis.

**Discussion**

We have made several novel observations in the present study. First, we have demonstrated for the first time that mammalian Trx-1, a single-tyrosine-containing molecule that has been shown to play an essential role in promoting cell survival, is susceptible to posttranslational nitrative modification. Second, we have provided the first evidence that nitrative modification inhibits Trx-1 activity in vitro and abolishes its cardioprotective effects in vivo. These results strongly suggest that with nitrative inactivation, Trx-1 may lose its antiapoptotic and cytoprotective property under those pathological conditions in which production of nitrating molecules (eg, ONOO⁻) is increased, thus contributing to cell death and tissue injury. Third, we have demonstrated that nitrative modification of Trx-1 results in its dissociation from ASK1 and subsequent ASK1 activation. This result identified a novel signaling mechanism by which reactive nitrogen species causes apoptotic cell death. Finally, we have demonstrated that in vivo MI/R resulted in significant Trx-1 nitration and inactivation. Treatment with a novel peroxynitrite decomposition catalyst shortly before reperfusion blocked nitration...
Recent studies have demonstrated that in addition to upregulation or downregulation of Trx expression at the gene level, Trx activity is regulated by posttranslational modification. Three forms of posttranslational modifications of Trx have been identified previously. These include oxidation, glutathionylation, and S-nitrosylation. Interestingly, all 3 forms of modification occur at cysteine residues but affect Trx function differently. Oxidation of the thiol groups of Cys-32 and -35 forms a disulfide bond that results in Trx inactivation. Glutathionylation occurs at Cys-73, and this posttranslational modification significantly inhibits Trx activity. S-nitrosylation occurs selectively at Cys-69. In contrast to oxidation and glutathionylation, S-nitrosylation markedly enhances Trx activity.22,26,35

In the present study, we have demonstrated for the first time that in addition to previously reported posttranslational Trx-1 modification that occurs at the cysteine residue, Trx-1 can also be modified at the tyrosine residue (protein nitration). Previous molecular and structural studies have demonstrated that, like cysteine, the content of tyrosine differs in different forms of Trx-1. Trx-1 from bacteriophage T4 contains 5 tyrosine residues, and 1 of them is located within its redox active center.36 Trx-1 produced by E. coli contains 2 tyrosine (Tyr49 and Tyr70) residues. Interestingly, mammalian Trx-1 only contains 1 tyrosine (Tyr49), and it is located within a region that is critical for Trx-1 folding.25,37,38 Because the function of Trx-1 critically depends on its proper folding,39,40 the present results strongly suggest that nitration of Tyr49 is a novel posttranslational modification that inhibits Trx-1 function by conformational change.

Previous biochemical studies have demonstrated that nitration often occurs with oxidation. In addition, we have observed that exposure of Trx to a peroxynitrite donor results in its functional inhibition, similar to that reported for Trx-1 oxidation. However, the present results strongly suggest that nitration is a novel posttranslational modification that regulates Trx-1 activity independent of oxidation. First, our in vitro study demonstrated that preincubation of H2O2-treated Trx-1 with DTT resulted in a complete recovery of its activity (data not shown). However, preincubation of SIN-1–treated Trx-1 with DTT failed to recover its activity. Second, previous studies have demonstrated that administration of oxidized Trx-1 exerts significant antioxidant and cytoprotective effects unless intracellular TrxR is inhibited, which suggests that exogenous oxidized Trx-1 is reduced by TrxR, with resultant recovery of its activity.41,42 However, the present study demonstrated that administration of nitrotyrosine dinitrase43 either does not exist in adult cardiomyocytes or its function is inhibited after ischemia/reperfusion. Further study to address this important question is warranted.

ASK1 is an MAPK kinase that functions as an upstream activator of JNK and p38 MAPK. Under physiological conditions, ASK1 activity is inhibited by several cellular factors, including Trx, glutaredoxin, and phosphoserine-binding protein 14-3-3.34 Trx-1 binds to the N-terminal domain of ASK1 with its cysteine 32 and/or 35 to inhibit ASK1 kinase activity, whereas glutaredoxin binds to the C-terminal domain of ASK1 to inhibit activity.44 Trx-1 binds to the N-terminal domain of ASK1 with its cysteine 32 and/or 35 to inhibit ASK1 kinase activity, whereas glutaredoxin binds to the C-terminal domain of ASK1 to inhibit activity.44 Trx-1 binds to the N-terminal domain of ASK1 with its cysteine 32 and/or 35 to inhibit ASK1 kinase activity, whereas glutaredoxin binds to the C-terminal domain of ASK1 to inhibit activity.44 Trx-1 binds to the N-terminal domain of ASK1 with its cysteine 32 and/or 35 to inhibit ASK1 kinase activity, whereas glutaredoxin binds to the C-terminal domain of ASK1 to inhibit activity.44 Trx-1 binds to the N-terminal domain of ASK1 with its cysteine 32 and/or 35 to inhibit ASK1 kinase activity, whereas glutaredoxin binds to the C-terminal domain of ASK1 to inhibit activity.44 Trx-1 binds to the N-terminal domain of ASK1 with its cysteine 32 and/or 35 to inhibit ASK1 kinase activity, whereas glutaredoxin binds to the C-terminal domain of ASK1 to inhibit activity.44 Trx-1 binds to the N-terminal domain of ASK1 with its cysteine 32 and/or 35 to inhibit ASK1 kinase activity, whereas glutaredoxin binds to the C-terminal domain of ASK1 to inhibit activity.44 Trx-1 binds to the N-terminal domain of ASK1 with its cysteine 32 and/or 35 to inhibit ASK1 kinase activity, whereas glutaredoxin binds to the C-terminal domain of ASK1 to inhibit activity.44 Trx-1 binds to the N-terminal domain of ASK1 with its cysteine 32 and/or 35 to inhibit ASK1 kinase activity, whereas glutaredoxin binds to the C-terminal domain of ASK1 to inhibit activity.44 Trx-1 binds to the N-terminal domain of ASK1 with its cysteine 32 and/or 35 to inhibit ASK1 kinase activity, whereas glutaredoxin binds to the C-terminal domain of ASK1 to inhibit activity.44 Trx-1 binds to the N-terminal domain of ASK1 with its cysteine 32 and/or 35 to inhibit ASK1 kinase activity, whereas glutaredoxin binds to the C-terminal domain of ASK1 to inhibit activity.44 Trx-1 binds to the N-terminal domain of ASK1 with its cysteine 32 and/or 35 to inhibit ASK1 kinase activity, whereas glutaredoxin binds to the C-terminal domain of ASK1 to inhibit activity.44 Trx-1 binds to the N-terminal domain of ASK1 with its cysteine 32 and/or 35 to inhibit ASK1 kinase activity, whereas glutaredoxin binds to the C-terminal domain of ASK1 to inhibit activity.44 Trx-1 binds to the N-terminal domain of ASK1 with its cysteine 32 and/or 35 to inhibit ASK1 kinase activity, whereas glutaredoxin binds to the C-terminal domain of ASK1 to inhibit activity.44 Trx-1 binds to the N-terminal domain of ASK1 with its cysteine 32 and/or 35 to inhibit ASK1 kinase activity, whereas glutaredoxin binds to the C-terminal domain of ASK1 to inhibit activity.44 Trx-1 binds to the N-terminal domain of ASK1 with its cysteine 32 and/or 35 to inhibit ASK1 kinase activity, whereas glutaredoxin binds to the C-terminal domain of ASK1 to inhibit activity.44 Trx-1 binds to the N-terminal domain of ASK1 with its cysteine 32 and/or 35 to inhibit ASK1 kinase activity, whereas glutaredoxin binds to the C-terminal domain of ASK1 to inhibit activity.44 Trx-1 binds to the N-terminal domain of ASK1 with its cysteine 32 and/or 35 to inhibit ASK1 kinase activity, whereas glutaredoxin binds to the C-terminal domain of ASK1 to inhibit activity.44 Trx-1 binds to the N-terminal domain of ASK1 with its cysteine 32 and/or 35 to inhibit ASK1 kinase activity, whereas glutaredoxin binds to the C-terminal domain of ASK1 to inhibit activity.44 Trx-1 binds to the N-terminal domain of ASK1 with its cysteine 32 and/or 35 to inhibit ASK1 kinase activity, whereas glutaredoxin bounds to the C-terminal domain of ASK1 to inhibit activity.44 Trx-1 binds to the N-terminal domain of ASK1 with its cysteine 32 and/or 35 to inhibit ASK1 kinase activity, whereas glutaredoxin binds to the C-terminal domain of ASK1 to inhibit activity.
ASK1 kinase activity.4,5,11 14-3-3 associates with ASK1 via the pSer567 site of ASK1 and inhibits ASK1-induced apoptosis.46 Previous studies have demonstrated that many cellular stress and apoptotic stimuli activate mitochondria-dependent apoptotic pathways by facilitating dissociation of ASK1 with its inhibitory proteins. Specifically, tumor necrosis factor-α has been reported to stimulate apoptosis by dissociating ASK1 from 14-3-3.47 Hyperglycemia has been shown to stimulate endothelial apopto-

sis by upregulating the expression of Trx-interacting protein (also termed vitamin D3-upregulated protein) and inducing dissociation of ASK1 from Trx-1.48 Recent in vitro studies have demonstrated that ROS results in Trx oxidation and subsequent dissociation from ASK1, thus facilitating apoptotic cell death.49 However, oxidized Trx can be reduced by Trx reductase and its ASK1 binding ability fully recovered. Moreover, administration of oxidized Trx-1 exerts significant antioxidant and cytoprotective effects unless intracellular TrxR is inhibited.31,42 Therefore, the in vivo pathological relevance of ASK1 activation by oxidative Trx-1 inactivation remains questionable. The present study demonstrated that nitrative modification of Trx-1 also resulted in its dissociation from ASK1 and subsequent ASK1 activation. Because an effective “denitration” system does not exist in adult cardiomyocytes, nitrated Trx-1 cannot be reactivated. Moreover, substantial evidence exists that production of reactive nitrogen species, particularly peroxynitrite, is markedly increased in a variety of diseased tissues. The results of the present study thus strongly suggest that nitrative Trx-1 modification is a novel pathological pathway by which reactive nitrogen species result in ASK1 activation and subsequent cardiomyocyte apoptosis.

Although numerous studies have been published demonstrating that protein nitration is increased in diseased tissues, we have identified a single protein that is nitrated in the ischemic/reperfused heart and have linked its nitrative inactivation with postischemic cardiomyocyte apoptosis, a primary form of cell death that is responsible for reperfusion injury. The present experimental results have broad clinical implications. Therapeutic interventions that inhibit Trx-1 nitration may have application in those diseases in which pathological apoptosis is increased, such as MI/R injury and neurological disorders. In contrast, promotion of posttranslational Trx-1 modification (nitration) may be a better approach than gene therapy (inhibition of Trx expression) for the treatment of cancer in which Trx expression is increased and physiological apoptosis is inhibited.

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Disclosures
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Apoptosis plays a critical pathogenic role in many cardiovascular diseases, including myocardial ischemia/reperfusion and heart failure. Identifying the signaling pathways that lead to myocardial apoptosis may open a new door toward preventing cardiomyocyte death and improving cardiac function in patients with cardiovascular diseases. Thioredoxin (Trx) is a small protein expressed in all living cells. Previous clinical results have demonstrated that Trx is markedly upregulated in cancer tissues, and molecules that inhibit Trx promote apoptosis and reduce cancer development, which suggests that Trx is a powerful antiapoptotic/cell-surviving molecule. Here, we have demonstrated for the first time that Trx is susceptible to nitrative modification and that this posttranslational modification inhibits Trx-1 activity in vitro and abolishes its cardioprotective effects in vivo. Additional studies demonstrated that nitrative modification of Trx-1 results in its dissociation from apoptosis signal-regulating kinase-1 (ASK1) and subsequent ASK1 activation. This result identified a novel signaling mechanism by which reactive nitrogen species cause apoptotic cell death. Finally, we have demonstrated that treatment with a peroxynitrite decomposition catalyst shortly before reperfusion blocked myocardial ischemia/reperfusion–induced nitrative Trx-1 inactivation, attenuated ASK1 activation, and reduced postsischemic myocardial apoptosis. The current experimental results have broad clinical implications. Therapeutic interventions that inhibit Trx-1 nitration may have application in those diseases in which pathological apoptosis is increased, such as myocardial ischemia/reperfusion injury and neurological disorders. In contrast, the promotion of posttranslational Trx-1 modification (nitration) may be a better approach than gene therapy (inhibition of Trx expression) for the treatment of cancer in which Trx expression is increased and physiological apoptosis is inhibited.
Nitrative Inactivation of Thioredoxin-1 and Its Role in Postischemic Myocardial Apoptosis
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