Antioxidant network expression abrogates oxidative posttranslational modifications in mice


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Antioxidant network expression abrogates oxidative posttranslational modifications in mice. Am J Physiol Heart Circ Physiol 300: H1960–H1970, 2011. First published February 18, 2011; doi:10.1152/ajpheart.01285.2010.—Antioxidant enzymatic pathways form a critical network that detoxifies ROS in response to myocardial stress or injury. Genetic alteration of the expression levels of individual enzymes has yielded mixed results with regard to attenuating in vivo myocardial ischemia-reperfusion injury, an extreme oxidative stress. We hypothesized that overexpression of an antioxidant network (AON) composed of SOD1, SOD3, and glutathione peroxidase (GSHPx)-1 would reduce myocardial ischemia-reperfusion injury by limiting ROS-mediated lipid peroxidation and oxidative posttranslational modification (OPTM) of proteins. Both ex vivo and in vivo myocardial ischemia models were used to evaluate the effect of AON expression. After ischemia-reperfusion injury, infarct size was significantly reduced both ex vivo and in vivo, ROS formation, measured by dihydroethidium staining, was markedly decreased, ROS-mediated lipid peroxidation, measured by malondialdehyde production, was significantly limited, and OPTM of total myocardial proteins, including fatty acid-binding protein and sarcoplasmic reticulum Ca2+-ATPase (SERCA2a), was markedly reduced in AON mice, which overexpress SOD1, SOD3, and GSHPx-1, compared with wild-type mice. These data demonstrate that concomitant SOD1, SOD3, and GSHPx-1 expression confers marked protection against myocardial ischemia-reperfusion injury, generating reactive oxygen species; superoxide dismutase; sarco(endo)plasmic reticulum Ca2+-ATPase 2a; reperfusion injury; myocardial ischemia

THE MECHANISMS RESPONSIBLE for altered susceptibility to ischemia-reperfusion injury are incompletely understood but involve the generation of ROS and reactive nitrogen species (RNS) and are influenced by cellular protective mediators such as SOD, catalase, and glutathione peroxidase (GSHPx) as well as abnormal Ca2+ handling. The accumulation of Ca2+ and the generation of reactive species, including nitric oxide (NO), superoxide (O2•−), and peroxynitrite (ONOO−), are believed to play an important role in I/R injury. In cardiovascular disease states, including I/R injury, increased protein tyrosine nitration, an irreversible detrimental protein modification mediated by ONOO−, is observed. Tyrosine nitration leads to decreased activity of several myocardial proteins, including mitochondrial and Ca2+-regulating proteins (24, 32, 46).

Myocardial cells are protected from minor oxidant challenges by an antioxidant network (AON) that includes enzymes such as SOD, catalase, and GSHPx (12, 20, 34, 49). Superoxide anions are readily dismutated by SOD into H2O2, which can then be converted into hydroxyl radicals by a Fenton-type reaction or scavenged by either catalase or GSHPx. Three SOD isoenzymes have been identified in mammalian cells: copper and zinc-containing SOD (Cu/Zn-SOD or SOD1), which is expressed as dimers in the cytoplasm; manganese-containing SOD (Mn-SOD or SOD2), which is expressed as tetramers in the mitochondria; and tetrameric extracellular SOD (EC SOD or SOD3). GSHPx-1, which is expressed in both the cytosol as well as the mitochondrial matrix, can use either lipid peroxides or H2O2 as substrates. Studies have suggested that hearts from SOD1 (56) and GSHPx-1 (57) knockout mice are more susceptible to I/R injury and that overexpression of either SOD1 (51), SOD3 (10), or GSHPx-1 (35, 42, 58) renders the heart more resistant to ex vivo myocardial I/R injury. However, in vivo, the efficacy of overexpression of individual antioxidant enzymes was not protective as either overexpression of GSHPx-1 or overexpression of SOD1 was found to be cardioprotective (23). One possibility is that neither enzyme alone is capable of full cardioprotective efficacy. Indeed, prior work has suggested that manipulation of the expression of one enzyme may lead to an imbalance in the antioxidant system (13, 38, 39). Therefore, the work reported here tested the hypothesis that concomitant overexpression of SOD1, SOD3, and GSHPx-1 attenuates myocardial I/R injury. We report that concomitant overexpression SOD1, SOD3, and GSHPx-1 conveys marked protection against myocardial ischemia-reperfusion injury, reducing ROS, ROS-mediated lipid peroxidation, and OPTM of critical cardiac proteins, including cardiac fatty acid-binding protein and SERCA2a.

MATERIALS AND METHODS

Transgenic mice. The generation of AON-overexpressing triple-transgenic mice has been previously described (31, 36). Constructs were prepared by cloning tagged cDNAs for human antioxidant enzymes (SOD1, SOD3, and GSHPx-1) into a vector containing the mouse H-2Kb promoter. AON mice were backcrossed for >10 generations onto the C57BL/6 background. The investigation described conformed with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and was approved by the

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Institutional Animal Care and Use Committee of The Ohio State University Medical Center.

Ex vivo myocardial I/R injury. Hearts isolated from AON-overexpressing mice or wild-type (WT) littermate controls were perfused using a nonworking heart Langendorff system with a constant perfusion pressure of 80 mmHg. Hearts were subjected to global ischemia of 30 min and reperfusion of 60 min as previously described (19). At the end of reperfusion, the heart was processed for the subsequent analysis of myocardial infarct size, ROS generation, and protein modification as described below. Ex vivo hearts were trimmed of all atria and fat while still cannulated and then perfused with 2 ml of 1.5% 2,3,5-triphenyltetrazolium chloride (TTC) at 37°C. Hearts were frozen, sectioned into 1-mm sections, fixed in 10% formalin, and weighed. To calculate infarct size (IS/weight, both sides of each section were digitally photographed and contoured with MetaVue software to delineate ischemic (red) and infarcted (white) tissue. Infarcts were reported as a percentage of the total left ventricular (LV) area multiplied by the total weight of that section.

In vivo myocardial I/R. The in vivo myocardial I/R mouse model was performed using previously described techniques (59). Mice were anesthetized with ketamine (55 mg/kg) plus xylazine (15 mg/kg). Animals were intubated and ventilated with room air (tidal volume: 250 μl, 120 breaths/min) with a mouse respirator (Harvard Apparatus, Holliston, MA). Rectal temperatures were maintained at 37°C by a thermoregulated heating pad. After a thoracotomy, an 8-0 silk suture was placed around the left coronary artery for ligation. After either a 20- or 60-min duration of ischemia, the occlusion was released, and reperfusion was confirmed visually. At 60 min of reperfusion [malondialdehyde (MDA) and protein analysis] or 24 h of reperfusion [infarct analysis], mice were reanesthetized, intubated, and ventilated as described above. The chest was reopened along the previous incision line to expose the heart, and the left main coronary artery was ligated in the same location as before. The heart was excised, and the aorta was cannulated. Three milliliters of 10% p-thalho blue (Heubach) was slowly injected directly into the aorta to stain the heart for delineation of the ischemic zone from the nonischemic zone. The area of the myocardium that did not stain with p-thalho blue was defined as the area at risk (AAR). Serial, short-axis, 1-mm-thick sections were cut and incubated at 37°C for 30 min. Infarcts were reported as a percentage of the total LV area multiplied by the total weight of that slice.

Immunoblot analysis. Hearts were homogenized in buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 0.5% Nonidet P-40, 1 mM sodium pyrophosphate, 5 mM sodium vanadate, 1 mM benzamidine, and 1 mM sodium fluoride with protease inhibitor cocktail (Sigma) for 10 s × 3 cycles. After 30 min of protein solubilization, samples were centrifuged at 13,000 rpm for 10 min. Supernatants were removed, transferred to nitrocellulose, and then subjected to SDS-PAGE gels, subjected to electrophoretic separation, and transferred onto nitrocellulose membranes for the subsequent Western protocol as described above.

Two-dimensional gel electrophoresis. Three replicates of control and transgenic heart homogenates, processed by the immunoblot protocol, were pooled in equal protein amounts. For two-dimensional gel electrophoretic comparison of total lysates, 100 μg of each pooled sample were combined with either Cy3 dye (WT, green) or Cy5 dye (AON, red) and incubated on ice for 30 min. Reactions were terminated with the addition of 10 mM lissamine (GE IP Ghor), which was protected from light. The IEF strip was focused using the manufacturer’s protocol and run on a 12% SDS-PAGE gel. The gel was removed and imaged on a GE Typhoon Phosphorimager for analysis.

3-NT detection. For 3-NT detection, 100 μg protein of each pooled sample were used to rehydrate a 3–10 pH 24-cm isoelectric focusing (IEF) strip overnight (GE IP Ghor). The IEF strip was focused using the manufacturer’s protocol and then electrophoresed on a 12% SDS-PAGE gel. The gel was removed, transferred to nitrocellulose, and then subjected to immunoblot analysis as described in Immunoblot analysis using
anti-3-NT antibody. Detection was performed using a Cy3-labeled secondary antibody, and Western blots were imaged using a Typhoon PhosphorImager.

**Protein identification.** Gels were digested with sequencing grade trypsin from Promega (Madison, WI) or sequencing-grade chymotrypsin from Roche (Indianapolis, IN) using Multiscreen Solvinent Filter Plates from Millipore (Bedford, MA). Briefly, bands were trimmed as close as possible to minimize background polyacrylamide material. Gel pieces were then washed in nanopure water for 5 min. The wash step was repeated twice before gel pieces were washed and or destained with 1:1 (vol/vol) methanol-50 mM ammonium bicarbonate for 10 min twice. Gel pieces were dehydrated with 1:1 (vol/vol) acetonitrile-50 mM ammonium bicarbonate. Gel bands were rehydrated and incubated with DTT solution (25 mM in 100 mM ammonium bicarbonate) for 30 min before the addition of 55 mM iodoacetamide in 100 mM ammonium bicarbonate solution. Iodoacetamide was incubated with the gel bands in the dark for 30 min. Gel bands were washed again with two cycles of water and dehydrated with 1:1 (vol/vol) acetonitrile-50 mM ammonium bicarbonate. The protease was driven into the gel pieces by rehydrating them in 12 ng/ml trypsin in 0.01% ProteaseMAX Surfactant for 5 min. Gel pieces were then overlaid with 40 ml of 0.01% ProteaseMAX surfactant and 50 mM ABC and gently mixed on a shaker for 1 h. Digestion was stopped with the addition of 0.5% trifluoroacetic acid. The mass spectroscopy (MS) analysis was performed immediately to ensure high-quality tryptic peptides with minimal nonspecific cleavage or frozen at −80°C until samples were analyzed.

**MS.** Capillary-liquid chromatography (LC)-nanospray tandem MS (Nano-LC/MS/MS) was performed on a Thermo Finnigan LTQ mass spectrometer equipped with a nanospray source operated in positive ion mode. The LC system was an UltiMate 3000 system from Dionex (Sunnyvale, CA). Solvent A was water containing 50 mM acetic acid, and solvent B was acetonitrile. Five microliters of each sample were first injected onto the μ-Precolumn Cartridge (Dionex) and then washed with 50 mM acetic acid. The injector port was switched to inject, and the peptides were eluted off of the trap onto the column. A 5-cm × 75-μm inner diameter ProteoPep II C18 column (New Object, Woburn, MA) packed directly in the nanospray tip was used for chromatographic separations. Peptides were eluted directly off the column into the LTQ system using a gradient of 2–80% solvent B over 45 min, with a flow rate of 300 nl/min. The total run time was 65 min. The MS/MS was acquired according to standard conditions established in the laboratory. Briefly, a nanospray source operated with a spray voltage of 3 kV and a capillary temperature of 200°C was used. The scan sequence of the mass spectrometer was based on the TopTen method; the analysis was programmed for a full scan recorded between 350 and 2,000 Da and a MS/MS scan to generate product ion spectra to determine the amino acid sequence in consecutive instrument scans of the 10 most abundant peaks in the spectrum. The CID fragmentation energy was set to 35%. Dynamic exclusion was enabled with a repeat count of 2 within 10 s, a mass list size of 200, an exclusion duration of 350 s, a low mass width of 0.5, and a high mass width of 1.5. The RAW data files collected on the mass spectrometer were converted to mzXML and MGF files using of MassMatrix data conversion tools (version 1.3, http://www.massmatrix.net/download). For low mass accuracy data, tandem MS spectra were acquired on an ion trap mass analyzer and the fragment mass accuracy of the precursor ions was set to 2.0 Da given that the data were acquired on an ion trap mass analyzer and the fragment mass accuracy was set to 0.8 Da.

**Dihydroethidium fluorescence.** Superoxide anion generation from I/R myocardium was determined using dihydroethidium (DHE) fluorescence (27). Ex vivo hearts were rapidly embedded in OCT and solidified in liquid nitrogen. Hearts were then sectioned at 5 μm and placed on slides. Sections were covered with 10 μM DHE (Sigma) in PBS (pH 7.4) and incubated in the dark for 30 min at 37°C. After being rinsed with PBS (pH 7.4), sections were fixed with 4% para-formaldehyde (pH 7.4) for 10 min, and fluorescence was visualized at 570 nm.

**MDA quantitation.** Hearts were homogenized in 20 mM PBS (pH 7.4) with 0.5 M butylated hydroxytoluene (Sigma) at 4°C for 10 s for three cycles. Samples were centrifuged at 4°C for 10 min at 13,000 rpm, and supernatants were stored at −80°C until the time of the MDA assay. Assays were performed according to the manufacturer’s protocol (MDA kit, OXIS International, Foster City, CA). MDA concentrations (in μM) were derived from the linear regression of known MDA standard concentrations and expressed per milligram of protein.

**Statistical analysis.** The results of the experiments were analyzed by several statistical methods (e.g., paired or unpaired t-tests, ANOVA, χ2-analysis, curve fitting functions, etc.) using standard software (e.g., GraphPad Prism, version 4.0). Results are expressed as means ± SE. For comparisons between two groups, significance was determined by paired or unpaired Student t-tests. For comparison of multiple groups, multifactorial ANOVA with a post hoc comparison was used to test for differences and to determine statistical significance. For all statistical evaluation, P values of <0.05 were considered significant.

**RESULTS**

**SOD1-, SOD3-, and GSHPx-1-overexpressing mice.** The generation and characterization of SOD1, SOD3, and GSHPx-1 (AON overexpressing) mice have been previously reported (31, 36). To examine the level of expression of these antioxidant enzymes, whole heart homogenates were analyzed by immunoblot analysis, which confirmed the expression of human SOD1 (Fig. 1A), human SOD3 (Fig. 1B), and human GSHPx-1 (Fig. 1C) in AON-overexpressing hearts. The relative level of expression appears quite exaggerated because of the minimal cross-reactivity of the anti-human enzyme antibodies with the native murine enzyme. However, comparable to what has been previously reported in pancreatic islet cells (36), the total activity of GSHPx-1 and SOD1/SOD3 was modestly but significantly increased (SOD1/SOD3: 1.8-fold and GSHPx-1: 1.2-fold; Fig. 1, D and E, respectively). To examine the effect of SOD1, SOD3, and GSHPx-1 overexpression on Ca2+ handling proteins, the levels of ryanodine receptor (Fig. 1F), SERCA2a (Fig. 1G), phospholamban (Fig. 1H), calsequestrin (Fig. 1I), and GAPDH (Fig. 1J) were also examined by immunoblot analysis. No differences in the level of any of these proteins were observed in AON-overexpressing hearts compared with WT hearts.

**AON expression protects against myocardial I/R injury.** Using an ex vivo myocardial I/R injury model, compared with hearts from WT animals, AON-overexpressing hearts demonstrated a 68% reduction in WT: 63.4 ± 4.8% vs. AON: 20.3 ± 3.7%, P < 0.05; Table 1) in myocardial IS after 30 min of global ischemia and 60 min of reperfusion (Fig. 1K).

Given the possibility for increased sensitivity of the Langendorff perfusion model to oxidative stress, an in vivo model of regional left coronary artery I/R injury was examined. WT or AON-overexpressing mice were subjected to in vivo myocardial I/R injury of either 20 or 60 min followed by 24 h of reperfusion. Compared with WT mice, AON-overexpressing mice, concomitantly overexpressing human SOD1, SOD3, and GSHPx-1, demonstrated a 92% reduction of IS after 20 min of...
Fig. 1. Characterization of antioxidant network-overexpressing (AON) hearts and responses to ex vivo and in vivo myocardial injury. Baseline myocardial samples were examined for the protein levels of human (h)SOD1 (A), hSOD3 (B), glutathione peroxidase-1 (hGSHPx-1; C), ryanodine receptor (F), sarco(endo)plasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a; G), phospholamban (H), calsequestrin (I), and GAPDH (J), or the enzyme activity of combined SOD1/ hSOD3 (D) or GSHPx-1 (E) as described in MATERIALS AND METHODS. Relative densitometry and representative images are shown for each. Enzyme activity is expressed per milligram of protein. K: hearts from either wild-type (WT) or AON animals were subjected to 30 min of global ischemia and 60 min of reperfusion as described in MATERIALS AND METHODS. Infarct size is expressed as a percentage of the total left ventricle. Representative stained myocardial images are displayed. L: WT or AON animals were subjected to 60 min of in vivo left coronary artery ligation-induced ischemia and 24 h of reperfusion as described in MATERIALS AND METHODS. Total infarct size is expressed as a percentage of the area at risk for infarction. Representative stained myocardial images are displayed. Values are means ± SE. NS, not statistically different.
ischemia (WT: 17.1 ± 1.8% vs. AON: 1.4 ± 0.6%, P < 0.05; data not shown) and a 55% reduction in IS after 60 min of ischemia and 24 h of reperfusion (WT: 40.0 ± 3.5% vs. AON: 18.1 ± 3.9%, P < 0.05; Fig. 1L and Table 2), indicating significant in vivo cardioprotective efficacy against prolonged ischemia with AON expression.

AON expression attenuates the formation of ROS and lipid peroxidation after myocardial I/R injury. To elucidate the mechanism of the cardioprotection conferred by AON expression, ROS generation, measured by DHE staining, was examined in WT and AON-overexpressing hearts subjected to 30 min of ex vivo global ischemia and 60 min of reperfusion. AON-overexpressing hearts displayed significantly less DHE staining after I/R injury than WT hearts (relative fluorescence: 887.4 ± 45.47 in WT hearts vs. 353.3 ± 39.18 in AON-overexpressing hearts, P < 0.0001, n = 4 hearts/group; Fig. 2A–C).

To further assess ramifications of diminished ROS generation in AON-overexpressing hearts, the formation of MDA, an indicator of ROS-mediated lipid peroxidation, was measured in WT and AON-overexpressing hearts exposed to 60 min of in vivo ischemia and 60 min of reperfusion. For both the ex vivo and in vivo experiments, this 60-min reperfusion time was chosen so that we could observe the modifications that occur within the initial oxidative burst and the sustained oxidative production during the recovery phase of reperfusion. WT and AON-overexpressing hearts displayed comparable levels of MDA at baseline (WT: 0.131 ± 0.015 μM/mg protein vs. AON: 0.130 ± 0.015 μM/mg protein, P > 0.05, n = 3 hearts/group; Fig. 2D). In contrast, after I/R injury, WT hearts demonstrated a twofold increase in the formation of MDA, whereas AON-overexpressing hearts displayed no significant difference in MDA formation from baseline (postischemia: 0.225 ± 0.022 μM/mg protein in WT hearts vs. 0.1215 ± 0.017 μM/mg protein in AON-overexpressing hearts, P < 0.001, n = 3 hearts/group; Fig. 2D). Together, these data indicate that AON expression significantly scavenges ROS produced during myocardial I/R injury, reducing its detrimental effects.

AON expression reduces OPTM. To further examine the ramifications of the observed reduction of ROS in AON-overexpressing mice after myocardial I/R injury, the “nitroproteome” of WT and AON-overexpressing hearts was examined for the specific peroxynitrite-mediated OPTM of tyrosine to 3-NT. Again, mice were subjected to 60 min of in vivo ischemia and 60 min of reperfusion so that we could observe the modifications that occurred within the initial oxidative burst and the sustained oxidative production during the myocardial recovery phase of reperfusion. WT hearts displayed a marked increase in OPTM formation after I/R compared with AON-expressing animals exposed to a similar experimental protocol (Fig. 2E). Furthermore, differences in the protein banding pattern suggested that concomitant SOD1, SOD3, and GSHPx-1 expression inhibits OPTM of multiple specific proteins.

To assess the specific nitrotyrosine OPTM of myocardial proteins, two-dimensional gel electrophoresis followed by 3-NT immunoblot analysis was conducted. While there were no significant differences in the intensity and distribution between WT and AON-overexpressing heart lysates at baseline (Fig. 3, A and B), the number and intensity of 3-NT modified proteins were greater in WT hearts compared with AON-overexpressing hearts after myocardial I/R injury (Fig. 3, C and D), suggesting that AON expression protects specific proteins from detrimental tyrosine nitration. To determine the proteins modified, MS/MS analysis was conducted. This analysis revealed the novel finding that 3-NT modified protein A in WT hearts is cardiac fatty acid-binding protein (FABP)-3, a key metabolic protein (Fig. 3E).

AON expression reduces the formation of peroxynitrite-mediated protein modification of SERCA2a. Given our interest in the role of oxidative stress in regulating Ca2+ handling and recent reports (2, 29, 33, 50) of OPTM of SERCA2a in models of heart disease, the effect of AON expression on tyrosine nitration of SERCA2a after myocardial I/R injury was evaluated. Immunoprecipitation with antibody to SERCA2a and detection with antibody to 3-NT (Fig. 4A) revealed OPTM of SERCA2a in WT hearts subjected to 60 min of ischemia and 60 min of reperfusion but significantly less modification of SERCA2a from AON-overexpressing hearts (relative densitometry: 3.386 ± 454.7 in WT hearts vs. 1.672 ± 63.8 in AON-overexpressing hearts, P = 0.0324, n = 3 hearts/group; Fig. 4B). Again, at baseline, there was no difference in the total level of SERCA2a between WT and AON-overexpressing hearts (Fig. 1G). These data suggest that AON expression protects SERCA2a from the detrimental OPTM of tyrosine nitration.

**DISCUSSION**

In the present study, we demonstrate that transgenic concomitant overexpression of human SOD1, SOD3, and GSHPx-1

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of Mice/Group</th>
<th>Sex, % male/female</th>
<th>Age, days</th>
<th>Body Weight, g</th>
<th>Heart Weight, mg</th>
<th>Heart Weight/Body Weight, mg/g</th>
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<tbody>
<tr>
<td>WT</td>
<td>6</td>
<td>83/17</td>
<td>194.67 ± 26.42</td>
<td>28.67 ± 0.42</td>
<td>71.12 ± 4.47</td>
<td>2.48 ± 0.18</td>
</tr>
<tr>
<td>AON</td>
<td>4</td>
<td>75/25</td>
<td>186.25 ± 30.44</td>
<td>29.5 ± 0.50</td>
<td>73.93 ± 9.28</td>
<td>2.51 ± 0.32</td>
</tr>
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Values are means ± SE. WT, wild-type control animals; AON, antioxidant network-expressing animals.

**Table 2. Baseline characteristics for WT and AON mice subjected to in vivo myocardial ischemia-reperfusion injury**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of Mice/Group</th>
<th>Sex, % male/female</th>
<th>Age, days</th>
<th>Body Weight, g</th>
<th>Heart Weight, mg</th>
<th>Heart Weight/Body Weight, mg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>8</td>
<td>62.5/37.5</td>
<td>120 ± 6.78</td>
<td>26 ± 1.29</td>
<td>67.76 ± 5.20</td>
<td>2.60 ± 0.18</td>
</tr>
<tr>
<td>AON</td>
<td>12</td>
<td>68/32</td>
<td>130 ± 1.29</td>
<td>26 ± 1.26</td>
<td>78.44 ± 5.23</td>
<td>2.98 ± 0.15</td>
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Values are means ± SE.
results in a marked reduction of ischemic injury, reducing ROS-mediated lipid peroxidation and RNS-mediated OPTM of a number of proteins, including cardiac FABP-3 and SERCA2a. The novelty of the present approach is that it uses the concomitant overexpression of several antioxidant enzymes en bloc to provide a network that can efficiently scavenge and neutralize ROS, thereby attenuating myocardial I/R injury. While only associative in nature, these novel data identifying OPTM of cardiac FABP and SERCA2a provide direction for future evaluation of the contribution of these specific protein modifications to myocardial I/R injury and may provide insights for the development of combined therapeutic approaches for the detection or treatment of acute ischemic heart disease, a major cause of morbidity and mortality in the United States and worldwide (55).

It is widely accepted that myocardial I/R injury induces the production of ROS (17, 61, 62). With coronary artery occlusion, myocardial ischemia quickly leads to decreased intracellular ATP levels and the accumulation of intracellular Ca\(^{2+}\) and hydrogen, resulting in bioenergetic and functional abnormalities (28). During ischemia, the redox status of the myocardium shifts to a more reduced state (60). With reperfusion, reoxygenation of the myocardium results in exaggerated metabolic shifts and worsening myocardial damage (8). Generation of ROS at the onset of reperfusion shifts the redox status of the myocardium to a more oxidized state (60). There is a significant burst of oxygen-derived free radicals generated within the first minutes of reperfusion (62), peaking 4–7 min after the onset of reperfusion, followed by a persistent generation of oxygen-derived free radicals (22, 26).

Generation of both ROS and RNS induces mitochondrial injury, sarcomplasmic reticulum dysfunction, and further Ca\(^{2+}\) accumulation (52). Indeed, several studies (4, 7, 22) support the concept that ROS, such as superoxide anions, H\(_2\)O\(_2\), and hydroxyl radicals, contribute to myocardial tissue injury secondary to ischemia and reperfusion (4, 7, 22). Myocardial cells are protected from minor oxidant challenges by an AON that includes enzymes such as SOD, catalase, and GSHPx (12, 20, 34, 49). A number of studies have used genetic modification of mice (knockout or transgenic overexpression of individual antioxidant enzymes) to explore the role of specific antioxidant enzymes in I/R injury. These results have yielded mixed interpretations regarding the role of individual antioxidant enzymes in myocardial I/R injury.

Experiments using knockout animals have demonstrated that after global ex vivo ischemia and reperfusion, hearts genetically devoid of GSH-Px displayed a reduced recovery of developed force, an increase in creatine kinase release, and larger myocardial IS compared with control hearts (57). Similarly, in SOD1 knockout hearts subjected to global ex vivo ischemia and reperfusion, a decreased recovery of LV developed pressure and larger myocardial higher IS were observed compared with control hearts (56). Complementing these
reports, compared with control hearts, transgenic hearts overexpressing GSH-Px-1 displayed improved recovery of contractile force, reduced creatine kinase release, and reduced IS after ex vivo global ischemia and reperfusion (58). In a separate model, SOD1 overexpression resulted in an increase in the recovery of contractile function, a decrease in IS, and improved recovery of high-energy phosphates after global ex vivo ischemia (51). Similarly, in an ex vivo working heart model of ischemia, SOD3 overexpression resulted in improved contractile recovery, stroke work, and stroke volume compared with control hearts (10). Thus, in ex vivo models, significant myocardial protection has been reported with genetic modulation of the expression of individual antioxidant enzymes.

In contrast, in vivo experiments demonstrated that neither deficiency nor overexpression of SOD1 affected myocardial IS after left coronary artery ischemia and reperfusion (23). Furthermore, overexpression of GSHPx did not convey in vivo myocardial protection in the same model (23). The conclusion from this study was that neither SOD1 nor GSHPx-1 modulates the susceptibility to in vivo myocardial I/R injury (23). However, given the fact that each of these enzymes acts as a critical component within an AON, one possibility for the disparate ex vivo and in vivo results is that neither enzyme alone is capable of full cardioprotective efficacy in vivo. This concept is supported by a prior study (36) using AON-expressing mice that demonstrated that AON pancreatic islets were maximally resistant to injury induced by hypoxanthine-xanthine oxidase-generated production of superoxide radicals ex vivo compared with WT, single-, or double-transgenic islets. Similarly, concomitant expression of SOD1, SOD3, and GSHPx-1 significantly protected pancreatic islets from hypoxia/reoxygenation.
injury ex vivo after an incubation under hypoxic conditions in nutrient-poor medium for 18 h followed by an incubation under normoxic conditions for 48 h in complete medium compared with WT islets. Additionally, single- or double-transgenic islets demonstrated no protection in this model. Alternatively, modulation of individual antioxidant enzyme levels may result in detrimental changes to the overall antioxidant system (13, 38, 39). Indeed, in models of induced oxidative stress, proteomic analysis revealed concomitant increases in SOD1, SOD3, and GSHPx-1 levels, likely representing an adaptive upregulation of the AON to combat oxidative stress (41). The current model attempted to mimic such a response by expression of an AON rather than individual antioxidant enzymes. While the immunoblots using anti-human antibodies exaggerate the level of expression of human SOD1, human SOD3, and human GSHPx-1 because of limited cross-reactivity to the murine enzymes, measurement of the level of enzymatic activity demonstrated a modest increase in GSHPx-1 and combined SOD1 and SOD3 activity, comparable to what has previously been reported (36).

Additional differences between the ex vivo and in vivo models are due to the fact that inflammatory cells, which are absent in ex vivo perfusion models, contribute to a prolonged generation of oxygen-derived free radicals during reperfusion in vivo (14). Also critical to the differences observed between ex vivo and in vivo studies is the fact that ex vivo perfusion with crystalloid solutions, which lack iron-binding proteins, may facilitate the production of hydroxyl radical by Fenton/Haber-Weiss reactions. Therefore, any approach that reduces oxidative stress may have a more profound effect in ex vivo perfused hearts but not on in vivo myocardial I/R injury. Here, we demonstrate that AON expression not only attenuates ex vivo I/R injury but also significantly reduces in vivo myocardial I/R injury, reducing infarct size and ROS-mediated lipid peroxidation.

ROS damage cells via a variety of mechanisms including peroxidation of polyunsaturated fatty acids within the membrane lipids (9). While studies (3, 11, 44) have suggested that ROS-mediated lipid peroxidation can occur with ischemia, upon restoration of blood flow, increased levels of ROS and RNS are generated, which contribute to lipid peroxidation. Peroxidation of membrane lipids results in the fragmentation of polyunsaturated fatty acids producing various aldehydes, alk(enals, and hydroxyalkenals, including MDA and 4-hydroxy-2-nonenal, that are reactive with proteins (15). Thus, by quenching ROS and limiting lipid peroxidation, AON expression conveys cardioprotection from OPTM of proteins after myocardial I/R injury.

With reperfusion of the myocardium, increased NO and superoxide results in the formation of ONOO⁻ and suppression of myocardial tissue O²⁻ consumption, causing a hypoxia-reoxygenation state that potentiates ROS generation (59). The nitration of tyrosine to 3-NT represents an OPTM secondary to oxidative stress that has been reported in a number of pathological conditions, including aging, hypertension, diabetes, and atherosclerosis (5, 18, 21). In animal models of sepsis, diabetes, or myocardial I/R injury, ONOO⁻-mediated tyrosine nitration appears restricted, as several studies (24, 25, 32, 46, 47, 53, 54) have demonstrated relatively small numbers of proteins that are modified.

Prior work has linked ROS production to oxidative modification of Ca²⁺-handling proteins such as SERCA2a (30, 43, 45); however, the identification of 3-NT modification of SERCA2a in response to I/R injury represents a novel finding. SERCA2a has been shown to be nitrated at two adjacent tyrosine residues (Tyr²⁰⁴ and Tyr²⁰⁵) in both skeletal and cardiac muscle from aged animals (29, 50). Tyrosine nitration of SERCA2a results in a decrease in activity (2, 33). Indeed, in myocardial samples from patients with dilated cardiomyopathy, nitrotyrosine modification of SERCA2a positively correlated with the time to half-relaxation in myocytes isolated from control and dilated cardiomyopathic hearts (33). These data demonstrate that SERCA activity is sensitive to the oxidative state of the myocardium. In the present study, SERCA2a appears to be a key protein susceptible to detrimental OPTM after myocardial I/R injury. AON overexpression not only results in a decrease in OPTM by tyrosine nitration of a number of myocardial proteins but also specifically attenuates tyrosine nitration of the key Ca²⁺-handling protein SERCA2a.
Characterization of additional proteins that underwent tyrosine nitration using two-dimensional immunoblot analysis coupled with LC-MS/MS identified heart-type FABP (hFABP or FABP-3) as one of the major proteins that underwent 3-NT modification after myocardial I/R injury in WT hearts but not in AON-overexpressing hearts. FABP-3 is expressed at high levels in the myocardium, where it serves to translocate fatty acids and fatty acid derivatives in the cytoplasm (16). In response to insulin stimulation, increased FABP phosphorylation has been observed in cardiac myocytes, suggesting metabolic regulation of FABP activity (37). In mice deficient in FABP-3, inefficient uptake of plasma long-chain fatty acids results in a switch to glucose utilization as an energy source (6). Thus, the activity of FABP appears critical in regulating energetic homeostasis. Of further interest, with myocardial injury, FABP-3 is released into the circulation and has been used as a biomarker for the diagnosis of acute coronary syndromes (1, 48). Whether detection of 3-NT modified FABP may increase the sensitivity and specificity for the detection of acute coronary syndromes is the focus of ongoing work. While speculative, specific ROS- or RNS-mediated modifications of specific myocardial proteins may serve to provide increased sensitivity and specificity for the detection of myocardial injury.

There are several limitations to the study that require acknowledgement. The present study did not differentiate between or specifically determine whether the early burst of oxidant generation during the first minutes of reperfusion or the sustained elevated levels during reperfusion were attenuated by AON expression. In addition, the present study did not identify the specific species or the source of oxidants that were potentially attenuated by AON expression. Furthermore, the examination of individual or dual antioxidant enzyme expression was not evaluated; therefore, the contribution of single- or double-transgene expression in this specific model cannot be stated. However, the prior work cited demonstrates that concomitant expression of SOD1, SOD3, and GSHPx-1 was required for maximal protection of pancreatic islets from superoxide-mediated injury and hypoxia/reoxygenation-mediated injury ex vivo. Indeed, future studies examining the efficacy of single, dual, or triple antioxidant enzyme expression in this model and models of increased oxidative stress (hypertension, diabetes mellitus, or hypercholesterolemia) will be critical to determining the applicability of the expression of an AON in conveying cardiovascular protection in vivo. Finally, these novel data identifying OPTM of cardiac FABP-3 and SERCA2a are associative in nature and do not prove that the reduction of these specific modification in AON-overexpressing hearts prevents I/R injury. Ongoing studies aimed at examining the contribution of the modification of these proteins to myocardial injury are underway.

In conclusion, a number of variables and pathways influence the susceptibility of the myocardium to ischemic damage. A key group of enzymes combine to form a network focused on the detoxification of ROS and RNS produced during I/R injury. The present study demonstrates that concomitant overexpression of SOD1, SOD3, and GSHPx-1 protects the murine myocardium from I/R-mediated oxidative injury, reducing lipid peroxidation and OPTM of tyrosine nitration of a number of myocardial proteins, including FABP and the critical Ca2+-handling protein SERCA2a.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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