Temporal Effects of Catalase Overexpression on Healing After Myocardial Infarction

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Background—Reactive oxygen species, such as hydrogen peroxide (H₂O₂), contribute to progression of dysfunction after myocardial infarction (MI). However, chronic overexpression studies do not agree with acute protein delivery studies. The purpose of the present study was to assess the temporal role of cardiomyocyte-derived H₂O₂ scavenging on cardiac function after infarction using an inducible system.

Methods and Results—We developed a tamoxifen-inducible, cardiomyocyte-specific, catalase-overexpressing mouse. Catalase overexpression was induced either 5 days before or after MI. Mice exhibited a 3-fold increase in cardiac catalase activity that was associated with a significant decrease in H₂O₂ levels at both 7 and 21 days. However, cardiac function improved only at the later time point. Proinflammatory and fibrotic genes were acutely upregulated after MI, but catalase overexpression abolished the increase despite no acute change in function. This led to reduced overall scar formation, with lower levels of Collagen 1A and increased contractile Collagen 3A expression at 21 days.

Conclusions—In contrast to prior studies, there were no acute functional improvements with physiological catalase overexpression before MI. Scavenging of H₂O₂, however, reduced proinflammatory cytokines and altered cardiac collagen isoforms, associated with an improvement in cardiac function after 21 days. Our results suggest that sustained H₂O₂ levels rather than acute levels immediately after MI may be critical in directing remodeling and cardiac function at later time points. (Circ Heart Fail. 2011;4:98-106.)

Key Words: myocardial infarction ■ oxidative stress ■ hydrogen peroxides ■ catalase

Congestive heart failure is a leading cause of morbidity and mortality in the United States and worldwide. The dominant cause of congestive heart failure is loss of myocardium as the result of coronary artery disease and subsequent occlusion. The resulting myocardial infarction (MI) induces apoptosis, an exacerbated immune response, fibroblast proliferation, and, ultimately, scar formation and myocyte hypertrophy. Although exact mechanisms behind these processes are still being examined, the increase in oxidative stress after MI is thought to play a critical role in many of these events. Some potential sources of the elevated reactive oxygen species after acute MI include cardiac fibroblasts, invading neutrophils, and cardiomyocytes. Regardless of the source, scavenging reactive oxygen species after acute MI remains an attractive therapeutic goal.

Clinical Perspective on p 106

Hydrogen peroxide (H₂O₂) is a potent signaling molecule generated mainly from the dismutation of 2 superoxide radicals by superoxide dismutase (SOD). The normal myocardium contains antioxidant proteins that act to scavenge H₂O₂, mainly catalase, glutathione peroxidase, and peroxiredoxin. Although it is not the only system that regulates H₂O₂ levels, catalase accounts for nearly 80% of all peroxidase activity in cardiomyocytes. Excess levels of H₂O₂ produce negative effects such as lipid peroxidation, apoptosis, and cardiac fibrosis in the myocardium, all of which aid in the progression to heart failure. Moreover, H₂O₂ stimulates the production of proinflammatory cytokines such as transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α), and interleukin 6 in activated cardiac fibroblasts. These cytokines alter the production of collagen deposited in the extracellular matrix in and surrounding the injured myocardium to replace the apoptotic cardiomyocytes. The rise in collagen deposition by H₂O₂ may impair chronic cardiac function.

After MI, there is a selective change in antioxidant levels. Catalase levels, although stable initially, decrease over time, whereas glutathione peroxidase remains unchanged. Because most of the peroxidase activity in myocytes is from catalase,
this significant change can drastically alter redox balance in the myocardium and makes a strong case for the restoration of catalase levels in the myocardium as an attractive therapeutic option. Indeed, many therapies that improve cardiac function after acute MI also increase catalase levels.5,6 Additionally, cardiac catalase overexpression was shown to prevent doxorubicin-induced cardiotoxicity and protect against ischemia/reperfusion injury in mice.1,7 Although the study demonstrated a critical role for H2O2-scavenging by catalase in the post–ischemia/reperfusion setting, the mice studied had supraphysiological (90-fold) overexpression of catalase from birth, 2 conditions quite unlikely in the human pathology. Adding to the controversy, when catalase protein was delivered directly after ischemia/reperfusion, there was no improvement noted.8,9 Finally, in a model of heat-shock–induced cardioprotection, inhibition of catalase with 3-aminotriazole had no effect on infarct size.10

To determine the role for catalase in postinfarct healing, we created a cardiomyocyte-specific, tamoxifen-inducible, catalase-overexpressing mouse to assess the role of H2O2 on cardiac function after MI at different times along the disease process. Our results demonstrate only modest protection at early time points when catalase is induced before MI. When catalase is induced a few days after MI, although there is no improvement in cardiac function, acute levels of inflammatory and fibrotic genes are decreased and there is a change in collagen isoforms expression ratio. These mechanistic changes led to an improvement in function at later time points and suggest timing must be carefully considered for sustained delivery of catalase.

Methods

Generation of Cardiac-Specific, Inducible Catalase-Overexpressing Mice

The laboratory of Dr W. Robert Taylor has generated a transgenic mouse that expresses human catalase under the control of the CX1 promoter. In front of the catalase gene, there is a floxed green fluorescent protein sequence with a stop codon.11 These mice were crossed with mice containing Cre recombinase under control of the α-myosin heavy chain promoter and a mutant estrogen receptor element (Mer-Cre-Mer, Jackson Labs; Stock No. 005650) to create a new double-transgenic mouse. All mice were on a C57BL6 background and genotyped for presence of both human catalase and Cre-recombinase as described.11,12

Induction of Catalase Overexpression

Adult male mice were injected intraperitoneally (40 mg/kg) with tamoxifen (Sigma), dissolved in sunflower oil as described,12 or sunflower oil alone for a period of 5 days.

Measurement of Catalase Activity

Protein extracts were incubated with 50 μmol/L hydrogen peroxide and subjected to kinetic readings of absorbance at 240 nm. Absorbance was converted to concentration using the Beer law, and rates of decomposition were determined by subtracting the 3-minute concentration from the initial reading to get a rate. One unit was defined as decomposing 1 μmol/L of hydrogen peroxide per minute.

Hydrogen Peroxide Measurements

Hydrogen peroxide levels were determined using amplex red assay (Invitrogen).

Myocardial Infarction

Adult male mice 8 to 12 weeks old were subjected to MI surgeries by ligation of the left anterior descending coronary artery for 30 minutes followed by reperfusion as described.13 All studies were performed in a randomized and blinded manner and were approved by the Emory University Institutional Animal Care and Use Committee.

Cardiac Function

Animals were placed under light anesthesia (Isoflurane, Webster Veterinary), and both M-mode and B-mode echocardiography were performed using a Vevo 770 microimaging system (VisualSonics). Measurements were taken from at least 5 cardiac cycles at peak systole and diastole to determine fractional shortening, ejection fraction, end-systolic and end-diastolic volume, and left ventricular (LV) mass.13

Real-Time Polymerase Chain Reaction

Tissue homogenates were prepared using Trizol (Invitrogen), and RNA was extracted per manufacturer’s protocol. Templates of cDNA were made with a commercially available kit (Applied Biosciences), and primers were designed using PrimerExpress (Applied Biosciences). Gene expression was determined using SYBRGreen and a thermal light cycler (Roche).

Collagen Evaluation

Collagen deposition was determined by picrosirius red (Sigma) staining, as previously described.14 Briefly, 5-μm tissue sections were stained with sirius red and imaged using a light microscope. Total collagen area (red staining) was normalized to total LV area in 3 separate sections per animal using ImageJ.

Statistics

All statistical analyses were performed using Graphpad Prism software, as described in the Figure legends. To determine differences between multiple groups, 1-way ANOVA was used. If significant differences were found, Tukey-Kramer post-tests were performed and reported. Data reported are mean±SEM.

Results

Development of the Inducible, Cardiac-Specific, Catalase-Overexpressing Mouse

We developed an inducible, cardiac-specific, catalase-overexpressing mouse by crossing tamoxifen-inducible, α-myosin chain–driven Mer-Cre-Mer mice with mice containing a transgene for human catalase driven by the chicken β-actin promoter. The transgene contained a floxed green fluorescent protein and stop codon before the catalase gene; thus, catalase overexpression is not active until Cre-mediated excision (Figure 1A). All mice were genotyped to confirm presence of the transgenese as previously described.11,12 Using this method, we were able to create 2 different conditions: preconditioned and delayed catalase induction (Figure 1B). Specifically, preconditioning indicates that tamoxifen was given for 5 days and surgery performed on the 7th day; delayed indicates that tamoxifen treatment was initiated immediately after MI.

To determine whether these mice inducibly overexpressed catalase in the heart, we injected adult male mice with either vehicle or tamoxifen (40 mg/kg) intraperitoneally once daily for 5 days. Mice were euthanized 3 and 7 days after cessation of tamoxifen treatment and assayed for catalase activity and protein expression. Although there was no change in catalase levels in noncardiac tissue (data not shown), catalase activity

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was significantly higher in the tamoxifen-treated transgenic mice compared with wild-type or vehicle-treated transgenic mice, and there was no significant increase between 3 and 7 days after tamoxifen cessation (Figure 2A). This increase was completely blocked by incubation of the sample with 3-aminotriazole, suggesting the increase was due to catalase and not another peroxidase (Supplemental Figure 1A). We also observed an increase in catalase protein expression in tamoxifen-treated transgenic mice, confirming that the increase in activity was due to protein overexpression (Figure 2B). To determine whether increases in catalase altered other antioxidant systems, we examined SOD activity in tamoxifen-treated animal hearts. As the data in Figure 2C demonstrate, cardiac SOD activity was equal among all groups, suggesting that alterations in cardiac catalase did not affect SOD levels. Taken together, our findings demonstrate that we developed a novel, inducible murine model that overexpresses human catalase specifically in cardiomyocytes to modest levels.

**H₂O₂ Production in Myocardium**

To determine whether increased cardiomyocyte catalase levels could reduce H₂O₂ levels, we subjected adult male mice to MI injury and examined H₂O₂ levels at 7 days in the LV free wall. Vehicle-treated transgenic mice subjected to MI surgery exhibited a significant increase in H₂O₂ over sham animals, as measured by Amplex Red (Figure 3). Cardiomyocyte-specific catalase overexpression significantly decreased cardiac H₂O₂ levels to sham values (Figure 3). These data suggest that cardiomyocyte-specific overexpression of catalase completely prevents increases in H₂O₂ levels in infarcted tissue after MI.

**Acute Protective Actions of Myocyte-Derived Catalase**

To evaluate the effect of timing for catalase induction, we assessed the outcome of preconditioned and delayed cardiac catalase induction on cardiac function after MI. Fractional shortening was measured as an index of cardiac function at 7 days after MI. Because a prior study has suggested that the presence of Cre may affect cardiac function, we examined vehicle-treated transgenic mice subjected to sham and MI surgery and found no significant difference in function compared with wild-type mice (Supplemental Figure 1B); thus, we used vehicle-treated transgenic mice as controls in subsequent studies. We observed a significant decrease in fractional shortening at 7 days in vehicle-treated transgenic mice subjected to MI (Sham/Tg: 49.8 ± 3.9% versus MI/Tg: 30.7 ± 2.4%; P < 0.01). Delayed catalase-overexpressing mice also demonstrated significantly reduced function compared with sham animals (MI/Tg: 50.9 ± 2.0% versus MI/Tg+ delayed: 32.2 ± 3.4%; P < 0.01) (Figure 4A). Interestingly, the preconditioning group demonstrated a trend toward improvement but was not statistically different from vehicle-treated MI mice (MI/Tg + preconditioned: 38.2 ± 2.8%; P > 0.05). Infarct size as measured by delayed contrast enhancement after MRI was not different among the groups (Supplemental Figure 2).

To determine whether delayed catalase induction had any biochemical benefits, we examined the regulation of proinflammatory and profibrotic genes in the LV homogenates using real-time polymerase chain reaction 7 days after MI. Both TNF-α and connective tissue growth factor (CTGF) were both significantly upregulated in vehicle-treated MI mice compared with sham animals (Figure 4B and 4C). Scavenging of H₂O₂ significantly abolished the increase of both TNF-α and CTGF by approximately 65% and 76%, respectively (Figure 4C). Taken together, these data demonstrate there is no statistically significant beneficial effect in cardiac function though even delayed reduction in H₂O₂ levels had underlying biochemical effects.

**Chronic Protective Actions of Myocyte-Derived Catalase**

To determine chronic H₂O₂ scavenging ability of delayed cardiomyocyte-catalase overexpression, we measured H₂O₂...
levels 21 days after infarction. Similar to the 7-day results, vehicle-treated transgenic MI mice at 21 days exhibited a significantly greater level of H₂O₂ compared with the sham animals (Figure 5). Whereas sham transgenic mice treated with tamoxifen had similar baseline levels, delayed catalase overexpression significantly (P<0.05) decreased MI-induced H₂O₂ levels, suggesting that catalase is still active at 21 days after treatment.

Because of the biochemical improvements seen early with a lack of functional improvements at 7 days, we sought to determine the functional effect of catalase overexpression at 21 days. Similar to 7 days, MI significantly decreased fractional shortening in vehicle-treated mice (P<0.001); however, both preconditioned (P<0.05) and delayed (P<0.01) catalase overexpression significantly improved this parameter (Figure 6A). Ejection fraction was also measured in these animals, and similar results were obtained (Figure 6B). Although there was a trend toward an improvement in end-diastolic volume, there were no significant changes with catalase overexpression; however, significant improvements in end-systolic volumes were seen with preconditioned (P<0.05) and delayed (P<0.05) catalase overexpression (Figure 6C and 6D). Finally, there was a significant improvement in LV mass in both preconditioned (P<0.05) and delayed (P<0.05) catalase overexpression (Figure 6E). These data demonstrate that despite no effect of catalase overexpression on acute function, both preconditioned and delayed catalase overexpression significantly improved function during the chronic phase of MI to the same degree.

Potential Mechanism of Chronic Improvement
To determine levels of fibrosis, heart sections were stained with sirius red 21 days after infarction and fibrotic scar area was normalized to total LV area. Representative sections for MI/Tg−, MI/Tg+ delayed, and MI/Tg+ preconditioned are shown in Figure 7A through 7C. Grouped data demonstrate a significant reduction in scar area with both catalase overexpressing mice (Figure 7D). Collagen 1A and 3A are the 2 major collagen isoforms expressed in the heart and newly deposited scar tissue. We observed that the vehicle-treated transgenic MI mice at 21 days had a significant increase in collagen 1A mRNA levels compared with the vehicle-treated transgenic sham mice (Figure 7E). Induction of catalase showed a trend for lower collagen 1A levels compared with the vehicle-treated transgenic mice at 21 days after MI (M/I/Tg−: 4.0±2.2 versus MI/Tg+: 2.1±0.56; P>0.05) (Figure 7E). On the other hand, the contractile collagen 3A mRNA expression in the tamoxifen-treated MI mice at 21 days was significantly (P<0.05) increased compared with vehicle-treated MI mice (Figure 7F). Taken together, these data demonstrate that chronic catalase overexpression not only reduces total collagen content but alters collagen isoforms expression as well.

Discussion
In the present study, we used an inducible, tissue-specific transgenic mouse model to determine temporal effects of phys-
We generated these new double-transgenic mice by cross-breeding 2 existing transgenic mice, the well-established α-myosin heavy chain Mer-Cre-Mer mouse, with a mouse containing a floxed stop codon between green fluorescent protein and the human catalase gene. We found no significant changes between wild-type mice and double transgenic mice treated with vehicle, indicating very little leakage of the gene. Additionally, prior studies from the Mer-Cre-Mer mice demonstrated very low levels of leakage by reporter gene detection, <1% at 2 months and <2% even after 1 year. Administration of tamoxifen for 5 days with a 3-day waiting period induced expression of catalase only in the heart and the increase in activity was completely abolished by 3-amino triazole, indicating no other peroxidase was involved. Longer time periods (7 days) produced no further changes, reinforcing that leakage was very low because recombination did not continue, and there was no regression of increased catalase activity. This increase in cardiac catalase was modest in comparison to prior studies with catalase overexpression in certain mouse models. Because this study generated a new double-transgenic mouse, Mer-Cre-Mer mice treated with tamoxifen were not examined alone. Despite this potential issue, all mice were compared with wild-type mice (Supplemental Figure 1), and recommendations of the prior study were followed including reduced dosing of tamoxifen (40 mg/kg used in this study, compared with 80 mg/kg reported to cause cardiomyopathy) and a waiting period. Despite our comparison of both tamoxifen and vehicle-treated MI mice to wild-type mice and reduced dosage, it may be possible that nonspecific tamoxifen effects may add confounding variables to the study; future experiments may include raloxifen-treated mice as well.

Using these mice, we created 2 conditions; the first we termed preconditioned as tamoxifen was given for 5 days (with a 3-day waiting period) before surgery and the second was termed delayed overexpression as tamoxifen treatment was initiated at the conclusion of surgery for 5 days thereafter.
Levels of LV H$_2$O$_2$ were significantly increased at 7 days after infarction, and delayed catalase induction was able to scavenge this potentially toxic species, suggesting that even delayed catalase overexpression in cardiomyocytes was sufficient to reduce H$_2$O$_2$ levels in the injured myocardium. However, despite this significant decrease in H$_2$O$_2$ concentrations, there was no acute improvement in cardiac function in either the delayed or preconditioned mice. This is in contrast to prior studies demonstrating acute functional improvements in cardiomyocyte catalase–overexpressing mice. Given the strong role of H$_2$O$_2$ as a vital second messenger in healthy tissues, it is quite possible that these studies overexpressing catalase to such high levels for prolonged periods of time abolished basal H$_2$O$_2$ signaling in the heart, leading to alterations in many pathways. In fact, many contractile and calcium-regulating proteins are redox-sensitive; thus, the potential for chronic alterations in myocyte function are significant. In keeping with our findings, catalase protein therapy studies demonstrated little effect of catalase protein delivery at acute time points in preventing myocardial injury. Supporting the lack of effect are studies showing improvements in function with postconditioning, with no effect on myocardial catalase levels.

Although we did not observe an improvement in cardiac function at 7 days with catalase overexpression, there were underlying biochemical changes evident in the delayed catalase overexpression group. An additional effect observed acutely after MI is the progression of inflammation localized in and around the infarcted zone. Infiltrating cells and cardiomyocytes contribute to the inflammation by synthesizing and releasing profibrotic and proinflammatory molecules. These inflammatory molecules are upregulated by H$_2$O$_2$ in a short and temporal manner to initiate the remodeling process.

Figure 6. Cardiac function 21 days after MI. A, Fractional shortening; B, ejection fraction; C and D, end-systolic and end-diastolic LV volume (LVESV and LVEDV); and E, LV mass (LVM) between various groups as measured by small-animal echocardiography. Both delayed and preconditioned (precon) catalase overexpression (Tg +) significantly improved indicated measurements of cardiac function at 21 days compared with vehicle-treated MI mice (Tg –). Statistical comparisons were made by ANOVA (A, $P<0.0001$; B, $P<0.0001$; C and D, $P=0.005$; and E, $P<0.0001$) followed by Tukey-Kramer post test. *$P<0.05$ versus respective sham; #$P<0.05$ versus vehicle-treated MI. $n=5$ animals per group. All data are mean±SEM.
abolished the increase in both of these markers and returned message levels to that of the sham group. H$_2$O$_2$ has been shown to be an important mediator of TNF-α–induced cellular damage and dysfunction in isolated ventricular myocytes. It was shown that in vitro, H$_2$O$_2$ is a potent stimulator of CTGF expression, and our data demonstrate a potential role for this regulation in vivo.

Next, we assessed chronic H$_2$O$_2$ scavenging ability and cardiac function with cardiomyocyte-specific overexpression of catalase after MI. Evaluation of cardiac H$_2$O$_2$ levels at 21 days after MI indicated that overexpression of catalase was still present, and the enzyme continued to significantly scavenge elevated levels of H$_2$O$_2$, seen in vehicle-treated mice. The delayed MI groups expressed significantly lower H$_2$O$_2$ concentrations than vehicle MI mice. Our data are in agreement with prior catalase overexpression studies in which catalase overexpressing mice continued to express active catalase long term. Interestingly, sham mice treated with tamoxifen demonstrated a reduction in basal H$_2$O$_2$ levels over the course of the study, though it did not reach statistical significance. These data underscore the need for inducible systems to examine the effect of catalase overexpression, as chronic basal reduction of H$_2$O$_2$ levels could serve as a confounding variable by altering normal signaling pathways. Chronic scavenging of H$_2$O$_2$ led to an improvement in several measures of cardiac function in both delayed and pretreated mice, suggesting that sustained scavenging may be more important than immediate reductions in H$_2$O$_2$. These data are in contrast with other oxidants such as superoxide, which peaks quite early in the injury process, and immediate...
scavenging may be beneficial.13 Mechanistically, early scavenging of H₂O₂ may play more of a role in the inhibition of proinflammatory and profibrotic signaling molecules than in acute cell death.

In addition, supraphysiological overexpression of cardiac-specific catalase from birth has been found to significantly attenuate age-induced contractile dysfunction and damage to the myocardium.19 We show that these early biochemical changes resulted in an improvement in cardiac remodeling by altering both the total collagen content of the tissue as shown by sirius red staining, as well as the collagen isoform content of the scar tissue. In fact, regulation of CTGF is critical in determining cardiac fibrosis during hypertrophy and plays an important role in upregulating collagen 1A levels after MI.27,31,33–35 Moreover, H₂O₂ may play an important role in regulating collagen cross-linking and tensile strength during catalase overexpression.33–35,37

We show in our inducible cardiomyocyte-specific, catalase-overexpressing mice that the improvement in cardiac function may be the result of the collagen 1A being replaced with the less stiff collagen 3A. Prior studies demonstrate that collagen 3A:1A ratios may play an important role in the contractile function of the heart in animal and human studies, and this may be a potential mechanism in the systolic functional improvements seen with catalase overexpression.33–35,37

In summary, we found that inducible, cardiomyocyte-specific catalase overexpression significantly upregulated the expression and activity of the enzyme to physiologically relevant levels. MI significantly elevated H₂O₂ concentrations that were lowered by catalase overexpression, persisting for the duration of the study. We also demonstrated that lower H₂O₂ levels significantly decreased proinflammatory and profibrotic markers acutely, although cardiac function was not improved 7 days after MI. In contrast, improvements in cardiac function were observed at 21 days after MI, which was associated with a change in collagen isoform expression. Cardiac-derived, catalase-overexpressing mice exhibited reduced scar tissue and higher amounts of the more contractile collagen 3A isoform, which may play a critical role in the improvement seen in systolic cardiac function at the later time point. We conclude that the temporal aspect of scavenging cardiac H₂O₂ after MI is a critical variable, and, despite lack of efficacy of protein delivery immediately after MI, efforts should be made for sustained scavenging over time to promote the healing process.

Sources of Funding
This work was supported by grants from the National Institutes of Health (HL094527, Dr Davis; HL007745, Dr Pendergrass and Dr Maiellaro-Rafferty; and HL090584, Dr Taylor) and as a postdoctoral fellowship from the American Heart Association (09POST2060589, Dr Maiellaro-Rafferty).

Disclosures
None.

References


**CLINICAL PERSPECTIVE**

Congestive heart failure is a leading cause of morbidity and mortality worldwide, and effective treatment options are greatly needed. Reactive oxygen species increase greatly after myocardial infarction and are thought to be a potential therapeutic target. Although some studies using genetic manipulation have shown promise, protein delivery studies of antioxidants have demonstrated mixed results. One potential reason could be that genetic overexpression studies examine chronically supraphysiological levels of antioxidant therapy and could include preconditioning effects. To negate this, we created a mouse model of catalase overexpression that was both tissue-specific and inducible. By using tamoxifen, we could turn on physiologically relevant levels of catalase at different time points only in cardiomyocytes. The data in the present study demonstrated that there was little benefit to acute cardiac function if catalase overexpression was induced at the same time of injury. However, there were underlying biochemical changes to fibrosis-related genes that manifested in chronic improvements in function. Regardless of when catalase was induced, overall scar formation was reduced, and more contractile collagen isoforms were present. This led to increases in ejection fraction, left ventricular mass, and end-systolic function. Taken together, our data showed that the sustained presence of catalase, rather than acute induction, could be an important modulator of postinfarction healing. Thus, for future therapy involving antioxidants, it may be prudent to improve the delivery to account for sustained release rather than focusing efforts on acute administration.
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_Circ Heart Fail._ 2011;4:98-106; originally published online October 22, 2010; doi: 10.1161/CIRCHEARTFAILURE.110.957712

_Circulation: Heart Failure_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1941-3289. Online ISSN: 1941-3297

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Supplemental Figure 1. Specificity of Cre recombinase upregulation on catalase activity and cardiac function over time. A. Addition of 3-aminotriazole (AT) abolished increase in catalase activity in catalase overexpressing mice (Tg) treated with tamoxifen (Tg+), indicating increase was not due to another peroxidase. All groups treated with AT unless indicated. B) Expression of Cre recombinase did not alter cardiac function. Wild-type mice were not statistically different from vehicle-treated transgenic (Tg-) mice after sham or MI surgery. Statistical comparisons were made by ANOVA (A, p<0.001; B, p=0.003) followed by Tukey-Kramer post test. n = 5-9 hearts per group.
Supplemental Figure 2. Infarct size measurements. Infarct size was measured acutely (1 day) following infarction with magnetic resonance imaging (delayed contrast enhancement of gadolinium). There were no significant differences between the groups by ANOVA (p=0.79; n≥3).