Scavenging Superoxide Selectively in Mouse Forebrain is Associated with Improved Cardiac Function and Survival Following Myocardial Infarction

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Abstract

Dysregulation in central nervous system (CNS) signaling that results in chronic sympathetic hyperactivity is now recognized to play a critical role in the pathogenesis of heart failure (HF) following myocardial infarction (MI). We recently demonstrated that adenovirus-mediated gene transfer of cytoplasmic superoxide dismutase (Ad-Cu/ZnSOD) to forebrain circumventricular organs, unique sensory structures that lack a blood-brain-barrier and link peripheral blood-borne signals to CNS cardiovascular circuits, inhibits both the MI-induced activation of these central signaling pathways and the accompanying sympathoexcitation. Here, we tested the hypothesis that this forebrain-targeted reduction in oxidative stress translates into amelioration of the post-MI decline in myocardial function and increase in mortality. Adult C57BL/6 mice underwent left coronary artery ligation or sham surgery along with forebrain-targeted gene transfer of Ad-Cu/ZnSOD or a control vector. The results demonstrate marked MI-induced increases in superoxide radical formation in the one of these forebrain regions, the subfornical organ (SFO). Ad-Cu/ZnSOD targeted to this region abolished the increased superoxide levels and led to significantly improved myocardial function compared to control vector-treated mice. This was accompanied by diminished levels of cardiomyocyte apoptosis in the Ad-Cu/ZnSOD but not the control vector-treated group. These effects of superoxide scavenging with Ad-Cu/ZnSOD in the forebrain paralleled increased post-MI survival rates compared to controls. This suggests that oxidative stress in the SFO plays a critical role in the deterioration of cardiac function following MI, and underscores the promise of CNS-targeted antioxidant therapy for the treatment of MI-induced HF.

Key Words: heart failure, antioxidant gene therapy, survival, sympathetic nervous system
Introduction

Coronary artery disease leading to myocardial ischemia and infarction (MI) is the primary cause of chronic heart failure (HF) (20). Early after an acute MI, neural and humoral compensatory mechanisms act to maintain adequate perfusion to vital organs (25). Over time, however, a gradual loss of cardiomyocytes due to apoptosis results in myocyte slippage and mural thinning, causing a chronic deterioration in cardiac function that culminates in HF (17, 22). Despite advances in the diagnosis and treatment of heart disease, MI-induced HF continues to be a leading cause of morbidity and mortality in the United States (20).

Abnormalities in central nervous system (CNS) signaling, resulting in excessive sympathetic drive, is strongly implicated in the post-MI decline to HF (7, 13, 35). This neuro-cardiovascular dysfunction increases the risk of cardiac arrhythmias during HF and is positively correlated with mortality (7). Increased plasma norepinephrine levels are a strong predictor of poor prognosis (7), and causal links between chronic sympathoexcitation, cardiomyocyte apoptosis and heart failure have recently been established (6, 17, 26).

Oxidative stress has received considerable attention recently as an important mechanism in the pathogenesis of HF. Elevated levels of reactive oxygen species (ROS) and decreased amounts of antioxidants have been observed in the plasma and tissues of HF patients (23). Excessive ROS are known to impair cardiac performance, and systemic antioxidants attenuate the development of HF in several different animal models (23).
important concept to emerge recently is that redox mechanisms in the CNS may play a
critical role in the pathophysiology of neuro-cardiovascular diseases (12, 13, 35).
Zanzinger et al. (29) first demonstrated that microinjection of the ROS scavenger
superoxide dismutase (SOD) into the brainstem decreased the exaggerated sympathetic
tone in pigs receiving chronic nitrate therapy. Our studies have shown that excessive
superoxide production in the CNS mediates neurogenic AngII-dependent hypertension in
mice (33), and others have demonstrated that this is due to ROS-mediated increases in
sympathoexcitation (15, 35). Furthermore, we have shown that ROS scavenging in
forebrain circumventricular organs (CVOs), particularly the subfornical organ (SFO),
diminishes the post-MI-induced sympathoexcitation in HF (14). Zucker and colleagues
have gone on to show that NADPH oxidase activity in the rostral ventrolateral medulla
(RVLM), a brainstem nucleus that links the forebrain CVOs with increased sympathetic
outflow, plays an important role in the sympathoexcitation observed in a rabbit model of
chronic heart failure (9).

Given the importance of sympathetic hyperactivity in the development of HF, along with
recent findings that central oxidative stress is involved in driving central autonomic
dysfunction, here we tested the hypothesis that selective forebrain-targeted scavenging of
ROS ameliorates the post-MI decline in myocardial function. Using a mouse model of MI
and adenoviral vectors to cause long-term modulation of the redox state, our results
demonstrate that scavenging ROS in the SFO led to improved myocardial function. This
was accompanied by diminished levels of cardiomyocyte apoptosis and increased post-
MI survival. These findings suggest that antioxidant strategies directed at CNS
cardiovascular control regions may provide a novel therapeutic approach for the
treatment of MI-induced HF.

**Materials and Methods**

**Animals**

Adult male C57BL/6 mice (8 wks) were used for all experiments. Animals were fed standard chow (Harlan Laboratories) and water *ad libitum*. All procedures were approved by the Animal Care and Use Committee at The University of Iowa and Cornell University. Care of the mice met or exceeded the standards set forth by the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, USDA regulations, and the AVMA Panel on Euthanasia.

**Myocardial Infarction and CNS Viral Gene Transfer**

MI was induced by ligation of the left anterior descending (LAD) coronary artery as described in detail previously (14). Briefly, mice were anesthetized, intubated, and ventilated. The heart was exposed via the third intercostal space, and the LAD was ligated using 8-0 ethilon suture. The thoracic wall was closed and the mice were extubated. Sham-operated animals underwent the same procedure, except the LAD was not ligated. During the same surgical session mice then underwent intracerebroventricular (ICV) injections of titer-matched stocks of adenoviral vectors (1x10^9 pfu/ml, 500nL) encoding either human cytoplasmic Cu/Zn superoxide dismutase (Ad-Cu/ZnSOD) or control β-galactosidase (Ad-LacZ). We have previously demonstrated that ICV delivery
results in robust gene transfer to SFO 100% of the time, with very occasional transduction of another forebrain CVO, the organum vasculosum of the lamina terminalis (OVLT) (<5% of the time)(21). At the conclusion of the experiments, SOD expression was localized by immunohistochemistry in brain sections using a sheep anti-human Cu/ZnSOD antibody (1:200, The Binding Site Limited, United Kingdom) as described (32). Images were collected digitally using a Nikon Labphot-2 Microscope equipped with epifluorescence and a Pixera 600 imaging system.

Analysis of Brain Superoxide Levels

A subset of mice were sacrificed 2 wks post-surgery and the brains were removed and immediately frozen on dry ice for measurement of superoxide levels as described previously (34). Briefly, brains were cryo-sectioned (30 µm) onto glass slides, rinsed in 1 M phosphate buffered saline (PBS) for 5 min, and incubated in 1 µM of dihydroethidium (DHE, in PBS) for 5 min in the dark. Slides were then rinsed in PBS for 2 min and imaged using confocal microscopy (Zeiss LSM 510) using an excitation wavelength of 543 nm and a rhodamine emission filter. DHE-treated tissues for all groups were processed and analyzed in parallel with identical detector and laser settings. Fluorescence intensity in the SFO was analyzed using Image J software as described (34). All data are expressed relative to sham animals.

Hemodynamic Measurements

Hemodynamic measurements were performed as described (16, 18) at 2 and 4 wks following MI or sham surgery. Mice were anesthetized with sodium pentobarbital (25
mg/kg, i.p.) and a 1.4F Millar catheter (Millar Instruments Inc., USA) was inserted into the left ventricle (LV) via the right common carotid artery under the guidance of the pressure signal as described (16). LV pressures were recorded for 10 min, data were digitized (Power Lab®, Chart Version 4.01) and dP/dt+ and dP/dt- were calculated as an index of myocardial contractility as described (18). The time series for LV pressure were interpolated using the cubic spline method (4) to a new sampling rate of 2000 Hz in order to obtain more accurate estimates of dP/dt+ and dP/dt-.

Determination of Cardiac Mass and Infarct Size

At the conclusion of the hemodynamic studies, mice were weighed and then sacrificed. Hearts were removed, blotted of excess blood, and weighed. Cardiac mass is expressed as a ratio of heart weight to body weight (mg/g). Hearts were then cryo-sectioned (short-axis, 20 µm), mid-LV sections were mounted onto glass slides and samples were counter-stained with hematoxylin and eosin. Digital images of the sections were acquired and Scion Image (Scion Corp.) was used to determine the infarct size as described previously (14).

Echocardiography

Transthoracic echocardiography was performed in a separate subset of mice using a Vevo 770 high resolution imaging system (VisualSonics, Toronto, Canada) under isoflurane (1%) anesthesia. Excess hair was removed from the thoracic region with chemical treatment (Nair, Church & Dwight Co. Inc., Princeton, NJ) to minimize signal attenuation. Images were acquired using a single-element mechanical transducer with
broadband frequency of 45 Mhz coupled to the chest using warmed Aquasonic 100
transmission gel (Parker Labs, Fairfield, NJ). Parasternal long and short axis (mid
papillary level) retrospective B-mode cineloops were acquired for analysis (1). Fractional
shortening (FS) was calculated as \[
\frac{(LV \text{ end diastolic dimension}-LV \text{ end systolic dimension})}{LV \text{ end diastolic dimension}} \times 100
\]. Due to LV asymmetry which occurs after infarction, LV end diastolic volume (LVEDV) and LV end systolic volume (LVESV) were measured from the parasternal long axis view and calculated using Simpson’s formula (3). Ejection fraction (EF) was calculated as \[
\frac{(LVEDV-LVESV)}{(LVEDV)} \times 100
\]. To calculate infarct size, length of infarcted myocardium was measured from the parasternal short axis view and expressed as a percentage of the left ventricle circumference.

Quantification of Apoptotic Nuclei using TUNEL Assay

Alternating mid-LV cryo-sections were mounted on poly-L-lysine coated slides (Sigma)
and TUNEL assays were performed using ApopTag Plus Peroxidase in Situ Apoptosis
Detection Kit (Serologicals Corporation) according to the manufacturer’s instructions. As a
positive control, sections were treated with DNase I to enzymatically induce the formation
of DNA strand breaks. Negative control samples underwent the same protocol except
biotin-16-dUTP or terminal deoxynucleotidyl transferase was not included in assays
performed on DNase I-treated sections. The apoptotic index was determined by dividing
the number of nuclei labeled by biotinylated dUTP by the total number of nuclei per unit
area of tissue (outside of the infarct and peri-infarct zones) in 3 randomly selected fields
per LV section (3 sections per sample) analyzed by a blinded observer. All data are
expressed relative to sham animals.
Quantification of Apoptosis using DNA Laddering

Hearts were removed from a separate cohort of mice from each of the treatment groups (2 wk time-point) and placed on ice. Peri-infarct and non-infarct LV tissue was collected using a dissection microscope and immediately frozen on dry ice. Genomic DNA was isolated and quantified (Nanodrop 1000). Apoptotic fragments were enriched using dephosphorylated adaptor molecules ligated to 0.3 µg DNA followed by hot start PCR according to manufacturer instructions (APO-DNA1, Maxim Biotech, San Francisco, CA). PCR products were run on a 1.5% agarose gel and visualized under UV light. 200, 400, 600, and 800bp band densities were quantitated using Image-J software.

Statistical Analyses

All data are expressed as mean ± SEM and were analyzed by ANOVA (after Bartlett’s test of homogeneity of variance) followed by the Newman-Keuls correction for multiple comparisons using Prism (GraphPad Software, Inc.). We compared the proportion of MI mice that survived between treatment groups using Fisher’s Exact Test.

Results

MI increases superoxide radical formation in the SFO

We first performed DHE fluorescence microscopy to directly examine superoxide levels in brains of MI and sham mice. As seen in the representative photomicrograph in Figure 1A and the summary data in Figure 1B, MI caused a nearly seven-fold increase in
superoxide-mediated fluorescence in SFO of control vector-treated mice compared to sham animals at 2wks. Comparable changes in ROS were not observed in any other brain region. Ad-Cu/ZnSOD abolished the ROS increase in SFO (Figure 1A,B), providing corroborating evidence that the free radical generated in this brain region is cytoplasmic superoxide. We further confirmed effective Ad-mediated gene transfer of Cu/ZnSOD to the SFO by performing immunohistochemistry following our experiments. As shown in Figure 1C, Cu/ZnSOD is expressed at a high level throughout the SFO of Ad-Cu/ZnSOD-treated mice compared to extremely low levels of background staining in Ad-LacZ-treated mice. Transduction of other forebrain structures was not observed.

Forebrain-targeted Superoxide Scavenging is Associated with Improved Myocardial Function Following MI

We next examined whether forebrain targeting of AdCu/ZnSOD translates into improved cardiac performance. Coronary ligation resulted in hemodynamic changes indicative of LV dysfunction (Figure 2A). Since there were no differences within groups at the two time-points, the data were combined. Compared to sham animals, MI mice exhibited a marked decrease in left ventricular peak systolic pressure (LVSP), as well as impairment in LV contraction and relaxation (± dP/dt). Gene transfer of the control vector Ad-LacZ had no effect on these responses (Figure 2B). In contrast, mice that had undergone forebrain-targeted gene transfer of Ad-Cu/ZnSOD at the time of coronary ligation had significantly improved cardiac performance, such that the LVSP and ± dP/dt were not significantly different from sham animals (Figure 2B). Interestingly, we did not observe any significant increases in LV end-diastolic pressure (LVEDP) in MI mice compared to sham-operated
controls, nor was this parameter affected by CNS gene transfer of Ad-Cu/ZnSOD (Figure 2B). Similarly, but less surprisingly, there were no significant differences in heart rate between groups (sham, 430±24; MI, 418±21; MI + AdLacZ 421±21; MI + Ad-Cu/ZnSOD 468±20 bpm, n=9-11 per group, p>0.05).

High-resolution ultrasound was also performed 48 hrs after surgery to confirm successful arterial ligation and to quantitate “baseline” infarct sizes, indicated by akinetic wall motion along the anterolateral border of the LV (images not shown). In addition, a similar extent of infarction was observed across the various treatment groups (percent of wall infarcted in short axis: MI, 26.6±1.6%, n=12; MI + Ad-LacZ, 28.9 ±2.6%, n=7; MI+Ad-Cu/ZnSOD, 28.7 ±0.9%, n=11; P>0.05). A summary of LV function data combined from measurements taken between 2 and 4 wks following sham or MI procedure are shown in Figure 3. It should be noted that as with the hemodynamic study above, the data were combined across the 2 to 4 wk period since there were no significant differences within the groups at the different time-points. LAD ligation caused marked reductions in FS (Figure 3A) and EF (Figure 3B), indicating severe impairment of LV contractility. Importantly, Ad-Cu/ZnSOD significantly improved both of these indices compared to the control vector, suggesting that increased redox signaling in SFO contributes to declining cardiac performance during HF. MI also caused significant increases in end systolic and diastolic volumes, (Figures 3C and 3D), further demonstrating impaired LV contraction as well as chamber enlargement. While LVESV was significantly reduced in MI mice treated with Ad-Cu/ZnSOD (Figure 3C), there was no significant effect of treatment on LVEDV (Figure 3D). Finally, similar to results from the catheterization studies, HR was not
significantly different (sham + Ad-LacZ, 431±9 BPM, MI+Ad-LacZ, 426±16 BPM, MI+Ad-Cu/ZnSOD 447±15 BPM, n=11-14 per group, P>0.05), suggesting that differences in LV performance between groups were not due to the effects of anesthesia.

Despite the improvement in function in Ad-Cu/ZnSOD-treated mice, MI-induced increases in cardiac mass (sham, 4.8±0.2, and MI, 6.8±0.5; n=9-11 per group, P<0.05) were not differentially affected in this group compared to Ad-LacZ-treated animals (MI+Ad-LacZ, 7.6±0.4, and MI+Ad-Cu/ZnSOD, 6.8±0.3; n=9-11 per group, P>0.05). Infarct sizes were also similar in all MI groups both by H & E staining of cardiac sections (MI, 53.9±3.6%, MI+Ad-LacZ, 53.8±2.8%, MI+Ad-Cu/ZnSOD, 55.4±2.5%; n=9-11 per group, P>0.05) and by echocardiography at 2 wks (MI, 43.9±1.3%, n=12, MI+Ad-LacZ, 43.9±3.1%, n=9, MI+Ad-Cu/ZnSOD, 42.6±1.3%, n=11, P>0.05).

**Overexpression of Cu/ZnSOD in the Forebrain Decreases Myocyte Apoptosis Following MI**

We hypothesized that decreased apoptosis in the surviving myocardium may account for the improved myocardial function in Ad-Cu/ZnSOD-treated MI mice, and therefore performed TUNEL assays to identify apoptotic nuclei. As shown in the representative photomicrographs and summary data in Figure 4, MI treatment markedly increased the cardiomyocyte apoptosis compared to sham, which was significantly reduced in MI treated mice receiving central gene transfer of Cu/ZnSOD.
We also performed DNA laddering experiments to confirm the results of the TUNEL assay. As shown in Figures 4C and 4D, MI caused ~3.5-fold increases in DNA laddering, which was significantly reduced by Ad-Cu/ZnSOD. These effects were largely restricted to the peri-infarct border of the left ventricle, as non-infarcted myocardium from all treatment groups exhibited virtually no DNA laddering (data not shown). Together, these results support a role for central redox signaling in the increased myocardial apoptosis seen after MI.

Viral Gene Transfer of Cu/ZnSOD to the Forebrain Decreases Mortality Following MI

There were dramatic differences in long-term post-MI survival between experimental groups. Of all the mice that underwent the MI procedure, 91.3% (63 of 69) survived the initial post-surgery period. However, by seven days post-surgery, approximately 30% (6 of 20) of MI-only treated mice had died (Figure 5). Similar mortality rates were observed in the MI mice that received control vector (6 of 21, 30%) (Figure 5). Interestingly, there was no additional mortality observed between two and four weeks in either MI treatment group (data not shown). Upon necroscopic inspection, all of the animals that died showed a large accumulation of blood in the thoracic cavity, suggesting left ventricular rupture. In contrast, only 5% (1 of 22) of the Ad-Cu/ZnSOD-treated MI mice died during this time period (Figure 5), with 21 of 22 of these mice surviving to the conclusion of the experiments suggesting that increased superoxide scavenging in the brain protected mice from the deleterious effects of MI.

Discussion
Developing heart failure is associated with unchecked neurohumoral excitation that progressively fuels cardiovascular deterioration (13, 25). Mounting evidence implicates the CNS as a primary culprit in driving this neural dysfunction (7, 13, 35). We have shown that superoxide radicals in the brain play a key role in the excessive sympatho-excitation that occurs following an MI in mice (14), and here we provide evidence that scavenging increased superoxide formation in the SFO - a pivotal brain region linking peripheral blood-borne signals with CNS cardiovascular circuits - is associated with marked improvement in cardiac performance following MI. Furthermore, we demonstrate that forebrain oxidant scavenging is associated with a decrease in cardiomyocyte apoptosis in the surviving myocardium and increased post-MI survival rates.

Emerging evidence suggests that certain CNS circuits serve as relay centers to integrate input from different organ systems in the periphery and regulate neurohumoral output that plays a crucial role in cardiac dysfunction and remodeling following MI (7, 13, 35). Our results further underscore this notion. Upon binding circulating factors, neurons in the SFO send extensive projections to the hypothalamic paraventricular nucleus (PVN), which plays an integral role in the regulation of vasopressin release and sympathetic tone, both directly and via projections to the RVLM (8, 13). Chronic over-activation of the SFO-PVN axis by Ang-II, aldosterone, interleukins, and tumor necrosis factor-α has been widely implicated in causing the MI-induced sympatho-excitation (7, 10, 28, 30, 31). For example, chronically blocking Ang-II signaling selectively in the brain reduces sympathetic activity, improves cardiac baroreflex function, decreases cardiac hypertrophy, and attenuates the subsequent development of HF following MI (12, 13, 31). Furthermore,
central mineralocorticoid blockade reduces sympathetic drive in HF rats (7, 30) and injection of TNF-α into the carotid artery increases sympathetic output (7). Together, these findings suggest that multiple signaling pathways may converge to increase oxidative stress in the SFO following MI, which may in turn powerfully influence sympathetic activity and the subsequent deterioration of cardiac function. Our results suggest that interruption of this cascade through targeted scavenging of ROS leads to improved cardiac performance. Further studies will be required to tease out the specific molecular substrates affected by central ROS scavenging and improved cardiac function after MI.

Because we observed a marked improvement in cardiac performance in Ad-Cu/ZnSOD-treated MI mice without observing a significant difference in infarct size or cardiac mass in these animals, we hypothesized that central antioxidant therapy may improve cardiac function by somehow preserving the integrity of the surviving myocardium. In support of this hypothesis, we observed a significant decrease in TUNEL-positive nuclei and DNA laddering in the LV of MI mice that had undergone gene transfer of Ad-Cu/ZnSOD compared to control vector-treated MI mice. Previous studies strongly suggest that apoptosis in the LV plays a critical role in the deterioration in cardiac function following MI, and apoptotic index is positively correlated with the degree of myocardial dysfunction in humans (reviewed in 17, 23). Recent studies have also suggested that therapies aimed at diminishing or replacing apoptotic cells in the surviving myocardium improves post-MI cardiac performance (17), even without significantly altering the infarct size. It is well established that increased sympathetic drive induces myocyte apoptosis, and treatment
with beta-receptor antagonists decrease apoptosis in the LV in animal models of HF (6, 25, 26). We have previously demonstrated that scavenging superoxide in the brain decreases sympathetic tone following MI in mice (14). Similarly, Gao et al. have demonstrated that ICV administration of the antioxidant Tempol or the NADPH oxidase inhibitor apocynin significantly attenuated resting renal sympathetic nerve activity in rabbits with HF (9). Together, these findings suggest central ROS scavenging may be linked to improved cardiac performance through decreasing norepinephrine-mediated cardiomyocyte apoptosis in the post-MI heart.

One interesting question is what advantages targeting SOD to the brain provides over systemic treatment with beta-receptor antagonists. Beta-blockade improves cardiac function and improves survival after MI in animal models (24), and is a mainstay of therapy for MI patients. However, optimal treatment is often limited by poor tolerance in humans due to the associated decrease in HR (5). In contrast, although we previously demonstrated that brain gene transfer of Cu/ZnSOD caused a decrease in markers of global sympathetic tone (14), resting heart rate was not affected. Thus, central ROS scavenging may allow for appropriate blockade of sympathetic tone to the LV without unwanted bradycardia due to direct blockade of beta-receptors in the heart.

Our findings that inhibition of sympathetic output by selectively scavenging central ROS paralleled improvements in cardiac performance and survival is intriguing when compared to recent clinical trials where chronic HF patients treated with the central sympathetic inhibitor moxonidine exhibited elevated mortality compared to
HF patients dosed with placebo (2, 19). It is possible that reducing sympathetic output by inhibiting specific redox circuits is beneficial to cardiac function following MI, however complete and/or non-specific ablation of central regulatory pathways may preclude autonomic functions that are beneficial to the failing heart. Finally, in addition to regulating sympathetic tone, targeting forebrain cardiovascular control regions may have further therapeutic effects. The SFO-PVN-RVLM axis is well known to play a key role in regulating other ROS-sensitive signaling molecules involved in the pathogenesis of HF including vasopressin, cytokines, and the renin-angiotensin system (7, 10, 13, 28, 30). Therefore, modulation of central ROS signaling may have an important effect on these targets, and this is the focus of ongoing investigations.

Another striking observation in this study is that gene transfer of Cu/ZnSOD to the forebrain improved survival following MI, apparently by decreasing the risk of LV rupture. Although the mechanisms through which central ROS scavenging would protect against MI-induced LV rupture are not understood, the potential interaction between increased sympathetic nerve activity, β-adrenergic signaling and activation of MMPs in the heart is one intriguing possibility (6, 17, 22, 23). For example, it has been demonstrated that there is increased survival after MI in transgenic mice overexpressing an inhibitor of β-adrenergic receptor kinase (24). Moreover, we have observed a rapid increase in MMPs (peak at 3 days) that precedes LV rupture and death in MI mice, and treatment of mice with the SOD-mimetic Tempol at the time of surgery markedly decreases MMP induction, urinary norepinephrine levels and MI-induced mortality (Lindley, Doobay, Bhalla, Sharma,
Davisson, unpublished data). Thus, it is possible that Ad-Cu/ZnSOD-mediated decreases in post-MI sympathetic drive may improve survival by decreasing $\beta$-adrenergic signaling and MMP induction in the heart.

While we made every attempt to include the appropriate controls for all experiments, there are particular variables which we could not control and therefore limit the conclusions which can be drawn. Hemodynamic and echocardiography measurements made under anesthesia can influence cardiac function, and while we observed no significant differences in heart rates between groups during measurements, the values were nonetheless diminished compared to conscious animals and this should be taken into account. In addition, there certainly are species-related limitations of our study in mice that need to be considered. For example, our observation of post-MI LV rupture is not reflective of the usual pattern of HF development in humans. Although a typical effect of LAD ligation in mice (11, 27), it should be noted that the beneficial effect of SOD on this particular end-point likely does not apply in humans. Similarly, although we have previously shown marked decreases in sympathetic activity (14), and now significant beneficial effects on myocardial function with SOD gene targeting to the forebrain, it should be noted that we cannot definitively conclude that SOD produces these myocardial improvements by only reducing sympathetic tone. As suggested above, it is certainly possible that other ROS-sensitive mechanisms are operating in parallel, and that interruption of these pathways by SOD could contribute to the improvement in cardiac performance. Similarly, while we have shown that ICV injection confers robust transgene expression in the SFO 100% of the time, but can cause transduction of the OVLT...
occasionally (<5% of the time), it is conceivable that ROS scavenging in this other region could influence the improved cardiac performance and decreased apoptosis we observed. However, because we did not observe increased ROS formation in the OVLT, we do not believe that this is likely.

Given the significant LV dysfunction we observed in our MI-treated mice, we were surprised to see no significant increases in LVEDP relative to sham controls at two- and four- weeks (Figure 2B). This could be a function of subsequent eccentric hypertrophy of the preserved myocardium over the 2-4 week post-infarction period, allowing LVEDP to return to near-normal levels at substantially increased LVED volumes (LVEDV). The magnitude of increase in LVEDV (almost three-fold) indicates that this was more than just a result of necrotic myocardium and stretch, but that compensatory eccentric myocardial hypertrophy was involved. Alternatively, this may have been a technical limitation of our intra-cardiac instrumentation, whereby a difference between groups of <5mmHg may exceed the sensitivity threshold. In support of this, our greatest degree of variability between mice of the same treatment group was observed in our LVEDP measurements (Figure 2B).

While ICV delivery of Ad-Cu/ZnSOD improved ± dP/dtmax in MI-treated mice to sham-treated levels, these mice still exhibited significantly reduced EF and elevated LVEDV. However, these can be mutually exclusive - a large EDV does not necessitate a decrease in dP/dtmax (or other measures of systolic function). Therefore, the rate of cardiomyocyte contraction (measured by dP/dTmax) can be similar between groups, although the
ventricular volumes at which these myocytes start to contract are different. LV contraction from a greater starting volume will result in a decreased EF for any specific stroke volume (which is the ultimate determinant of cardiac output). This explains the minimal change in EF despite an improvement in dP/dtmax. Similarly, for the LVEDV to decrease, one would need to invoke cardiomyocyte atrophy to a pre-infarction state - an unlikely explanation in the face of an infarction involving 45% of the LV wall. The compensatory eccentric hypertrophy may allow reduction of LVEDP to sub-critical levels, with a consequent reduction in end-diastolic wall stress. Indeed, our data demonstrate the independence of these 2 different measures of cardiac function. Finally, the volumes involved in our model may be too small to allow small but significant differences to be detected using echocardiographic estimates of cardiac volume (Simpson's method).

**Perspectives and Significance**

In conclusion, our results suggest that oxidative stress in the SFO play a prominent role in the neural dysfunction that accompanies HF. The current study provides evidence for the first time that interfering with redox pathways selectively in the forebrain is associated with improved myocardial function, amelioration of cardiac apoptosis, and increased survival following MI. These findings underscore the critical role central redox signaling plays in the deterioration of cardiac function following MI, and suggest that antioxidant therapy directed to certain regions of the CNS may provide a novel strategy for the treatment of HF.
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References


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**Figure Legends**

**Figure 1. MI increases superoxide formation in the SFO of mouse brain.**
A) Representative photomicrographs of DHE staining in the SFO of sham mice and MI mice that underwent ICV microinjection of Ad-Cu/ZnSOD or Ad-LacZ. B) Summary of DHE fluorescence intensity in the SFO demonstrating a ~7-fold increase in superoxide-mediated fluorescence in this brain region of MI mice (MI+Ad-LacZ, n=5) compared to shams (n=3). Ad-Cu/ZnSOD (n=5) abolished this increase. Three coronal sections (30µMm) through the SFO from each animal were analyzed. *P<0.05 vs sham; †P<0.05 vs MI+Ad-LacZ. C) Typical immunostaining of SFO showing robust human Cu/ZnSOD expression in mice that underwent ICV injections of Ad-Cu/ZnSOD (left) 2 wks earlier, but not in Ad-LacZ-injected mice (right); scale bar=50 µm.

**Figure 2. Increased scavenging of superoxide in the forebrain improves myocardial function following MI.** A) Representative high fidelity recordings of LV pressure obtained with a 1.4f Millar catheter in sham, MI and MI mice that underwent ICV injection of Ad-Cu/ZnSOD or Ad-LacZ. B) Summary data showing that MI resulted in significant reductions in LV peak systolic pressure (LVSP) and impairment in LV contraction/relaxation (+/- dP/dt) (MI, n=9 or MI+AdLacZ, n=10 vs shams, n=9). Myocardial function was significantly improved in MI mice that underwent gene transfer of Ad-Cu/ZnSOD (n=11). There were no differences in LVEDP. *P<0.05 vs sham; †P<0.05 vs MI or MI+Ad-LacZ.
**Figure 3. Forebrain-targeted overexpression of Cu/ZnSOD improves LV function following MI.** Summary of echocardiography data collected 2-4 wks following MI or sham and SFO gene transfer. MI (n=27) caused significant decreases in fractional shortening (A) and ejection fraction (B) compared to shams (n=17), and overexpression of Cu/ZnSOD in the forebrain (n=28) caused significant improvement in these two endpoints. The MI-induced increases in end-systolic volume (LVESV) were significantly improved by Cu/ZnSOD (C), whereas the LV dilation at end-diastole (LVEDV) was not (D). * P<0.05 vs. sham+Ad-LacZ; † P<0.05 vs. MI+Ad-LacZ.

**Figure 4. Central antioxidant therapy is associated with decreased apoptosis in the heart following MI.** A) Representative photomicrographs of TUNEL staining performed on tissue sections from the surviving myocardium of sham, MI and MI mice treated ICV with Ad-LacZ or Ad-Cu/ZnSOD. B) Summary data demonstrating that MI (n=4) caused a nearly 8-fold increase in the apoptotic index in myocardial sections compared to shams (n=6). Forebrain-targeted gene transfer of Ad-Cu/ZnSOD (n=9) reduced the number of apoptotic nuclei to near sham levels, whereas Ad-LacZ (n=10) had no effect. *P<0.05 vs sham; †P<0.05 vs MI or MI+Ad-LacZ; scale bar=25 µm. C) Apoptotic DNA fragmentation was examined by ligation-mediated PCR and visualized by agarose gel electrophoresis. Shown are fragments isolated from peri-infarct myocardium 2 wks after sham (lane 2), MI (lane 3), MI + Ad-Cu/ZnSOD (lane 4), or MI + Ad-LacZ (lane 5). Positive-control DNA provided by the manufacturer is shown in lane 6; lanes 1 and 7 contain molecular weight ladder. D) Summary graph of densitometry analysis performed on 200-, 400-, 600- and 800-base-pair fragments shows an increase in MI-induced (n=4) apoptosis compared to
sham (n=4) which was significantly reduced with targeting of Ad-Cu/ZnSOD to the forebrain (n=5). *P<0.05 vs. sham; † P<0.05 vs. MI or MI+Ad-LacZ.

**Figure 5. Ad-mediated gene transfer of Cu/ZnSOD to the forebrain reduces mortality following MI.** Summary of percentage of animals surviving the first 2 wks after sham or MI surgery. By days 4-7 post-MI, approximately 30% of MI mice had died. ICV injection of Ad-Cu/ZnSOD increased survival in MI mice to nearly the same level as shams, whereas Ad-LacZ had no effect on mortality. There was no mortality among the sham animals. *P<0.05 vs MI alone or MI+Ad-LacZ; (n given in figure).
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5