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Silencing Nox4 in the Paraventricular Nucleus Improves Myocardial Infarction–Induced Cardiac Dysfunction by Attenuating Sympathoexcitation and Periinfarct Apoptosis

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Rationale: Myocardial infarction (MI)-induced heart failure is characterized by central nervous system–driven sympathoexcitation and deteriorating cardiac function. The paraventricular nucleus (PVN) of the hypothalamus is a key regulator of sympathetic nerve activity and is implicated in heart failure. Redox signaling in the PVN and other central nervous system sites is a primary mechanism of neuro-cardiovascular regulation, and excessive oxidant production by activation of NADPH oxidases (Noxs) is implicated in some neuro-cardiovascular diseases.

Objective: We tested the hypothesis that Nox-mediated redox signaling in the PVN contributes to MI-induced sympathoexcitation and cardiac dysfunction in mice.

Methods and Results: Real-time PCR revealed that Nox4 was the most abundantly expressed Nox in PVN under basal conditions. Coronary arterial ligation (MI) caused a selective upregulation of this homolog compared to Nox1 and Nox2. Adenoviral gene transfer of Nox4 (AdsiNox4) to PVN (bilateral) attenuated MI-induced superoxide formation in this brain region (day 14) to the same level as that produced by PVN-targeted gene transfer of cytoplasmic superoxide dismutase (AdCu/ZnSOD). MI mice treated with AdsiNox4 or AdCu/ZnSOD in the PVN showed marked improvement in cardiac function as assessed by echocardiography and left ventricular hemodynamic analysis. This was accompanied by significantly diminished sympathetic outflow and apoptosis in the periinfarct region of the heart.

Conclusions: These results suggest that MI causes dysregulation of Nox4-mediated redox signaling in the PVN, which leads to sympathetic overactivation and a decline in cardiac function. Targeted inhibition of oxidant signaling in the PVN could provide a novel treatment for MI-induced heart failure. (Circ Res. 2010;106:1763-1774.)

Key Words: heart | reactive oxygen species | NADPH oxidase | brain | sympathetic nerves

Despite recent decline in the incidence of many cardiovascular diseases in the United States, that of heart failure (HF) continues to rise. HF is characterized by progressively deteriorating cardiac function, in part because of persistent sympathetic overactivation that is initiated in response to impaired cardiac function. Whereas increased sympathetic drive aids perfusion acutely following a cardiac insult such as myocardial infarction (MI), chronic sympathoexcitation contributes to fluid retention, as well as cardiac arrhythmias, hypertrophy, and apoptosis, all of which contribute to declining cardiac function over time.

The paraventricular nucleus (PVN) of the hypothalamus is a critical site of autonomic and neuroendocrine regulation. The nucleus of the tractus solitarius (NTS) relays afferent information to the PVN, which, in turn, sends efferent projections to the rostral ventral lateral medulla (RVLM) and spinal cord to modulate sympathetic outflow. Neurons of the forebrain circumventricular subfornical organ (SFO) (a structure outside the blood–brain barrier that interacts with peripheral circulating factors) also project to the PVN, which can further stimulate sympathoexcitation as well as the production and secretion of vasopressin. Indeed, the PVN is strongly implicated in the neurohumoral dysregulation observed in HF. Recently, our studies in mice showed that PVN neurons are chronically activated after MI, and that this parallels the sustained elevations in sympathetic outflow during MI-induced HF.

Substantial evidence implicates reactive oxygen species (ROS) as key signaling molecules in PVN and other central nervous system (CNS) nuclei in maintaining cardiovascular homeostasis and in mediating cardiovascular diseases such as hypertension and HF. For example, increased redox...
Non-standard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>Ad</td>
<td>adenovirus</td>
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<tr>
<td>Ang</td>
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<td>AR</td>
<td>adrenergic</td>
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<td>Cat</td>
<td>catalase</td>
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<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>DHE</td>
<td>dihydroethidium</td>
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<td>EF</td>
<td>ejection fraction</td>
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<td>FS</td>
<td>fractional shortening</td>
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<tr>
<td>HF</td>
<td>heart failure</td>
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<td>LAD</td>
<td>left anterior descending coronary artery</td>
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<td>LV</td>
<td>left ventricular</td>
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<td>MI</td>
<td>myocardial infarction</td>
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<tr>
<td>NE</td>
<td>norepinephrine</td>
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<tr>
<td>NFκB</td>
<td>nuclear factor κB</td>
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<td>Nox</td>
<td>NADPH oxidase</td>
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<td>NTS</td>
<td>nucleus tractus solitarius</td>
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<tr>
<td>O$_2^-$</td>
<td>superoxide</td>
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<td>PVN</td>
<td>paraventricular nucleus</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>RVLM</td>
<td>rostral ventral lateral medulla</td>
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<td>SFO</td>
<td>subfornical organ</td>
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<td>SOD</td>
<td>superoxide dismutase</td>
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Abundant evidence implicates the NADPH oxidase (Nox) family of enzymes as a key source of free radicals in the CNS during normal signaling processes and in the oxidative stress that contributes to some neuro-cardiovascular diseases.8,16,17 Tremendous diversity exists in the distribution and regulation of the Nox homologs18 and indeed we have previously reported a distinct pattern of Nox homolog expression in murine fore-, mid-, and hindbrain regions involved in cardiovascular regulation.8 Although several studies have shown that MI-induced cardiac dysfunction is significantly improved in transgenic mice in which various subunits of the NADPH oxidase complex have been globally deleted,19 these knockout models do not permit analysis of the impact of specific Nox-mediated redox signaling in specific brain regions such as the PVN.

Given the importance of the PVN in regulating sympathetic outflow and its involvement in HF, combined with the key role NADPH oxidases play in central neuro-cardiovascular regulation and disease, we tested the hypothesis that dysregulated Nox signaling in the PVN contributes to the enhanced oxidative output and deteriorating cardiac function induced by MI. Using viral gene transfer to modulate Nox expression and ROS levels selectively in PVN, our results demonstrate that Nox4-generated O$_2^-$ in the PVN contributes to the persistent sympathoexcitation and cardiac dysfunction that accompanies the post-MI decline to HF.

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Animals

Adult C57BL/6 mice (~18 to 20 g, 8 to 10 weeks old) were used for all experiments. All procedures were approved by the Animal Care and Use Committee at Cornell University. Care of the mice met or exceeded the standards set forth by the NIH Guide for the Care and Use of Laboratory Animals, US Department of Agriculture regulations, and the American Veterinary Medical Association Panel on Euthanasia.

Adenoviral Vectors

Construction and characterization of recombinant E1-deleted adenoviral vectors encoding human copper-zinc superoxide dismutase (AdCu/ZnSOD), catalase (AdCat),21 bacterial β-galactosidase (AdLacZ),22 and siRNA targeted against eGFP (AdsiGFP) or Nox4 (AdsiNox4)23 have each been described previously. All vectors were obtained from the University of Iowa Gene Vector Core.

Targeted Gene Transfer to the PVN and Induction of MI

Mice were anesthetized with sodium pentobarbital and placed in a stereotoxic apparatus. Viruses were delivered bilaterally to PVN (200 nL total) using titer-matched adenoviral stocks ($10^9$ pfu/mL). Dual virus injections (AdCat experiments) used titer-matched stocks that were mixed before delivery (100 nL each).

MI was induced by surgical ligation of the left anterior descending coronary artery (LAD) as described.6 Briefly, mice were anesthetized with sodium pentobarbital and intubated. The heart was accessed from the third intercostal space and the LAD was ligated with 8-0 Ethilon suture. Sham procedure was identical to MI except without LAD ligation.

Quantitative Real-Time PCR Detection of Nox Homologs

Mice were decapitated and brains flash frozen. PVN tissue was isolated by micropunch, total RNA was harvested, and cDNA was generated using random hexamers. Templates (25 ng) were subjected to triplicate to real-time quantitative PCR using ABI 7500FAST system. Power SYBR Green and Nox1-, Nox2-, or Nox4-specific primers.23,25 β-actin was used for relative quantitation by ΔΔCt method, expressed as $2^{-\Delta\Delta C_t}$, or with 18S RNA for absolute quantitation (expressed as copy number relative to 18S copy number in 100 ng RNA).25

In Situ Hybridization and Western Blot Analysis for Nox4 Detection

Serial cryosections of OCT-embedded brains containing PVN from sham and MI mice were labeled with antisense cRNA probes containing the 225- to 901-bp region of mouse Nox4 mRNA (NM_015760). In situ hybridization was performed using digoxigenin-labeled probes as described previously.26 Nox4 protein was measured by Western blot performed on PVN micropunches, and lysates were subjected to SDS-PAGE as described.23

Measurement of ROS in PVN

Dihydroethidium (DHE) staining was performed as described previously.6,11 Fresh frozen sections containing PVN were mounted onto glass slides, incubated in 1 μmol/L DHE, rinsed, and imaged using a Zeiss LSM510 confocal microscope. DHE fluorescence intensity was analyzed using NIH ImageJ and normalized to sham animals.6,9
Detection of Cu/ZnSOD in Brain Sections
SOD immunohistochemistry was performed as described. Mice were perfused transcardially, brains removed, and cryosections containing PVN were mounted directly onto glass slides. Samples were incubated with a sheep anti-human Cu/ZnSOD antibody followed by donkey anti-sheep rhodamine-conjugated secondary antibody. Images were obtained using a Zeiss Axio Imager D1 fluorescence microscope.

Hemodynamic and Echocardiography Measurements
Hemodynamic measurements were performed as described previously. Left ventricular (LV) pressures were recorded at 4 kHz using a 1.4f Millar catheter and +dP/dt and −dP/dt were calculated. A subset of mice was instrumented with jugular catheters for intravenous injection of isoproterenol over 30 seconds to a final dosage of 1 ng/g. Peak +dP/dt was measured over the subsequent 30 seconds and expressed relative to baseline just before injection.

Transtracheal echocardiography was performed under light anesthesia using a VIVO 770 high resolution imaging system and 707B transducer with 45-MHz broadband frequency (VisualSonics). Retrospective B-mode cineloops obtained from parasternal long and short axes were used to calculate fractional shortening (FS) and ejection fraction (EF).

Measurement of Urinary Norepinephrine
Urine was collected and norepinephrine (NE) measured by ELISA (IBL) according to the instructions of the manufacturer and as previously described.

Power Spectral and Pharmacological Analysis of Autonomic Tone
A separate cohort of mice underwent implantation of radiotelemeters before sham/MI surgery to evaluate autonomic tone both by power spectral analysis and arterial pressure responses to ganglionic blockade. Briefly, mice were anesthetized and instrumented with telemetry devices as described. A subset of mice also underwent PVN-targeted viral gene transfer of AdsiNox4 at this time. Following 3 to 5 days recovery, telemeters were activated for continuous sampling of arterial pressure waveforms at 2 kHz in conscious mice. After 3 days baseline recording, mice underwent MI or sham procedure. High-frequency sampling of pressure waveforms in conscious mice resumed the next day for an additional 14 days, at which time mice received acute hexamethonium injections (5 mg/kg, IP) and arterial pressure responses were recorded. Power spectral analysis of arterial pressure variability before and after MI/sham surgery was performed as described. Spectra were divided into low frequency (0.4 to 1.0 Hz) and high frequency (1.0 to 3 Hz). Data were calculated as a percentage in relation to baseline and presented as low frequency/high frequency relative to sham animals.

Quantification of Apoptosis by DNA Laddering
Mice were euthanized by decapitation, hearts removed and immediately placed on ice, and peri- and noninfarct tissues were dissected. DNA was isolated, apoptotic fragments enriched, and the DNA laddering assay was performed (APO DNA1) as previously described.

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 Bonferroni post test for multiple comparisons using Prism 5 (GraphPad Software, Inc.).

Results
Nox4 Is the Most Abundant Nox Homolog in the PVN and Is Selectively Upregulated Following MI
Excessive Nox1, Nox2, or Nox4 activation contributes to a number of cardiovascular diseases, however the expression profiles of these homologs in the PVN have not been investigated. Using real-time RT-PCR in PVN micropunches, we show that Nox4 is the predominant isoform in PVN under basal conditions (Figure 1A). Nox1 and Nox2 are detectable, but Nox4 is expressed at levels 25 to 30-fold higher than these.
other homologs. This is consistent with our earlier findings that Nox4 is the most abundant Nox homolog in midbrain, a region that encompasses PVN and other regions. We next measured levels of these homologs 2 weeks after MI, a time when there is significant sympathoexcitation and cardiac function is impaired. MI mice exhibited a two-fold increase in Nox4 expression in the PVN compared to sham animals (Figure 1B), whereas levels of Nox1 and Nox2 were unaffected (Nox1: 0.7 fold-sham; Nox2: 0.9 fold-sham; *P*<0.05 vs sham, *n* = 4 to 8 biological samples per group).

To provide more precise spatial resolution of Nox4 expression in PVN, and to verify the efficacy of AdsiNox4 in this brain region, we performed in situ hybridization using Nox4-specific cRNA probes. Sham animals showed punctate staining of Nox4 mRNA in PVN, and this was markedly increased at 14 days post-MI (Figure 1C). We have previously reported that AdsiNox4 effectively and selectively silences Nox4 transcription in cultured neurons and in SFO in vivo, however here we sought to confirm its fidelity in PVN using in situ hybridization. As shown in Figure 1C, bilateral PVN microinjection of AdsiNox4 attenuated the MI-induced increase in Nox4 signal, confirming the accurate targeting of our transgene and its ability to knock down Nox4 in this brain region. Importantly, injection of control siRNA (AdsiGFP) had no effect. Together these results demonstrate that Nox4 is the most abundant Nox homolog in the PVN, it becomes selectively upregulated after MI, and it can be silenced by injection of AdsiNox4.

**MI Causes a Robust Increase in Nox4-Mediated Superoxide Radical Formation in PVN**

To begin to examine the functional consequences of MI-induced Nox4 upregulation in PVN, we analyzed the intracellular ROS profile in PVN using DHE fluorescence confocal microscopy at 3, 7, and 14 days after MI or sham surgery. As shown in representative photomicrographs in Figure 2A (day 14) and summary data in Figure 2B, ROS were not altered at day 3 post-MI but were significantly increased by 7 days and became even more pronounced by 2 weeks post-MI. Bilateral PVN-targeted AdsiNox4 attenuated the MI-induced increase in DHE fluorescence at 14 days to the same level as that produced by viral gene transfer of the O$_2^-$ scavenger Cu/ZnSOD (AdCu/ZnSOD) to this brain region (Figure 2A and 2C). Importantly, these effects were not attributable to a nonspecific effect of the adenoviruses or indiscriminate activation of RNA silencing machinery because MI mice injected with the control vectors AdsiGFP or AdLacZ exhibited DHE intensities that were nearly identical to untreated MI mice (Figure 2A and 2C). We have shown previously that AdCu/ZnSOD not only causes robust and localized Cu/ZnSOD expression in CNS at the 2-week time point, but it also results in a robust increase in SOD activity. To confirm robust PVN-localized transgene expression here, Cu/ZnSOD immunohistochemistry was performed in AdLacZ- and...
AdCuZn/SOD-treated mice. Figure 2D shows high level Cu/ZnSOD expression in PVN of AdCu/ZnSOD- but not AdLacZ-treated mice. Collectively, these results strongly suggest that MI-mediated upregulation of Nox4 in PVN results in a marked increase in O$_2^-$ in this brain region.

**AdsiRNA-Mediated Silencing of Nox4 in PVN Improves MI-Induced Cardiac Dysfunction**

To investigate the cardiovascular consequences of MI-induced Nox4 upregulation in PVN, we measured the effects of PVN-targeted AdsiNox4 on echocardiographic and LV hemodynamic end points in mice 2 weeks after MI or sham surgery. High-resolution ultrasonographic analysis demonstrated normal cardiac function in sham animals as evidenced by unaltered EF and FS compared to naïve mice (EF: naïve, 59.1 ± 2.7% versus sham, 54.7 ± 3.4%; FS: naïve, 37.5 ± 2.0% versus sham, 35.5 ± 1.2%; P > 0.05; n = 11 and 15, respectively). In contrast, a marked diminution in EF (Figure 3A) and FS (Figure 3B) was observed in mice that underwent MI surgery. These findings are consistent with what we and others have reported previously in this model of MI. Importantly, AdsiNox4 microinjected into PVN led to significant improvement in both EF and FS in MI-treated animals, whereas the control AdsiGFP did not alter either parameter (Figure 3A and 3B).

Because AdsiNox4 inhibits ROS formation in PVN (see above), these data suggest that the beneficial effect of AdsiNox4 on cardiac function is attributable to this antioxidant action. However, to provide further evidence for this, we compared these AdsiNox4 ultrasound data to those obtained in a separate cohort of MI mice injected with AdCu/ZnSOD.
in the PVN. As shown in Figure 3, the positive effects of AdCu/ZnSOD on EF and FS were similar to those produced by AdsiNox4, suggesting that Nox4-mediated O$_2^-$ production in the PVN is involved in the post-MI decline in heart function. Furthermore, because AdLacZ, the control for AdCu/ZnSOD, had no effect on EF and FS in MI mice, we could exclude nonspecific side-effects of adenoviral gene transfer. It is also important to note that infarct sizes, as estimated by ultrasound at 48 hours postsurgery, were similar between all MI groups (MI alone: 27.8 ± 2.1%; MI + AdsiGFP: 31.1 ± 2.1%; MI + AdsiNox4: 30.1 ± 1.8%; MI + AdCu/ZnSOD: 26.9 ± 1.5%; MI + AdLacZ: 28.9 ± 2.1%; P > 0.05, n = 7 to 9 per group), confirming that the differences in cardiac function observed with AdsiNox4 and AdCu/ZnSOD were not attributable to different degrees of infarction at the start of the experiment.

To further evaluate the effects of PVN-targeted Nox4 inhibition on cardiac function using another method, separate cohorts of mice were instrumented with cardiac catheters to measure basal LV hemodynamic parameters 14 days post-MI or sham surgery. Similar to the MI-induced cardiac dysfunction observed with high-resolution ultrasound, coronary artery ligation caused significant LV impairment as indicated by diminished LV peak contractility (+dP/dt max, Figure 4A), relaxation (=dP/dt max, Figure 4B) and systolic pressure (Figure 4C) compared to shams. Transduction of the PVN with either AdsiNox4 or AdCu/ZnSOD restored these parameters toward normal (sham) levels (Figure 4). Collectively, these data provide evidence that chronic cardiac dysfunction caused by MI can be significantly improved by inhibiting Nox4-generated O$_2^-$ in the PVN.

Heart rate was not different between groups during the hemodynamic studies (sham: 416 ± 29; MI alone: 400 ± 44; MI + AdsiGFP: 441 ± 27; MI + AdsiNox4: 411 ± 32; MI + AdCu/ZnSOD: 444 ± 30; MI + AdLacZ: 441 ± 35 bpm; P > 0.05, n = 6 to 9 per group) or during the echocardiography experiments (sham: 439 ± 18; MI alone: 460 ± 14; MI + AdsiGFP: 485 ± 19; MI + AdsiNox4: 464 ± 15; MI + AdCu/ZnSOD: 442 ± 21; MI + AdLacZ: 455 ± 21 bpm; P > 0.05, n = 7 to 14 per group). This suggests that improvement in cardiac function after transduction of the PVN with either AdsiNox4 or AdCu/ZnSOD is not explained by a differential cardio-depressant effect of anesthesia in these experiments.

**Figure 5.** Nox4 knockdown in the PVN attenuates MI-induced sympathoexcitation and restores LV responsiveness to bAR challenge. A, Summary of urinary NE concentrations 14 days after sham (n = 11), MI (n = 9), or MI combined with PVN-targeted AdsiGFP (n = 12), AdsiNox4 (n = 12), AdCu/ZnSOD (n = 9), or AdLacZ (n = 11). B, Power spectral analysis of arterial pressure variability 14 days after sham (n = 4), MI (n = 4), or MI combined with PVN-targeted AdsiGFP (n = 4). Data were calculated as percentages in relation to baseline and presented as low frequency/high frequency (LF/HF) relative to sham animals. C, Summary of peak decreases in mean arterial pressure (MAP) to hexamethonium 14 days after sham (n = 4), MI (n = 4), or MI + AdsiNox4 (n = 4). D, Summary of peak LV contractility following acute isotropin stimulation (expressed as percentage of baseline) 14 days after sham (n = 6), MI (n = 5), or MI combined with PVN-targeted AdsiGFP (n = 7), AdsiNox4 (n = 6), AdCu/ZnSOD (n = 7), or AdLacZ (n = 6). *P < 0.05 vs sham; †P < 0.05 vs MI alone, MI + AdsiGFP, or MI + AdLacZ; n.s., not significant.

**PVN-Targeted Nox4 Silencing Attenuates MI-Induced Sympathoexcitation and Restores LV Responsiveness to b-Receptor Challenge**

Given the evidence that PVN plays a key role in the sympathoexcitatory response in various models of HF,5,30 we hypothesized that a mechanism by which AdsiNox4 improves cardiac performance in postinfarct mice is by modulating sympathetic outflow. First, urinary NE levels were measured in naive and sham mice (naïve: 168.5 ± 15.8 versus sham: 162.8 ± 24 ng/mL, P < 0.05 vs MI (n = 7) and others,31 NE concentrations were not different between naïve and sham mice (naïve: 168.5 ± 15.8 versus sham: 162.8 ± 24 ng/mL, P > 0.05, n = 5 per group), but were significantly elevated following MI (Figure 5A). These MI-induced increases in NE were nearly normalized either by silencing Nox4 (AdsiNox4) or by scavenging O$_2^-$ (AdCu/ZnSOD) in the PVN (Figure 5A). Neither of the control vectors (AdsiGFP or AdLacZ) had any significant effects on MI-induced increases in urinary NE.

To further investigate the contributions of Nox4 in the PVN on sympathetic outflow following MI, we next performed power spectral analysis of arterial pressure variability as an additional index of sympathetic tone. Increased low frequency/high frequency oscillations of arterial pressure
reflect increased sympathetic activity. By 2 weeks postsurgery, MI caused a 3-fold increase in the low frequency/high frequency ratio compared to sham animals (Figure 5B), providing additional evidence that MI induces marked sympathoexcitation. Bilateral injection of AdsiNox4 into the PVN significantly attenuated the MI-induced increase in low frequency/high frequency, suggesting that Nox4-mediated signaling in the PVN contributes to increased sympathetic activity following MI.

A third strategy was used to evaluate the role of Nox4 signaling in the PVN on sympathetic drive. Increased depressor responses to ganglionic blockade with hexamethonium are taken to indicate enhanced contribution of the sympathetic nervous system to basal blood pressure. As seen in Figure 5C, mice that had undergone MI 14 days earlier exhibited augmented decreases in mean arterial pressure to hexamethonium compared to sham animals. PVN-targeted silencing of Nox4 normalized this response, suggesting that Nox4 contributes to enhanced sympathetic control of basal blood pressure in MI-treated mice. It is important to note that baseline mean arterial pressures were not different among the groups early after sham/MI surgery (day 3, sham: 89±9; MI: 88±15; MI + AdsiNox4: 89±10 mm Hg; P>0.05, n=4 per group) nor at 14 days postsurgery just before injection of hexamethonium (sham: 88±6; MI: 88±3; MI + AdsiNox4: 84±11 mm Hg; P>0.05, n=4 per group).

Persistent sympathetic stimulation has been shown to decrease myocardial catecholamine sensitivity via downregulation of cardiac β-adrenergic receptors (βARs). This translates to diminished LV contractility on acute βAR challenge. To provide a final marker of MI-induced sympathoexcitation and LV function in these studies, we examined LV contractile responses to the nonselective βAR agonist isoproterenol in the various groups. As shown in Figure 5D, isoproterenol caused a 30% increase in peak contractility (+dp/dt) in sham animals, and this response was significantly blunted in mice that had undergone MI. This is consistent with previous reports in mice. AdsiNox4 injected into the PVN restored the LV contractile response to βAR stimulation in MI mice, and did so to the same extent as scavenging O$_2^-$ with AdCu/ZnSOD in this brain region. It is important to note that heart rates immediately before and after isoproterenol injection were not statistically different among any of the treatment groups (data not shown), eliminating the possibility of this variable altering cardiac preload and therefore LV contractility. These results provide additional evidence that Nox4-mediated O$_2^-$ formation in the PVN contributes to sustained sympathoexcitation following MI, which leads to a loss of inotropic reserve.

**Nox4 Silencing in the PVN Ameliorates Perifascet Apoptosis**

Because high sympathetic drive to the heart has been linked to cardiomyocyte apoptosis, we next sought to determine whether MI-induced upregulation of Nox4 in PVN (and concomitant sympathoexcitation) is involved in myocardial apoptosis. Two weeks following MI, there was a ≈3-fold increase in apoptotic DNA fragment density in LV tissue compared to sham controls (Figure 6). This was mostly restricted to the perifascet border of the LV, as noninfarcted myocardium from all treatment groups exhibited very little DNA laddering (data not shown). PVN-targeted AdsiNox4 or AdCu/ZnSOD caused significant attenuation of the apoptotic response, whereas the control vectors had little effect. This suggests that Nox4-mediated O$_2^-$ signaling in the PVN is involved in the increased myocardial apoptosis observed after MI.

**Hydrogen Peroxide Scavenging in PVN Has No Impact on ROS Formation or Cardiac Performance Postinfarct**

Our evidence thus far suggests that inhibition of O$_2^-$ formation in PVN by AdsiNox4 or AdCu/ZnSOD attenuates cardiac dysfunction, sympathoexcitation and cardiac apoptosis following MI. However, because H$_2$O$_2$ is a byproduct of Cu/ZnSOD dismutation of O$_2^-$ and Nox4 has been linked to H$_2$O$_2$ production, we considered the possibility that H$_2$O$_2$ in the PVN may be involved in the effects we observed. To test this, we performed bilateral PVN injections of adenoviral vectors encoding the H$_2$O$_2$ scavenger catalase (AdCat) alone or in combination with AdCu/ZnSOD before the MI procedure. As shown in Figure 7A, the MI-induced increase in DHE fluorescence intensity in PVN at 14 days was significantly attenuated by AdCu/ZnSOD but not by AdCat. Furthermore, the coinjection of AdCat with AdCu/ZnSOD had no further effect on ROS formation than AdCu/ZnSOD alone. Similarly, scavenging O$_2^-$ with AdCu/ZnSOD was the most effective intervention for improving post-MI EF (Figure 7B) and FS (Figure 7C), because AdCat had little impact on these parameters when administered either alone or in combination with AdCu/ZnSOD. These results suggest that the functional benefit observed in these studies is attributable to diminished O$_2^-$ signaling and not enhanced H$_2$O$_2$ levels in the PVN.

**Discussion**

It is well established that CNS-driven autonomic dysfunction participates in the post-MI decline to HF, however the precise sites and molecules involved still have yet to be fully elucidated. Here we report that MI causes an upregulation of...
Nox4 expression in the PVN, which leads to increased O$_{2}^{•−}$ formation in this brain region. Inhibiting this response selectively in the PVN, either by silencing Nox4 or by increasing Cu/ZnSOD levels in this region, improves postinfarct cardiac performance by ameliorating chronic sympathoexcitation and concomitant loss of LV βAR responsiveness. This was associated with reduced apoptosis in the perifunction zone. Importantly, PVN-targeted delivery of the H$_{2}$O$_{2}$ scavenger catalase, either alone or in combination with CuZnSOD, had no effect on ROS formation in the PVN or MI-induced cardiac dysfunction, suggesting that Nox4-generated O$_{2}^{•−}$ in this brain region, and not H$_{2}$O$_{2}$, is the likely culprit in this model of HF.

Increasing evidence supports a role for NADPH oxidases as key signaling intermediates in a variety of CNS sites and centrally-mediated cardiovascular responses. For example, we have demonstrated that dominant-negative inhibition of a Rac1-containing NADPH oxidase in the SFO abolishes the pressor, bradycardic, and dipsogenic responses to central angiotensin II (Ang II) administration, and that Nox2 and Nox4 each play distinct roles in mediating these responses. Recently, NADPH oxidase activation in the PVN has been shown to be required for activation of the cardiac sympathetic afferent reflex, although the specific Nox homologs involved were not identified. Moreover, Wang et al have shown that Nox2-derived ROS in the NTS are critical in mediating the effects of Ang II on calcium signaling and neuronal activation.

NADPH oxidase–derived ROS signaling is also strongly implicated in HF, with Nox2 emerging as a critical homolog thus far. Global knockout of Nox2 or related subunits improves cardiac function and autonomic dysfunction when HF is induced in these mice. A correlation between upregulation of Nox2 in RVLM and rising sympathetic outflow in a rabbit model of HF has been reported, and this is thought to contribute to enhanced carotid body chemoreceptor sensitivity observed in these animals. In other studies by this group, chronic central delivery of Ang II has been shown to increase expression of Nox2 and related subunits in the RVLM, which is associated with an increase in O$_{2}^{•−}$ production and enhanced renal sympathetic activity. Because Ang II levels are increased in the CNS during HF, the implication is that Nox2 activation in RVLM is important in the sympathoexcitation that accompanies this disease.

To the best of our knowledge, this is the first report that Nox4 activation in the CNS is also critical in the post-MI deterioration in cardiac function. Our studies focused on the PVN, which like the RVLM, is strongly implicated in the autonomic dysfunction associated with HF. We showed that although Nox1 and Nox2 were detectable in the PVN, they were expressed at low levels and were not altered following MI. Nox4, on the other hand, was the most abundant homolog in the PVN under basal levels, and its expression was increased two-fold after ligation of the LAD coronary artery. This is consistent with evidence that Nox4 may be regulated at the transcriptional level because it does not require assembly of cytosolic subunits to confer enzymatic activity.

There are conflicting reports regarding the oxidant species (O$_{2}^{•−}$ or H$_{2}$O$_{2}$) produced by Nox4 in different cells and tissues. For example, Nox4 produces mainly H$_{2}$O$_{2}$ in rat aortic smooth muscle cells, whereas Nox4-dependent O$_{2}^{•−}$ generation has been reported in intracellular compartments of human embryonic kidney cells. Recent evidence demonstrates that siRNA-mediated knockdown of Nox4 inhibits both O$_{2}^{•−}$ and H$_{2}$O$_{2}$ production in kidney glomerular mesangial cells, suggesting that the active enzyme is capable of producing multiple oxidant species. In our studies, injection
of either AdsiNox4 or the $\text{O}_2^{\cdot-}$ scavenger AdCu/ZnSOD normalized DHE fluorescence in the PVN of MI-treated mice, suggesting that intracellular $\text{O}_2^{\cdot-}$ is the oxidant species produced by Nox4. Moreover, our findings that $\text{H}_2\text{O}_2$ scavenging in the PVN with AdCat had no effect on DHE staining or cardiac function, either alone or in combination with AdCu/ZnSOD, further supports a role for $\text{O}_2^{\cdot-}$ as the Nox4-generated ROS in this model. However, depending on the level of $\text{H}_2\text{O}_2$ and its subcellular localization, catalase may not be the most appropriate scavenger and therefore we cannot entirely rule out a role for $\text{H}_2\text{O}_2$. Studies are ongoing in our laboratory using adenoviruses encoding other $\text{H}_2\text{O}_2$ scavengers, including glutathione peroxidase-1.

Progressive deterioration of cardiac function following MI is the result of multiple events, including ventricular hypertrophy, cardiac arrhythmias, and increased cardiac apoptosis.\textsuperscript{32,43} Our findings of a significant increase in periinfarct apoptosis accompanying the LV dysfunction after MI are consistent with reports that LV apoptosis correlates with declining cardiac function in humans with HF.\textsuperscript{19,44} Furthermore, our data showing that PVN-targeted Nox4 silencing or $\text{O}_2^{\cdot-}$ scavenging reduced LV apoptosis and was associated with marked improvement in cardiac function are in line with studies showing a correlation between reduced cardiomyocyte apoptosis after MI and improved LV function in other animal models of HF.\textsuperscript{45} Because excessive NE stimulation induces cell death in cardiomyocytes through activation of protein kinase A and excessive calcium influx via voltage-dependent calcium channels,\textsuperscript{43,46} and apoptosis can be attenuated by blockade of cardiac $\beta$ARs,\textsuperscript{47} we speculate that the sympathoinhibitory effects of Nox4 silencing in the PVN of postinfarct mice may help explain the reduction in periinfarct apoptosis in this study. However, further studies will be required to fully understand how Nox4 silencing in the PVN impacts key elements of the cardiac apoptotic cascade.

In addition to increased sympathetic outflow, we also observed decreased LV responsiveness to $\beta$AR stimulation with isoproterenol in postinfarct mice. These results are consistent with human studies in which cardiac $\beta$ARs are downregulated in response to elevated catecholamine concentrations.\textsuperscript{43,48} This is thought to be protective, because chronic catecholaminergic stimulation increases cardiac hypertrophy and cardiomyocyte apoptosis, which lead to further cardiac dysfunction.\textsuperscript{49} The success of clinical $\beta$-blocker therapy in HF patients is largely attributable to competition for available $\beta$-receptors in the surviving myocardium, which not only reduces the detrimental effects of chronic catecholamine stimulation but also inhibits further downregulation of these receptors as levels of NE remain elevated.\textsuperscript{32} In our model, Nox4 silencing or $\text{O}_2^{\cdot-}$ scavenging in the PVN resulted in improved contractile reserve, which we speculate was attributable to attenuation of CNS-driven sympathetic outflow to the heart and preservation of the chronotropic and inotropic effects of NE. Although clinical trials (MOXCON) using pharmacological inhibition of central sympathetic outflow have not had favorable outcomes in HF patients, it should be noted that the drug used (moxonidine) caused global inhibition of CNS sympathetic activity, which may have precluded extracardiac autonomic functions that are necessary to pre-

Figure 8. Schematic depicting possible Nox4-mediated signaling mechanisms in PVN following MI. 1, PVN neurons are activated either by way of upstream circumventricular organs (eg, SFO) and the circulating factors that interface with them post-MI, or through stimulation of cardiac afferent pathways that use Ang II and perhaps other signaling factors. 2, Stimulation of PVN neurons leads to increased expression and activation of Nox4, causing elevated $\text{O}_2^{\cdot-}$ in this nucleus. 3 and 4, This could lead to changes in firing of RVLMinjecting PVN neurons through modulation of $\text{Ca}^{2+}$, $\text{K}^{+}$, or other currents (3) and/or activation of redox-sensitive transcription factors such as activator protein-1 or NF-B (4). 5, Increased activator protein-1 or NF-B-mediated transcription of many possible targets in PVN, including tyrosine hydroxylase (TH), the Ang II type 1 receptor (AT$_{1}$R), or even Nox4 itself, could lead to neuronal changes that sustain increased sympathetic output through projections to the RVLm. Solid blue lines indicate established findings, whereas dotted lines depict pathways still under investigation.

serve post-MI homeostasis. It is possible that targeted disruption of selective central regulatory circuits such as the PVN, the neurons of which project to T$_{1}$ to T$_{3}$ and T$_{9}$ to T$_{11}$ segments of the spinal cord, which in turn give rise to sympathetic efferents projecting to the heart and kidney, respectively,\textsuperscript{5} may provide a more specific approach to attenuating post-MI autonomic dysfunction.

Questions that remain unanswered by our studies are what brain region(s) and molecular signaling event(s) are upstream of Nox4 activation in the PVN following MI, and what are the downstream effectors that lead to altered sympathetic outflow (see Figure 8). Myocardial ischemia, cardiac cell death and declining cardiac output collectively evoke the release of numerous vaso- and neuroactive substances from multiple cells and tissues, including Ang II, vasopressin, catecholamines, and proinflammatory cytokines, all of which help to sustain tissue perfusion and cardiac output.\textsuperscript{16,39,49} These peptides act locally in peripheral tissues, but some of them can further influence cardiovascular regulation by interfacing with regions of the brain that lack a blood–brain barrier (circumventricular organs), including the SFO and organum vasculosum of the lamina terminalis.\textsuperscript{39,49} These centers have robust projections to the PVN and, therefore, serve as circuits through which circulating signals can evoke CNS-driven responses.\textsuperscript{35,49,50} Indeed, we have previously shown that MI-induced redox-signaling events in the SFO result in chronic activation of PVN neurons.\textsuperscript{8} In addition to actions at circumventricular organs, peripheral circulating and neural signals can evoke Ang II and cytokine production in CNS sites inside the blood–brain barrier,\textsuperscript{51} including the PVN,\textsuperscript{39} which also leads to modulation of autonomic outflow in
Given that it is now well established that the actions of Ang II in the CNS (both inside and outside the circumventricular organs) require redox signaling,7,16 and that Ang II is known to activate Nox4,34 we speculate that Ang II may be a critical upstream stimulus of MI-induced Nox4 activity in the PVN. Whether this would occur through circulating Ang II interfacing with the SFO,6,11,49 causing subsequent activation of angiotensinergic projections from SFO to PVN,49,52 or stimulation of local Ang II production within the PVN itself will require further investigation. In addition, because intrinsic reflexes such as the cardiac sympathetic afferent reflex involve neural signaling from the NTS to the PVN through Ang II-mediated ROS formation in PVN neurons,13 and given that the cardiac sympathetic afferent reflex is enhanced during HF15 and involves NADPH oxidase activation,14 it is also possible that augmented Ang II signaling via this pathway contributes to the increased Nox4 activity we report.

The downstream effectors that are activated by Nox4/ROS in the PVN and lead to long-term changes in sympathetic activity are also up for speculation (see Figure 8). It is now well established that ROS can modulate neuronal signaling through alterations in Ca2+/CaM,53 or K+ channels,54 so it is possible that the MI-mediated Nox4/ROS induction leads to such changes in RVLM-projecting PVN neurons. In addition, because there is compelling evidence that activation of transcription factors such as activator protein-1 and nuclear factor (NF)κB in CNS nuclei contribute to cardiovascular disease,55,56 and these transcription factors are redox-sensitive,57 we are currently using in vivo bioluminescence58 to test the hypothesis that Nox4-mediated activation of one or both of these transcription factors in PVN is causally linked to MI-induced sympathoexcitation and cardiac decline. It is interesting to note that Zucker and colleagues have recently demonstrated increased activator protein-1 binding in RVLM of rabbits with HF,51 and activation of brain NFκB in PVN has been observed in response to Ang II infusion.56

Finally, although our studies implicate Nox4-mediated redox signaling in the PVN in the autonomic dysfunction that occurs after MI, we did not investigate the effect of ROS scavenging in this site on the synthesis and secretion of vasopressin. The PVN is comprised of 2 distinct sets of neurons that serve diverse roles in cardiovascular regulation: parvocellular neurons that regulate autonomic outflow via nerve outflow.59 Elevated plasma levels of vasopressin have been reported after MI50; however, to date, a link between redox signaling in the PVN and increased vasopressin production or release has not been investigated. Because it is likely that our method of gene transfer results in transduction of both PVN neuronal subtypes,60 treatment with AdCu/ZnSOD or AdsiNox4 could alter the redox state of magnocellular neurons, which could, in turn, influence vasopressin levels. Further investigation into the effects of PVN-targeted Nox4/ROS inhibition on plasma vasopressin levels after MI will be necessary to address this possibility.

In conclusion, we report that Nox4-mediated oxidative stress in the PVN plays a causal role in the cardiac dysfunc-

**Disclosures**

None.

**References**


11. Lindley TE, Infanger DW, Rashin M, Zhou Y, Doobay MF, Sharma RV, Davison RL. Scavenging superoxide selectively in mouse forebrain is...


Novelty and Significance

What Is Known?

- Overactivation of the sympathetic nervous system and declining cardiac function are hallmarks of myocardial infarction (MI)-induced heart failure.
- The paraventricular nucleus (PVN) of the hypothalamus in the brain is involved in sympathoexcitation during heart failure.
- Oxidative stress in the PVN and other brain sites may be involved in the pathogenesis of certain cardiovascular diseases.

What New Information Does This Article Contribute?

- There are direct causal links between oxidative stress in the PVN, sympathetic overactivity, and declining cardiac function after myocardial infarction (MI).
- Nox4-containing NADPH oxidase is the primary source of free radicals in the PVN after MI.
- Selective silencing of Nox4 in the PVN improves MI-induced cardiac dysfunction by diminishing sympathoexcitation and apoptosis in the heart.

Morbidity and mortality associated with acute MI and heart failure are linked to unchecked neurohumoral excitation that eventually fuels a downward spiral of cardiovascular deterioration. Several discrete regions of the CNS have emerged as primary culprits in driving this neural dysfunction, although the underlying molecular mechanisms remain poorly understood. Here, we report, for the first time, that the Nox4 homolog of NADPH oxidase in the hypothalamus is a key molecule underlying MI-induced neural excitation and cardiac decline. Although NADPH oxidase has been implicated in MI-induced autonomic and cardiac dysfunction, the studies have relied on nonspecific inhibitors and/or global null mutations in mice. Our experiments are unique in that they combine brain site-selective gene transfer of Nox4-specific siRNA with sophisticated in vivo cardiovascular assessment in mice. This strategy allowed us to link the MI-induced upregulation of Nox4 in the hypothalamus with declining cardiac function and other cardiovascular parameters in vivo. Further investigation into antioxidant therapies targeted to the hypothalamus may provide a novel strategy for the treatment of MI-induced heart failure.
Materials and Methods

Animals

Adult C57BL/6 mice (~18-20g, 8-10 weeks old) were used for all experiments. Mice were fed standard chow (Harlan Laboratories) and water ad libitum. All procedures were approved by the Animal Care and Use Committee at 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.

Adenoviral vectors

Construction of recombinant E1-deleted adenoviral vectors encoding human copper-zinc superoxide dismutase (AdCu/ZnSOD),1 catalase (AdCat),2 and bacterial β-galactosidase (AdLacZ)3 have been described previously and were obtained from the University of Iowa Gene Vector Core.

To achieve stable knockdown of Nox4 in the PVN, we employed adenoviral vectors expressing siRNA targeted against Nox4 or control message (eGFP), which were constructed and purified by the University of Iowa Gene Vector Core as previously described.4 Briefly, 21 base-pair short hairpin RNA targeting sequences specific for Nox4 or eGFP were engineered under the control of the mouse U6 promoter. The hairpin was situated next to the U6 transcription start site (within 6 base pairs) and followed by a synthetic, minimal polyA cassette. A separate CMV promoter drives expression of a reporter gene (GFP for siNox4 and LacZ for siGFP).

Targeted gene transfer to the PVN and induction of MI

PVN-targeted injections of adenoviral vectors were performed using methods described previously5,6. Briefly, mice were anesthetized with sodium pentobarbital (5.0 mg/kg, i.p.) and placed in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA). After leveling between lambda and bregma, a burr hold was drilled and a glass barreled pipette was inserted at the following coordinates (relative to bregma): 0.3 mm each side of midline, 0.7 mm caudal, 5.0 mm ventral. Bilateral PVN injections were performed (200nl total) using a nitrogen gas pressure injector (MicroData Instrument, Inc., PM2000B, S. Plainfield, NJ) and titer-matched (1x10⁹ pfu/mL) viral stocks. Dual virus injections (e.g. AdCat + AdCu/ZnSOD) utilized titer-matched stocks that were mixed prior to injection and delivered simultaneously. Injections were performed 2-3 days prior to subsequent procedures to ensure robust transgene expression at the time of MI or sham treatment.

MI was induced by surgical ligation of the left anterior descending (LAD) coronary artery as previously described.7 In brief, mice were anesthetized with sodium pentobarbital (5.0 mg/kg, intraperitoneal) and intubated with a rodent ventilator. The heart was accessed from the third intercostal space and the LAD was ligated with 8-0 ethilon suture. The thoracic wall was closed and the mice were extubated. Sham procedure was identical to MI with the exception of LAD ligation.

Quantitative real-time PCR detection of Nox homologues

Mice were decapitated and brains were removed and immediately placed on dry ice. PVN was isolated by micropunch (0.75mm, Stoelting Co., Wood Dale, IL) from 3-5 mice per biological sample. Total RNA was isolated by Trizol® (Invitrogen, Carlsbad, CA) extraction and reverse
transcribed using random hexamer primers. 25ng template samples were subjected in triplicate to real-time qPCR (ABI 7500FAST system) using Power SYBR Green (Applied Biosystems, Foster City, CA) and Nox- or β-actin-specific primers for relative quantitation or with primers for 18S RNA for absolute quantitation. Standard PCR amplification conditions were used according to manufacturer’s suggestions (Applied Biosystems). Primers were designed with Primer Express Software (v1.5, Applied Biosystems) and targeted to nonhomologous regions of mRNA sequences for mouse as follows: Nox1 sense, 5'-CTACAGTAGGAAGCCACAGGCACT-3'; Nox 1 anti-sense, 5'-ACTGTCAGTTTGAGACTGATG-3'; Nox2 sense, 5'-CCCTTTGGTGATACGGCATGAGAT-3'; Nox2 anti-sense, 5'-CAATCCGCTCAGCTAATCA-3'; Nox4 sense, 5'-GGATCACAGAAGGTCCTAGCAG-3'; Nox4 anti-sense, 5'-GCAGCTACATGACACCTGAGA-3'; -actin sense, 5' CATCCTCTTCTCCGAGGAAGA-3'; -actin anti-sense 5' ACAGGATTTCCATACCAAGGAGG-3'; 18S sense, 5'-GTAACCCGTTGAACCCCATT-3'; and 18S anti-sense 5'-CCATCCAATCGTGACG-3'. Primer sets were obtained by Integrated DNA Technologies (IDT, Coralville, IA). Copy numbers of Nox1, Nox2, and Nox4 transcript were determined from standard curves generated using pure plasmid templates as previously described and expressed as copy number relative to 18S copy number contained in 100ng total RNA. We used the ddCt method for relative quantification of Nox1, Nox2, and Nox4 in sham and MI-treated groups. In this case, data are expressed as 2^{(-ddCt)}.

In situ hybridization and Western blot analysis for detection of Nox4

In situ hybridization was performed essentially as described by Rodriguez et al. Antisense cRNA probes against Nox4 were created with 677-bp coding sequences for mouse Nox4 (NCBI Accession number: NM_015760). Digoxigenin (DIG)-labeled cRNA probes were synthesized using Sp6 RNA polymerases as well as the DIG RNA Labeling Mix (Roche Molecular Biochemicals). Mice were sacrificed by decapitation and brains immediately frozen in O.C.T. embedding medium (Miles, Inc.) in a dry ice-cooled bath of ethanol for 15 minutes and stored at -80°C until use. Coronal sections through the PVN (20 μm) were obtained and mounted on RNase-free glass slides. Brain section from each of the various treatment groups were mounted on the same slides and assayed together. Tissues were then fixed with 4% paraformaldehyde in RNase-free 0.1M PBS for 10 min. Sections were treated with acetylation solution (0.1M triethanolamine, 0.65% HCl, 0.25% [v/v] acetic anhydride) for 10 min at room temperature followed by PBS washes. A pre-hybridization step was then performed by incubating the sections in hybridization buffer (50% formamide, 5x SSC, 5x Denhardts, 250 μg/ml yeast tRNA, 500μg/ml salmon sperm DNA, 50mg/ml heparin) for 2 hours at room temperature. Heat-denatured anti-sense NOX4 cRNA probe was then applied to the slides at 800ng/ml and hybridization was performed at 60°C for 48 hours. After hybridization, sections were washed with serial dilutions of SSC buffer at 65°C for 2 hours. Tissues were then incubated with alkaline phosphatase-conjugated antibodies to DIG (1:1000, Roche) at 4°C overnight. Signals were revealed by incubating the sections with nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate (Roche) at room temperature. Staining was visualized using a Leica DMI 6000B microscope (Leica Microsystems, Inc.).

Nox4 protein levels were assessed by Western blot performed on PVN tissue punch lysates subjected to SDS-PAGE. Samples were incubated with rabbit polyclonal anti-Nox4 antibody (#NB110-58849, Novus Biologicals;1:500 in PBS with 1% non-fat dry milk and 0.1% Tween-20) followed by donkey anti-rabbit HRP (#sc-2313, Santa Cruz Biotechnology, Inc., 1:2000) and subjected to chemiluminescence. Band intensity was quantified by densitometry using NIH ImageJ and normalized to β-actin loading controls.
Measurement of ROS in PVN

Dihyrdroethidium (DHE) staining was performed 3, 7, and 14 days after MI or sham procedure as described previously. Briefly, brains were cryo-sectioned in the PVN-containing coronal plane (20 μm) onto glass slides and incubated in 1 μM of DHE (in PBS) for 6 min at room temperature in the dark, rinsed in PBS for 2 min, and imaged using a Zeiss LSM 510 confocal microscope with an excitation wavelength of 543 nm and a rhodamine emission filter. All sections for control and experimental groups were processed and analyzed in parallel using identical detector and laser settings. Fluorescence intensity was analyzed using NIH Image J software as described previously and normalized to fluorescence levels observed in sham animals.

Detection of Cu/ZnSOD in brain sections

SOD immunohistochemistry was performed as described previously. Briefly, mice were anesthetized with pentobarbital and perfused transcardially with 4% paraformaldehyde (in 0.1M phosphate buffered saline, PBS). Brains were removed and placed in 20% sucrose overnight. PVN-containing brain samples were sectioned in the coronal plane (20μm) and mounted directly onto glass slides. Slides were incubated with 10% normal horse serum for 1 hour at room temperature prior to incubation with a sheep anti-human Cu/ZnSOD antibody (#574597, Calbiochem-Novabiochem, 1:120 in PBS) for 24 hours at 4°C. Slides were washed with PBS and then incubated with a donkey-anti-sheep rhodamine-conjugated secondary antibody (#6897, Abcam, 1:200) for 1 hour at room temperature in the dark and washed in PBS before being coverslipped. Images were obtained using a Zeiss Axio Imager D1 microscope.

Hemodynamic and echocardiography measurements

Hemodynamic measurements were performed 14 days after MI or sham procedure as described previously. Briefly, left ventricular (LV) pressures were recorded at 4KHz sampling frequency using a 1.4f Millar catheter (Millar Instruments Inc., USA), digitized (Power LabR, Chart Version 4.01), and dP/dt+ and dP/dt- were calculated as an index of myocardial contractility as described. A subset of mice was also instrumented with jugular catheters (MicroRenathane MRE-025, Braintree Scientific, Inc.) for intravenous injection of isoproterenol. Isoproterenol HCl (Sigma-Aldrich) was prepared at 0.2ng/μL (in PBS) and injected over 30 seconds to a final dosage of 1ng/g mouse weight. Peak ±dP/dt was measured over the 30 seconds following injection and expressed relative to baseline values just before injection.

Transthoracic echocardiography was performed at 2 and 14 days post-MI or sham surgery using a VEVO 770 high resolution imaging system and 707B single-element transducer with 45MHz broadband frequency (VisualSonics, Toronto, Canada). Data were acquired in mice under light anesthesia (1.0% isoflurane) as described previously. Retrospective B-mode cineloops obtained from parasternal long and short axes were used to calculate fractional shortening (FS) [(LV end diastolic dimension-LV end systolic dimension)/LV end diastolic dimension*100] and ejection fraction (EF) [(LV end diastolic volume-LV end systolic volume)/(LV end diastolic volume)*100].

Measurement of urinary norepinephrine

Urine was collected 14 days after MI or sham procedure and norepinephrine (NE) measured by ELISA (#RE59261, IBL, Hamburg, Germany) as described previously. ELISA was performed according to manufacturer’s instructions. Optical densities from colorimetric reaction of
antibody-conjugated alkaline phosphatase with p-Nitrophenyl phosphate substrate were obtained on a photometer at 405nm, and NE concentrations were derived from a cubic spline standard curve generated with NE standards.

*Spectral and pharmacologic analysis of autonomic tone*

A separate cohort of mice underwent implantation of radiotelemeters prior to sham/MI surgery to evaluate autonomic tone both by power spectral analysis and arterial pressure responses to ganglionic blockade. Briefly, mice were anesthetized (ketamine, 117 mg/kg in combination with xylazine, 17 mg/kg intraperitoneally, i.p.) and instrumented with TA11PA-C10 radiotelemetry devices (Data Sciences International, Arden Hills, MN) as described. Briefly, the telemeter catheter was inserted into the thoracic aorta via the left common carotid artery. The telemeter body was placed in a subcutaneous pouch created along the right flank. A subset of mice also underwent PVN-targeted viral gene transfer of AdsiNox4 or AdsiGFP at this time. After 3-5 days of surgical recovery in individual home cages, telemeters were activated for continuous sampling of arterial pressure waveforms at 2KHz (Dataquest A.R.T., Data Sciences International) in conscious mice. After 3 days baseline recording, mice underwent MI or sham procedure. High-frequency (2KHz) recording of pressure waveforms in conscious mice resumed the following day for an additional 14 days, at which time mice received acute injections (i.p.) of the ganglionic blocker hexamethonium (Sigma-Aldrich, 5mg/kg). Arterial pressure responses to hexamethonium were recorded for an additional hour.

For assessment of autonomic function, power spectral analysis of arterial pressure variability before and after MI/sham surgery was performed as described. Briefly, spectral power of mean arterial pressure (MAP) in the frequency domain was determined using custom-written functions in HemoLab Analyzer and Batch Processor software (version 9.3, provided by Dr. Harald Stauss, University of Iowa, Iowa City, IA). An average spectrum using 4096 point Fast Fourier Transforms (FFT) with 50% overlap was computed for a compact spectrum display. Spectra were divided into the following frequency ranges: low frequency (LF: 0.4-1.0 Hz) and high frequency (HF: 1.0-3 Hz) as described. Increased LF/HF oscillations of arterial pressure reflect increased sympathetic activity. Data were expressed as a percentage in relation to baseline and presented as LF/HF relative to sham animals.

*Quantification of apoptosis by DNA laddering*

At 14 days post-MI or sham surgery, mice were sacrificed by decapitation, hearts removed and immediately placed on ice, and peri- and non-infarct tissues were dissected and frozen on dry ice. DNA was isolated and apoptotic fragments enriched according to manufacturer instructions (APO-DNA1, Maxim Biotech, San Francisco, CA). DNA laddering assay was performed as previously described.

*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 Bonferroni post-test for multiple comparisons using Prism 5 (GraphPad Software, Inc.).
References


