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Superoxide Is Involved in the Central Nervous System Activation and Sympathoexcitation of Myocardial Infarction–Induced Heart Failure

Timothy E. Lindley, Marc F. Doobay, Ram V. Sharma, Robin L. Davisson

Abstract—Increased angiotensin II signaling in the brain has been shown to play a critical role in the excessive sympathoexcitation and development of heart failure (HF) after myocardial infarction (MI). We have recently demonstrated that reactive oxygen species mediate the actions of angiotensin II in the brain. In this study, we tested the hypothesis that increased redox signaling in central cardiovascular control regions is a key mechanism in the neurocardiovascular dysregulation that follows MI. Ligation of the left coronary artery induced a large MI and subsequent HF in adult C57BL/6 mice, as demonstrated by cardiac hypertrophy, hydrothorax, and ascites. Immunohistochemical analysis of Fos, a marker of neuronal activation, revealed a significant increase in the number of Fos-positive neurons in the paraventricular nucleus and supraoptic nucleus at 2 and 4 weeks after MI compared with sham mice. Intracerebroventricular injection of an adenoviral vector encoding superoxide dismutase (Ad-Cu/ZnSOD) caused a significant decrease in the number of Fos-positive neurons in the paraventricular nucleus and supraoptic nucleus at 2 weeks after MI compared with mice receiving either saline or a control vector (Ad-LacZ). There was also a diminished role of sympathetic drive in post-MI mice treated centrally with Ad-Cu/ZnSOD, as demonstrated by significantly attenuated falls in heart rate and mean arterial pressure to the ganglionic blocker hexamethonium and decreased urinary norepinephrine levels in these mice compared with Ad-LacZ–treated MI mice. These results suggest that superoxide plays a key role in the central activation and sympathetic hyperactivity after MI in mice and that oxygen radicals in the brain may be important new targets for therapeutic treatment of heart failure. (*Circ Res.* 2004;94:402–409.)

Key Words: reactive oxygen species ■ angiotensin II ■ superoxide dismutase ■ brain ■ adenovirus

Chronic heart failure (HF) is a leading cause of morbidity and mortality in the United States, and despite improvements in treatment, the incidence and prevalence of the disease have continued to increase in recent years.¹ The leading cause of HF in this country is coronary artery disease leading to myocardial ischemia and infarction.² Excessive activation of central neurohumoral systems after myocardial infarction (MI) is thought to play a critical role in the pathogenesis of HF,³ and it has been demonstrated that plasma norepinephrine levels in patients with HF are positively correlated with mortality.⁴ However, the molecular mechanisms underlying the increased central activation and chronic sympathoexcitation remain poorly understood.

During the development of HF, there is an elevation in plasma angiotensin II (Ang II) levels, which is thought to be involved in increasing sympathetic drive and altering cardiac baroreflex function through its effects both centrally and peripherally.^{3,5} One mechanism by which circulating Ang II

exerts its effects centrally is by binding to its receptors on neurons in specialized brain regions that lack a blood-brain barrier, known as circumventricular organs (CVOs).^{5,6} Neurons in these regions, such as the subfornical organ (SFO) and organum vasculosum of the lamina terminalis (OVLT), then signal to other downstream cardiovascular control regions of the brain, including hypothalamic nuclei, such as the paraventricular nucleus (PVN) and supraoptic nucleus (SON), which additionally activate several neurohumoral pathways.^{5,7,8} Recent work has demonstrated that interrupting this positive-feedback loop by selectively blocking central Ang II signaling reduces the sympathetic hyperactivity, improves cardiac baroreflex function, and attenuates the subsequent development of HF after MI in rats.^{9–11} Therefore, understanding the mechanisms underlying Ang II signaling in the brain is critical in elucidating the pathogenesis of MI-induced HF.

Reactive oxygen species (ROS), such as superoxide and hydrogen peroxide, once thought only to be harmful byprod-

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From the Department of Anatomy and Cell Biology (T.E.L., M.F.D., R.V.S., R.L.D.), Free Radical and Radiation Biology Program; Department of Radiation Oncology (R.L.D.); and The Cardiovascular Center (R.V.S., R.L.D.), The University of Iowa Roy J. and Lucille A. Carver College of Medicine, Iowa City, Iowa.

Correspondence to Robin L. Davisson, PhD, Department of Anatomy and Cell Biology, 1-251 Bowen Science Building, The University of Iowa Roy J. and Lucille A. Carver College of Medicine, Iowa City, IA 52242. E-mail robin-davisson@uiowa.edu

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ucts of oxidative metabolism, are now recognized as critical second messengers in a wide range of cellular processes.^{12,13} Recently, we have identified superoxide as a key mediator of the central actions of Ang II on blood pressure and drinking behavior.¹⁴ Given the link between central Ang II and neurodysregulation after MI, the goal of this study was to test the hypothesis that superoxide signaling in the brain is involved in driving neuronal activation and sympathoexcitation in MI-induced HF. Combining a modified procedure for inducing MI and HF in mice, genetic modulation of the redox state of the brain using adenoviral vectors, and *in vivo* analysis of cardiovascular function, our results suggest that oxidant signaling in key cardiovascular control regions of the brain may play an important role in the pathogenesis of HF after MI.

Materials and Methods

The redox state of the brain in mice that had undergone MI or sham surgery was manipulated by central microinjection of recombinant adenoviral vectors encoding cytoplasmic superoxide dismutase (Cu/ZnSOD). The effect of increased superoxide scavenging on MI-induced neuronal activation and sympathetic nervous system activity was examined. What follows is a brief summary of the experimental strategy. A detailed description of methods and experimental protocols can be found in the online data supplement available at <http://www.circresaha.org>.

Fos Expression in the Central Nervous System After MI

Adult C57BL/6 mice were anesthetized with pentobarbital (1 mg/kg) and methoxyflurane, the heart was exposed, and the left anterior descending (LAD) branch of the coronary artery was ligated. Sham mice underwent the same procedure except for coronary ligation. At 2 or 4 weeks, mice were perfused transcardially with 4% paraformaldehyde. Brains were removed and cryosectioned (10 to 30 μ m), and immunohistochemistry was performed using an antibody that recognizes c-Fos, FosB, Fra-1, or Fra-2 (c-Fos K25, Santa Cruz). Fos-positive neurons were counted in three serial sections taken from cardiovascular control regions, and data were expressed as a ratio relative to controls.

Effect of Ad-Cu/ZnSOD and Losartan on Fos Expression in Brains of Post-MI Mice

At 7 days after MI, mice received an intracerebroventricular (ICV) injection of saline or adenoviral vectors (500 nL, 1×10^9 pfu/mL) encoding human Cu/ZnSOD (Ad-Cu/ZnSOD) or β -galactosidase (Ad-LacZ). A separate cohort of MI mice was instrumented with osmotic minipumps (model 1007D, Alzet) for chronic ICV administration of losartan (1 μ g/h) or vehicle. At 2 weeks after MI, brains were removed and Fos immunohistochemistry was performed as above. Transgene expression was localized by immunohistochemistry using an anti-human Cu/ZnSOD antibody (The Binding Site Limited, Birmingham, England).

Effect of Ad-Cu/ZnSOD on Central Fos Expression Elicited by Hypertonic Saline

One week after ICV administration of Ad-Cu/ZnSOD or Ad-LacZ, healthy C57BL/6 mice were injected subcutaneously with 6% or 0.9% saline (500 μ L). Two hours later, Fos immunohistochemistry was performed as above.

Effect of Ad-Cu/ZnSOD on Sympathetic Tone in Post-MI Mice

C57BL/6 underwent MI or sham surgery and received an ICV injection of Ad-Cu/ZnSOD or Ad-LacZ. At 2 weeks after MI, urinary norepinephrine (NE) levels were determined by ELISA (IBL). In a separate cohort at 4 weeks after MI, changes in mean

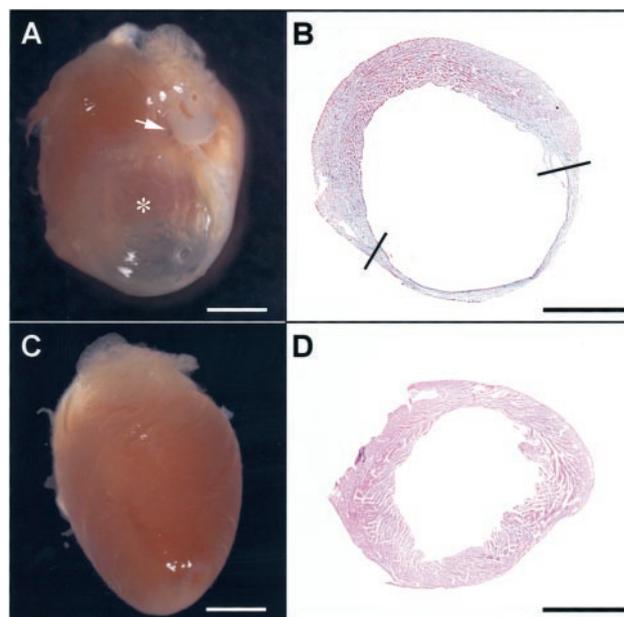


Figure 1. Ligature of the LAD branch of the left coronary artery in mice causes a large MI. A, Representative sample demonstrating that ligation of the LAD in a mouse (white arrow shows ligature) causes an infarction of the anterolateral wall of the left ventricle, including the apex, which became thin and transparent by 2 weeks after MI (indicated by asterisk). B, H&E-stained cross section of the heart shown in A, with the margins of the infarcted region indicated by the black lines. Such images were used to quantitate infarct sizes. C and D, Whole heart and cross section for a sham animal. Scale bar=2 mm.

arterial pressure (MAP) and heart rate (HR) after ganglionic blockade (hexamethonium, 5 mg/kg IP) were recorded in conscious mice from indwelling femoral artery catheters using a pressure transducer-coupled Powerlab set-up (Chart version 4.0.1).

Results

Mice Develop Congestive HF Within 4 Weeks of MI

Adult C57BL/6 mice underwent an MI or sham procedure. Approximately 95% of mice survived the ligation procedure and the subsequent 24 hours, whereas 30% died between postoperative days 4 and 7, most with evidence of left ventricular rupture. All MI mice surviving beyond day 7 lived until the conclusion of the experiment. All sham-operated mice survived the initial procedure and throughout the experiment. Ligation of the LAD caused a large infarction of the anterolateral wall and apex of the left ventricle (LV). As shown in Figure 1A, the infarcted region was thin, dilated, and nearly transparent. Quantitative analysis of H&E-stained sections (Figure 1B) revealed that the infarcted region encompassed $49.1 \pm 5.4\%$ ($n=4$) of the circumference of the LV at 2 weeks after MI, and this was not significantly different at 4 weeks ($53.1 \pm 3.1\%$, $n=6$, $P>0.05$). Although body weight did not differ between MI and sham animals, MI mice demonstrated significant cardiac hypertrophy (total heart weight) at both 2 and 4 weeks (Table). However, it was not until 4 weeks after MI that LV and lung weights were significantly greater than sham animals (Table) and the accumulation of ascites became evident. Taken together,

Organ Weights of MI and Sham-Operated Mice

	n	Body, g	Heart, mg/g	Left Ventricle, mg/g	Lungs, mg/g
Sham	5	24.8±0.3	5.3±0.1	3.8±0.1	10.6±0.9
2 Weeks After MI	4	24.6±0.4	7.4±0.8*	4.3±0.3	11.4±1.6
4 Weeks After MI	6	25.5±0.4	8.7±0.9*	5.4±0.5*	15.1±4.3*

Values are mean±SEM. n indicates the number of mice included in each group. *P<0.05 vs sham.

these findings demonstrate that coronary artery ligation causes a large MI that leads to congestive HF within 4 weeks after MI, as indicated by hypertrophy accompanied by hydrothorax and ascites.

Neurons in the PVN and SON Are Chronically Activated After MI in Mice

The expression of the Fos family of transcription factors FosB, Fra-1, and Fra-2 has been used as a marker for chronic neuronal activation, including in studies of rats with MI-induced HF.¹⁵ Using an antibody that recognizes all of these Fos members, immunohistochemical analysis of Fos-positive neurons was performed to identify chronically activated brain regions after MI in mice. Sections from throughout the brain were analyzed, and although several brain regions stained positively for Fos, only the PVN and SON showed differences in the number of Fos-positive neurons in MI and sham animals. As such, quantitative analyses focused on these regions. As shown in representative photomicrographs in Figure 2A, MI mice had markedly

greater numbers of Fos-positive neurons in the PVN and SON at both 2 and 4 weeks after ligation compared with sham animals. Summarized in Figure 2B, these data demonstrate a 3- to 4-fold increase in positively stained neurons for both regions in mice that had received an MI. This increase in Fos-positive neurons was observed as early as 2 weeks after MI and was sustained through 4 weeks. This suggests that neurons within the PVN and SON are chronically activated even before the onset of congestive HF and remain so once HF is established. It should be noted that in sham mice, the numbers of Fos-positive neurons were not different at 2 and 4 weeks after surgery and thus were grouped for data summary and statistical analysis.

ICV Ad-Cu/ZnSOD Decreases MI-Induced Neuronal Activation in the PVN and SON

We next tested the hypothesis that superoxide is involved in the chronic activation of neurons in the PVN and SON after MI. One week after MI, separate cohorts of mice received an ICV injection of Ad-Cu/ZnSOD, Ad-LacZ, or saline, and 1

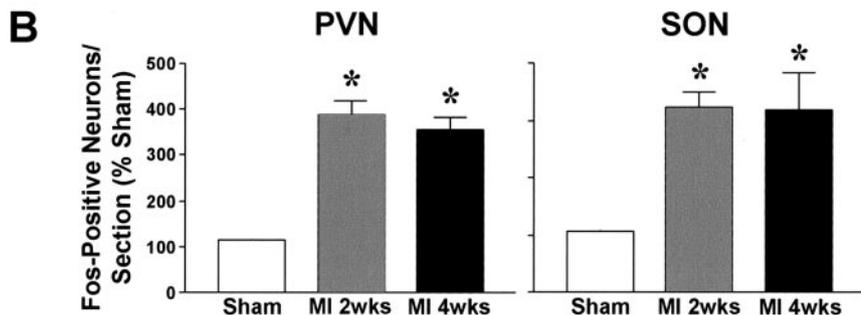
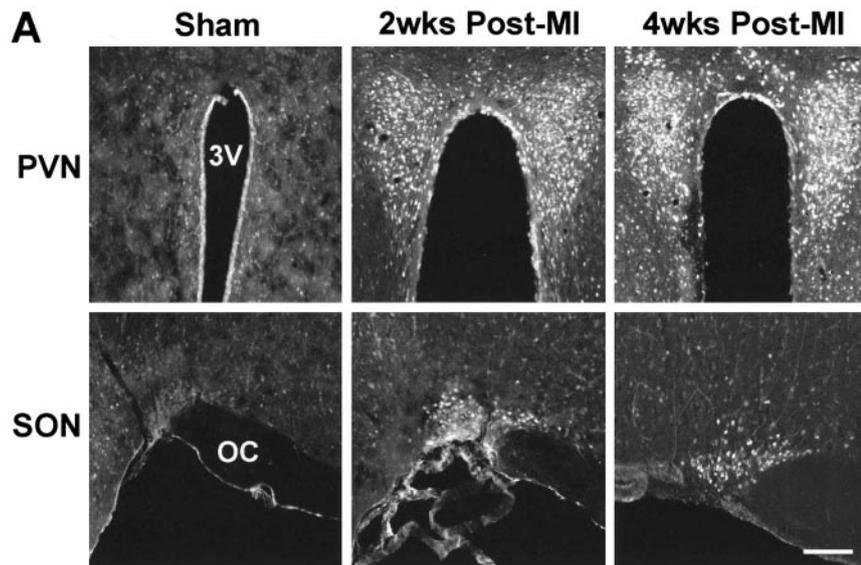


Figure 2. Mice exhibit chronic neuronal activation in PVN and SON after MI. A, Representative photomicrographs demonstrating an increase in Fos-positive neurons in the PVN and SON at 2 and 4 weeks after MI compared with sham animals. B, Summary of quantification of Fos-positive neurons in PVN and SON at 2 weeks (n=6) and 4 weeks (n=5) after MI compared with sham mice (n=5). Data are mean±SEM. *P<0.05 vs sham. 3V indicates third ventricle; OC, optic chiasm. Scale bar=100 μm.

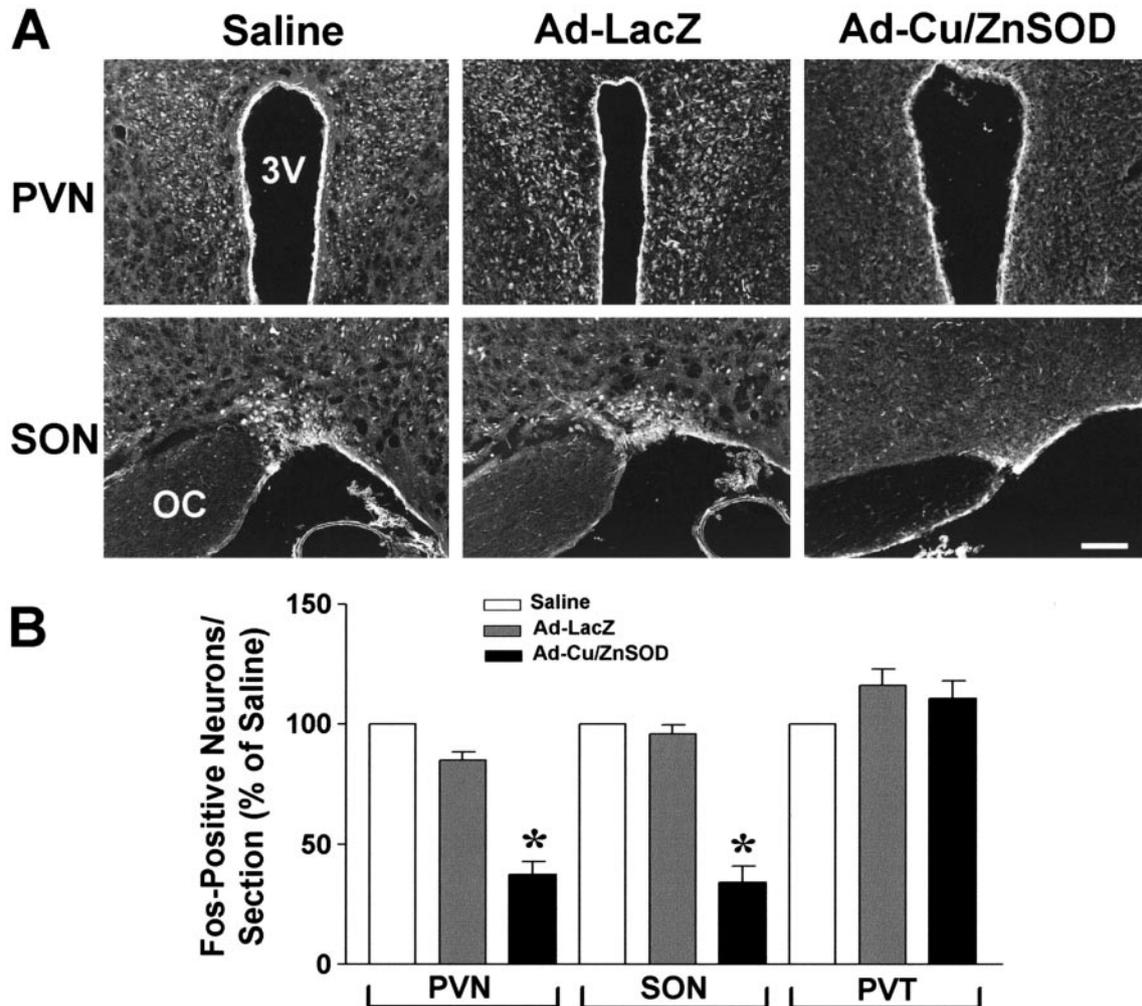


Figure 3. Modulation of the redox state of the brain with Cu/ZnSOD decreases MI-induced neuronal activation in PVN and SON. A, Representative photomicrographs demonstrating that Fos-positive staining in the PVN and SON of MI mice is markedly reduced in Ad-Cu/ZnSOD-injected mice, whereas the response is intact in saline-treated and Ad-LacZ-treated mice. B, Summary of quantification of Fos-positive neurons in PVN, SON, and PVT in mice that had received an ICV injection of saline (n=3), Ad-LacZ (n=6), or Ad-Cu/ZnSOD (n=7) 1 week earlier. All data were obtained 2 weeks after MI. Numbers of Fos-positive neurons in PVT were similar in MI and sham mice (see text). Bright-field DAB images were inverted to make them comparable to Figures 2 and 5. * $P < 0.05$ vs saline and Ad-LacZ. 3V indicates third ventricle; OC, optic chiasm. Scale bar=100 μm .

week after ICV injections (2 weeks after MI), Fos immunohistochemistry was performed. As shown in representative photomicrographs in Figure 3A, MI mice that had received ICV injections of either saline or the control vector Ad-LacZ showed similar robust Fos-positive staining in both PVN and SON as that shown for MI mice in Figure 2. In contrast, this MI-induced increase in Fos staining was markedly reduced in the Ad-Cu/ZnSOD-injected mice. The data are summarized in Figure 3B, and the results demonstrate that modulation of the redox state of the brain results in a significant decrease in the numbers of chronically activated neurons in PVN and SON after MI. The adenovirus itself did not affect MI-induced neuronal activation, because there were similar numbers of Fos-positive neurons in these brain regions of Ad-LacZ-treated and saline-treated animals (Figures 3A and 3B). Also important to note is that Ad-Cu/ZnSOD did not indiscriminately decrease Fos expression in all brain regions. For example, Fos expression in the paraventricular thalamic

nucleus (PVT), a region found to contain similar numbers of Fos-positive neurons in both MI and sham animals (sham, 108.3 ± 6.9 ; n=6; MI, 107.5 ± 7.0 ; n=6; $P > 0.05$), was not altered in response to ICV Ad-Cu/ZnSOD (Figure 3B). Therefore, the inhibitory effects of Ad-Cu/ZnSOD on neuronal activation were not generalized and widespread but rather selective for MI-induced Fos activation in the PVN and SON.

Ang II has been shown to activate neurons in the PVN and SON in rats,^{8,16} and we have recently demonstrated that central Ang II signaling is mediated by AT₁ receptor-dependent increases in superoxide production.¹⁴ Therefore, we also examined the role of Ang II in chronic neuronal activation of the PVN and SON after MI using the AT₁ receptor antagonist losartan. These studies were performed exactly as described above, except mice were chronically infused ICV with losartan in place of Ad-Cu/ZnSOD. Central infusion of losartan significantly attenuated the number of Fos-positive neurons in the PVN (vehicle, 383.3 ± 14.7 ;

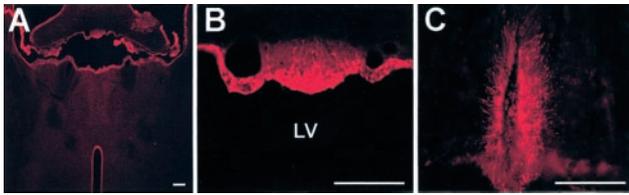


Figure 4. Recombinant human Cu/ZnSOD transgene expression is high in the SFO and OVLT. Representative photomicrographs of human Cu/ZnSOD immunoreactivity in coronal brain sections 1 week after ICV injection of Ad-Cu/ZnSOD. A, Cu/ZnSOD was confined to tissue surrounding the ventricular system, particularly the ependymal layer and choroid plexus. However, 2 periventricular regions lacking a blood-brain barrier, the SFO (B) and OVLT (C), exhibited intense Cu/ZnSOD staining. LV indicates lateral ventricle. Scale bar=200 μ m.

losartan, 308.0 ± 17.3 ; 19.7% decrease; $n=4$ per group; $P<0.05$) but not in the SON (vehicle, 99.0 ± 6.8 ; losartan, 86.0 ± 13.6 ; 13.1% decrease; $n=4$ per group).

Cu/ZnSOD Transgene Expression Is Localized to the SFO and OVLT

To confirm transgene expression in the brain and also to determine potential sites where ICV-administered Ad-Cu/ZnSOD could be acting to inhibit MI-induced Fos expression in the PVN and SON, immunohistochemical analyses were performed on brain sections using human Cu/ZnSOD-specific antibodies. As shown in Figure 4A, Cu/ZnSOD transgene expression was highest in the tissue surrounding the ventricles, particularly the ependymal layer and choroid plexus. In addition, two of the circumventricular organs (CVOs), the SFO and OVLT, exhibited very robust Cu/ZnSOD expression (Figures 4B and 4C). Importantly, no other CVOs, nor other regions of the brain parenchyma, including the PVN and SON, were positive for the transgene. It should be noted that the same robust transgene expression as shown in Figure 4 was observed as early as 3 days after injection (data not shown). This is consistent with our earlier report demonstrating that Ad-mediated transgene expression reaches a maximum by 3 days after injection and remains stable for at least 3 weeks.¹⁷ It should also be noted that no human Cu/ZnSOD immunoreactivity was detected in peripheral tissues nor in brains of saline-treated or Ad-LacZ-treated mice (data not shown). These results demonstrate that transgene expression is robust at the time that experimental protocols were performed and that key cardiovascular control regions that project to the PVN and SON—the SFO and OVLT—express high levels of the Cu/ZnSOD transgene.

Ad-Cu/ZnSOD Does Not Interfere With the Ability of PVN and SON Neurons to Be Activated

To verify that Cu/ZnSOD did not cause an indiscriminate disruption of neuronal networks necessary for activating PVN and SON neurons, we studied Fos expression in response to injection of hypertonic (6%) saline. This stimulus is known to cause a similar pattern of Fos activation, including increased PVN and SON staining, as that elicited by MI.¹⁸ One week after ICV injections of Ad-lacZ or Ad-Cu/ZnSOD, mice received a subcutaneous injection of 6% saline or 0.9% saline (500 μ L). Two hours later, mice were killed and Fos immunohistochemistry was performed. As shown in the

representative example in Figure 5A, subcutaneous administration of hypertonic saline caused a similar robust increase in Fos-positive neurons in the PVN and SON of both Ad-Cu/ZnSOD-injected and Ad-LacZ-injected mice. These data are summarized in Figure 5B and demonstrate that modulation of the redox state of the brain does not affect the hypertonic saline-dependent increase in neuronal activation in the PVN and SON. These results suggest that PVN and SON neurons are still functionally intact and able to respond to other activating stimuli after ICV Ad-Cu/ZnSOD.

Ad-Cu/ZnSOD Diminishes Basal Sympathetic Tone in Mice With HF

Because activation of the PVN has been linked to sympathetic excitation in HF,^{16,19} we examined the hypothesis that Ad-Cu/ZnSOD, in addition to decreasing neuronal activation, could also attenuate enhanced sympathetic drive in mice with HF. As an index of sympathetic tone, we first examined the depressor and bradycardic responses to ganglionic blockade. Four weeks after MI or sham surgery, separate cohorts of mice received an ICV injection of Ad-Cu/ZnSOD or Ad-LacZ and were instrumented with femoral arterial catheters. Three days later, changes in MAP and HR were recorded in response to the ganglionic blocker hexamethonium (IP). As seen in Figure 6A, sham animals that had received the control vector exhibited modest decreases in MAP (before hexamethonium, 103.0 ± 2.6 mm Hg; after hexamethonium, 96.0 ± 4.2 mm Hg) and HR (before hexamethonium, 618.3 ± 88.9 bpm; after hexamethonium, 578.3 ± 88.8 bpm) to hexamethonium, and Ad-Cu/ZnSOD had no significant effect on these responses (MAP: before hexamethonium, 100.7 ± 6.0 mm Hg; after hexamethonium, 94.0 ± 10.6 mm Hg; HR: before hexamethonium, 732.7 ± 10.4 bpm; after hexamethonium, 686.0 ± 30.5 bpm; $P>0.05$). In contrast, mice with congestive HF that had received the control vector (Ad-LacZ) showed markedly augmented depressor (before hexamethonium, 107.0 ± 2.0 mm Hg; after hexamethonium, 80.2 ± 4.1 mm Hg; $P<0.05$) and bradycardic (before hexamethonium, 742.6 ± 12.6 bpm; after hexamethonium, 588.2 ± 30.5 bpm; $P<0.05$) responses to hexamethonium, suggesting an enhanced contribution of sympathetic drive to basal MAP and HR in these mice. Interestingly, ICV Ad-Cu/ZnSOD caused a significant attenuation of the fall in MAP (before hexamethonium, 106.5 ± 1.8 mm Hg; after hexamethonium, 95.7 ± 3.6 mm Hg; $P<0.05$) and HR (before hexamethonium, 750.8 ± 12.4 bpm; after hexamethonium, 670.7 ± 21.2 bpm; $P<0.05$) in response to hexamethonium compared with MI-AdLacZ animals (Figure 6A). To confirm these findings using a different index of sympathetic tone and also to determine the effects of Ad-Cu/ZnSOD on sympathetic tone at an additional time point (2 weeks after MI), urinary NE levels were measured in mice that were given an ICV injection of either Ad-LacZ or Ad-Cu/ZnSOD at the time of MI surgery. As shown in Figure 6B, MI mice with the control vector demonstrated a significant increase in urinary NE levels compared with sham animals at 2 weeks. Ad-Cu/ZnSOD prevented the rise in urinary NE in MI mice such that levels were not different from sham animals (Figure 6B). These results suggest that increased superoxide production in

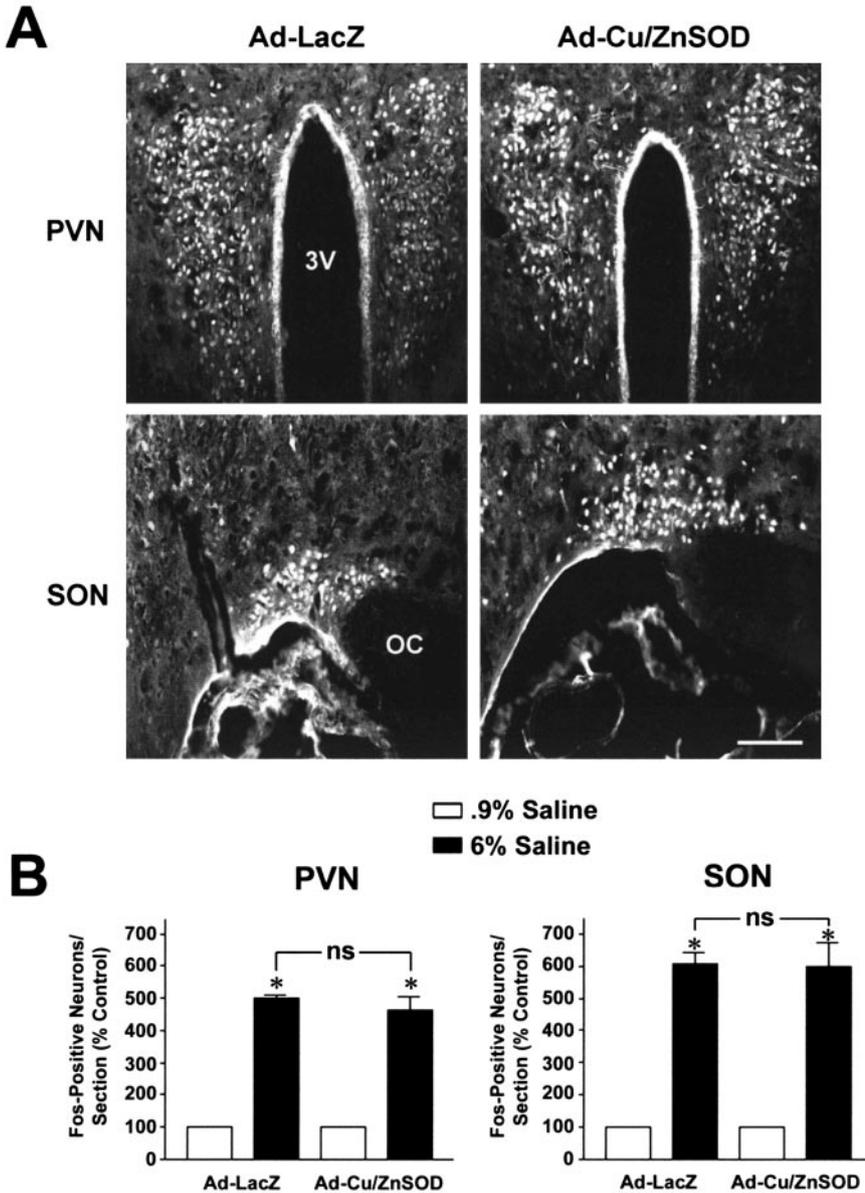


Figure 5. Overexpression of Cu/ZnSOD in the brain does not affect hypertonic saline-induced increases in Fos activation. A, Representative photomicrographs demonstrating similar levels of Fos activation in PVN (top) and SON (bottom) of Ad-LacZ-treated and Ad-Cu/ZnSOD-treated animals after subcutaneous injection of 6% saline. B, Quantification of Fos-positive neurons in the PVN and SON of Ad-LacZ-treated and Ad-Cu/ZnSOD-treated animals after subcutaneous injection of 0.9% or 6% saline. Data are mean \pm SEM. * $P < 0.05$ vs 0.9% saline; $n = 3$ per group. 3V indicates third ventricle; OC, optic chiasm. Scale bar = 100 μ m.

the brain after MI plays a critical role in driving the sympathoexcitation of the post-MI decline to HF.

Discussion

Activation of central cardiovascular control regions leading to sympathoexcitation is thought to play a critical role in the progression of HF after MI,³ although the molecular mechanisms driving this neurodysregulation are poorly understood. Using a mouse model of MI-induced HF, we have demonstrated that neurons in the hypothalamic nuclei PVN and SON are chronically activated during the development of HF and superoxide radicals play a key role in driving the excitation of these regions. Furthermore, our studies suggest that oxidant signaling in the central nervous system (CNS) is critical in the sympathetic hyperactivity of these mice after MI. To the best of our knowledge, this is the first report to demonstrate a role for ROS in the central neurohumoral activation that follows MI and suggests that oxidative stress in the brain may be involved in the pathogenesis of HF.

In the present study, we first determined the pattern of neuronal activation in central cardiovascular control regions after MI in mice. We used immunohistochemistry to detect induction of members of the Fos family of immediate early genes, including c-Fos, FosB, Fra1, and Fra2. Although c-Fos expression is transiently increased after neuronal stimulation, expression of FosB, Fra1, and Fra2 are well recognized to remain elevated under conditions of chronic stimulation, such as those occurring with various drug addictions²⁰ and during the post-MI period.¹⁵ Using an antibody that recognizes all four Fos family members in an effort to map chronic neuronal activation, we observed a dramatic increase in the number of Fos-positive neurons in the PVN and SON at both 2 and 4 weeks after MI. This is consistent with previous findings in rats with MI-induced HF. Using the same antibody as in the present study, Vahid-Ansari et al¹⁵ demonstrated an increase in Fos-positive neurons in these hypothalamic nuclei at both 2 and 4 weeks after MI in rats. In addition, Patel et al²¹ demonstrated an increase in neuronal activity in the PVN of HF rats using hexokinase

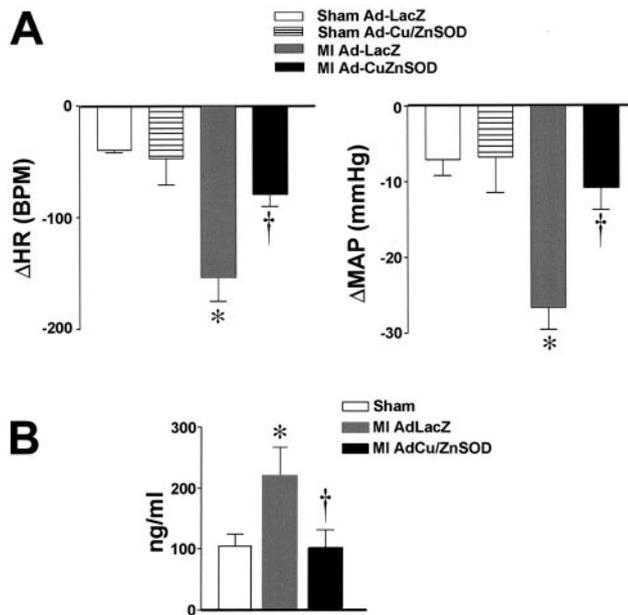


Figure 6. ICV administration of Cu/ZnSOD diminishes sympathetic drive in mice with HF. A, Summary of changes in MAP and HR in response to the ganglionic blocker hexamethonium (5 mg/kg, IP) in sham or mice with HF (5 weeks after MI) treated 3 days earlier with ICV Ad-LacZ or Ad-Cu/ZnSOD. MI mice injected with Ad-LacZ (n=5) had markedly greater decreases in HR and MAP in response to hexamethonium than sham animals injected with either Ad-LacZ (n=3) or Ad-Cu/ZnSOD (n=3). ICV Ad-Cu/ZnSOD (n=6) significantly attenuated the depressor and bradycardic responses in MI mice. B, Summary of urinary NE levels at 2 weeks after MI. MI mice injected ICV with Ad-LacZ (n=6) had significantly increased urinary NE levels compared with sham animals (n=9), and this was normalized by ICV Ad-Cu/ZnSOD (n=6). Data are mean \pm SEM. * P <0.05 vs sham; † P <0.05 vs MI Ad-LacZ.

activity assays, and increased neuronal firing in these areas by *in vivo* electrophysiological recording has also been reported.¹⁶

The time course of Fos activation relative to the development of HF in the present study in mice is consistent with what others have found in different species.¹⁵ Chronic neuronal activation was detected as early as 2 weeks after MI, but overt HF did not develop until 4 weeks after coronary ligation. This suggests that central activation precedes the development of HF rather than being a consequence of it and thus may play a causal role in the pathogenesis of the disease.

Despite general agreement concerning the time course and localization of neuronal activation in the CNS after MI, the molecular mechanisms remain poorly understood. Findings in the present study demonstrate that overexpression of Cu/ZnSOD in the brain abrogated the Fos activation. Because Fos expression is linked to sympathoexcitation and the development of HF,¹⁵ this suggests that increased superoxide levels in critical brain regions may play an important role in the pathogenesis of HF. Importantly, the inhibitory effect of SOD could not be attributed to the viral delivery system, because Fos staining was entirely intact in animals treated with the control vector. Nor was it attributable to a generalized interference with relevant neuronal networks, because SOD had no effect on Fos staining after injection with hypertonic saline, a stimulus known to elicit Fos expression in PVN and SON.¹⁸ Rather, the data suggest that redox mechanisms in the CNS are selectively activated in

response to MI and play a role in the neuronal excitation that ensues.

The immunohistochemical data demonstrating uptake and expression of the Cu/ZnSOD transgene in two of the CVOs—SFO and OVLT—may provide a possible clue as to the mechanisms involved in MI-induced oxidant signaling and neuronal activation in PVN and SON. Indeed, it is very interesting that the inhibitory effects of SOD on neuronal activation were observed in regions that were not directly transduced by the virus. However, this may not be so surprising given that the SFO and OVLT are known to send extensive projections to the PVN and SON as part of the so-called visceral neuraxis, the central circuitry that links circulating factors in the periphery with neurohumoral effector systems in the CNS.²² In fact, activation of the hypothalamic nuclei by way of sensory CVOs is one prominent theory to explain the pathways underlying MI-induced neurohumoral activation and sympathoexcitation.¹⁶ As such, our data lead us to speculate that circulating factors induced by MI act on redox systems in CVOs, such as the SFO and OVLT, which in turn lead to excitation of downstream PVN and SON neurons. The hypothesis of oxidant signaling via CVOs is particularly attractive in light of a recent study by Oury et al²³ demonstrating that brain regions lacking a blood-brain barrier are among the richest sources of endogenous SOD in the mammalian brain. Together with our findings, this raises the intriguing possibility that CVOs are pivotal sites in the CNS for redox-regulated information processing.

It is interesting to speculate which MI-induced circulating factors may be interacting with redox systems in the CNS. Given that plasma Ang II levels are elevated during HF,³ that circulating Ang II has been shown to induce Fos activation in the PVN and SON via binding to SFO and OVLT neurons,⁸ and our recent studies showing that superoxide radicals in the brain play a key role in mediating the central vasopressor effects of Ang II,¹⁴ we hypothesized that Ang II may play an important role in the redox-mediated neuronal activation after MI. In the present study, we demonstrated that chronic inhibition of central Ang II signaling with ICV infusion of losartan significantly attenuated neuronal activation in the PVN, albeit to a lesser degree than Ad-Cu/ZnSOD treatment. These results suggest that although Ang II may contribute to the redox-mediated central activation, other ROS-sensitive mechanisms may also be important. Indeed, there are several other molecules that are known to be involved in the pathogenesis of MI-induced HF and are also linked to ROS. For example, aldosterone is increased in the plasma during HF,³ facilitates Ang II binding in the brain,²⁴ and has recently been shown to be involved in increasing neuronal firing in the PVN of HF rats.¹⁶ In addition, central aldosterone increases plasma tumor necrosis factor- α levels in rats with HF,²⁵ which in turn activates forebrain regions²⁶ and increases ROS production in some cell types.²⁷ Finally, NO is also known to act as a critically important inhibitory neuromodulator in the SFO and PVN, as well as other cardiovascular control regions.^{28,29} The increased scavenging of NO by MI-induced superoxide generation in the brain could act to reduce the inhibitory influences of NO, thus contributing to the neuronal activation. Indeed, a delicate balance between NO and superoxide has been shown to be critical for normal physiological responses in other cells of the cardiovascular system.¹² The role of these various factors in

central redox signaling and HF, alone and in combination, is the subject of ongoing investigations in our laboratory.

Finally, our data confirm that mice with MI-induced congestive HF exhibit enhanced sympathetic drive, as demonstrated previously in other species,¹¹ and strongly suggest that superoxide radicals are involved in driving this sympathoexcitation. Coupled with our findings that Cu/ZnSOD inhibits MI-induced Fos expression in PVN and SON, these results suggest that oxidant signaling via the hypothalamus may be involved in the sympathetic dysregulation that is a hallmark of the post-MI decline to HF. It has been known for some time that the SON has links to sites involved in sympathetic control, with one third of its afferent projections originating in brain stem nuclei.³⁰ The PVN is also well-established as a key integrative center for regulating sympathetic activity via its inputs from the NTS³¹ and efferent projections to the rostral ventrolateral medulla and spinal cord.³² This certainly does not rule out the possibility that redox mechanisms in other CNS sites are involved in the post-MI neurodysregulation; however, at least within the limits of detection of the assays used in these studies, the PVN and SON are most strongly implicated. Interestingly, Cu/ZnSOD did not alter the response to ganglionic blockade in sham animals, suggesting that redox mechanisms may play a more predominant role in regulating sympathetic outflow during disease states such as HF than under basal conditions. This is supported by recent studies by Zanzinger et al³³ showing that microinjection of SOD into the rostral ventrolateral medulla decreased renal sympathetic nerve activity and MAP to a greater extent in pigs under chronic oxidative stress.

In conclusion, our studies suggest that oxidative stress in the brain may play an important role in the neurodysregulation that accompanies the post-MI decline to HF. Although understanding the precise pathways and intracellular mechanisms will require additional investigation, these studies lead us to speculate that oxygen radicals in the brain may be important new targets for the treatment of HF induced by MI.

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Methods and Experimental Protocols

Animals

Adult C57BL/6 mice (8-12wks, 17-28g) were used for all experiments. Animals were fed standard mouse chow (Harlan Laboratories) and water ad libitum. All procedures were approved by the Animal Care and Use Committee at the University of Iowa. Care of the mice used in the experiments met or exceeded the standards set forth by the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Myocardial Infarction

Mice were anesthetized with pentobarbital (1 mg/kg, i.p.) and supplemental anesthesia was provided using the inhaled anesthetic, methoxyflurane (Medical Developments, Australia). Mice were intubated and ventilated with a mixture of room air and methoxyflurane (Harvard Apparatus, 200 μ l tidal volume, 150 cycles/min). A lateral incision (0.5 cm) was made to expose the third intercostal space. The ribs were retracted and the pericardial sac was opened to expose the free surface of the heart. The left anterior descending (LAD) branch of the coronary artery was ligated along the anterolateral border of the heart using 8-0 ethilon suture (Ethicon). Successful ligation of the LAD resulted in blanching of the myocardium. A chest tube, constructed of PE 50 polyethylene tubing connected to a 3ml syringe, was placed in the thorax and the chest was closed to create a seal around the tubing. The skin was then closed over the chest tube using 6-0 silk suture (Ethicon). Negative pressure was applied to the chest tube

to reduce the pneumothorax, mice were extubated, and the chest tube was removed. Mice receiving the sham procedure underwent the same protocol, except the artery was not ligated.

Determination of Organ Weights

Mice were weighed and then sacrificed at either 2 or 4 wks following MI or sham procedure. The peritoneum was examined for ascites. The heart and lungs were removed, blotted to remove excess blood, and weighed. The atria and right ventricle were removed from the heart and the left ventricle (LV) was weighed separately. Organ weights were expressed as a ratio versus body weight (mg/g).

Determination of Infarct Size

Hearts were cryo-sectioned (short-axis) and mounted onto glass slides. Three 20 μ m sections were taken at 250 μ m intervals starting at the apex. The sections were then stained with hematoxylin and eosin (H &E) and coverslipped with Permount (Fisher Scientific). Digital images of the sections were obtained using a Kodak DCS760 digital camera with a Nikon Micro Nikkor 105mm lens. Using Scion Image (Scion Corp.), the length of the infarcted region (see Figure 1B, lines demarcate margins of infarcted myocardium) was measured and expressed as a percentage of the total circumference of the left ventricle.

Intracerebroventricular Injection of Adenoviral Vectors and Localization of Transgene Expression

Construction of recombinant E1-deleted adenoviral vectors encoding human cytoplasmic Cu/Zn superoxide dismutase (Ad-Cu/ZnSOD) or bacterial β -galactosidase (Ad-LacZ) (kind gift from Dr. John F. Engelhardt, University of Iowa) has been detailed previously^{1,2}. Titer-matched stocks of Ad-Cu/ZnSOD and Ad-LacZ were used (1×10^9 pfu/ml). Mice received intracerebroventricular (ICV) injections of Ad-CuZnSOD, Ad-LacZ or saline as previously described². At the conclusion of the experimental protocols, SOD expression was localized by immunohistochemistry in brain sections as described previously² using a sheep anti-human Cu/ZnSOD antibody (1:200, The Binding Site Limited, United Kingdom). Images were collected digitally using a Nikon Labphot-2 Microscope equipped with epifluorescence and a Pixera 600 imaging system.

Experimental Protocols

Experiment 1: Fos Expression in the CNS Following MI. At 2 or 4 wks following MI or sham procedure, mice were anesthetized with pentobarbital and perfused transcardially with 4% paraformaldehyde. Brains were removed, post-fixed for 1hr, saturated in 20% sucrose overnight and then cryo-sectioned (coronal, 10-30 μ m). Sections were incubated in 10% normal horse serum (NHS) for 30 min before incubation with rabbit polyclonal c-Fos (K25) antibody (1:200, Santa Cruz Biotechnologies, recognizes c-Fos, FosB, Fra-1, and Fra-2) in phosphate buffer containing 2% NHS and 0.3% Triton for 48 hrs at 4°C. Sections were then incubated in either Alexafluor 488 goat anti-rabbit IgG conjugate (1:200, Molecular Probes) for immunofluorescence or biotinylated goat anti-rabbit

IgG (1:200, Vector Laboratories) for diaminobenzidine (DAB) staining for 2hrs as described¹. All brain sections were examined and images were collected digitally as described above. Quantification of Fos staining was performed by counting positively stained neurons in 3 serial sections taken through various regions of the brain for each mouse. All data are expressed relative to controls for each individual immunohistochemical experiment.

Experiment 2: Effect of Ad-Cu/ZnSOD and Losartan on Fos Expression in Brains of Post-MI Mice. One wk following MI, separate cohorts of mice were anesthetized with pentobarbital and received an ICV injection of Ad-Cu/ZnSOD, Ad-LacZ, or saline (500 μ l) as described above. One wk after ICV injections (2wks post-MI), mice were perfused and brains processed for Fos immunohistochemistry as described in Experiment 1. In a separate experiment, the role of central AngII in Fos expression was also examined. Studies were performed exactly as described above, except mice were infused ICV with the AT₁ receptor antagonist losartan rather than Ad-Cu/ZnSOD. One wk following MI, mice were implanted with ICV cannulae as described previously³. Osmotic minipumps (model 1007D, Alzet) filled with losartan (1 μ g/hr) vehicle were connected to cannulae with PE50 tubing and implanted subcutaneously on the back of the mouse. After one wk of continuous infusion (2wks post-MI), mice were perfused and brains processed for Fos immunohistochemistry as described above.

Experiment 3: Effect of Ad-Cu/ZnSOD on Central Fos Expression Elicited

by Hypertonic Saline. To verify that Cu/ZnSOD overexpression did not have a generalized inhibitory effect on central circuitry involved in neurohumoral activation, we studied Fos activation in response to subcutaneous injections of hypertonic saline in separate groups of mice that had received either Ad-Cu/ZnSOD or Ad-LacZ (ICV) 1 wk earlier. The rationale for using hypertonic saline was based on findings that changes in plasma osmolality elicit neuronal activation in many of the same brain regions as MI does, e.g. PVN and SON⁴. One wk following viral transduction of the brain, mice received a subcutaneous injection of 6% saline or 0.9% saline (500 μ l). Two hrs later, mice were anesthetized with pentobarbital, perfused transcardially, and brains were processed for Fos immunohistochemistry as described in Experiment 1.

Experiment 4: Effect of Ad-Cu/ZnSOD on Sympathetic Tone in Post-MI Mice. Four wks following MI or sham procedure, separate cohorts of mice received an ICV injection of Ad-CuZnSOD or Ad-LacZ as described above, and during the same surgical session, were instrumented with femoral arterial catheters as described previously⁵. Three days after viral injections, changes in mean arterial pressure (MAP) and heart rate (HR) were recorded in response to the ganglionic blocker hexamethonium (5mg/kg, ip) as described previously³. The mice were conscious and freely moving in their home cages. Data were acquired and analyzed on a pressure transducer-coupled computer using Powerlab® (Chart version 4.0.1). In a separate experiment using an additional index of sympathetic tone, we examined the effect of ICV Ad-Cu/ZnSOD on urinary

norepinephrine levels. Separate cohorts of mice received an MI or sham surgery and an ICV injection of either Ad-LacZ or Ad-Cu/ZnSOD. Two wks later, urine was collected according to the method of Oliverio *et al.*⁶ and immediately frozen on dry ice and stored in the dark at -80C until quantification. Norepinephrine (NE) levels were quantified by ELISA (IBL, Hamburg, Germany) according to the manufacturer's instructions.

Statistical Analysis

All data are expressed as mean \pm SEM and were analyzed by Student *t* test or ANOVA (after Bartlett's test of homogeneity of variance), followed by the Newman-Keuls correction for multiple comparisons. Statistical analyses were performed using Prism (GraphPad Software, Inc.).

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