Brain Perivascular Macrophages and the Sympathetic Response to Inflammation in Rats After Myocardial Infarction

Yang Yu, Zhi-Hua Zhang, Shun-Guang Wei, Jordi Serrats, Robert M. Weiss, Robert B. Felder

Abstract—Inflammation is associated with increased sympathetic drive in cardiovascular diseases. Blood-borne proinflammatory cytokines, markers of inflammation, induce cyclooxygenase 2 (COX-2) activity in perivascular macrophages of the blood-brain barrier. COX-2 generates prostaglandin E₂, which may enter the brain and increase sympathetic nerve activity. We examined the contribution of this mechanism to augmented sympathetic drive in rats after myocardial infarction (MI). Approximately 24 hours after acute MI, rats received an intracerebroventricular injection (1 μL/min over 40 minutes) of clodronate liposomes (MI+CLOD) to eliminate brain perivascular macrophages, liposomes alone, or artificial cerebrospinal fluid. A week later, COX-2 immunoreactivity in perivascular macrophages and COX-2 mRNA and protein had increased in hypothalamic paraventricular nucleus of MI rats treated with artificial cerebrospinal fluid or liposomes alone compared with sham-operated rats. In MI+CLOD rats, neither perivascular macrophages nor COX-2 immunoreactivity was seen in the paraventricular nucleus, and COX-2 mRNA and protein levels were similar to those in sham-operated rats. Prostaglandin E₂ in cerebrospinal fluid, paraventricular nucleus neuronal excitation, and plasma norepinephrine were less in MI+CLOD rats than in MI rats treated with artificial cerebrospinal fluid or liposomes alone but more than in sham-operated rats. Intracerebroventricular CLOD had no effect on interleukin 1β and tumor necrosis factor-α mRNA and protein in the paraventricular nucleus or plasma interleukin-1β and tumor necrosis factor-α, which were increased in MI compared with sham-operated rats. In normal rats, pretreatment with intracerebroventricular CLOD reduced (P<0.05) the renal sympathetic, blood pressure, and heart rate responses to intracarotid artery injection of tumor necrosis factor-α (0.5 μg/kg); intracerebroventricular liposomes had no effect. The results suggest that proinflammatory cytokines stimulate sympathetic excitation after MI by inducing COX-2 activity and prostaglandin E₂ production in perivascular macrophages of the blood-brain barrier. (Hypertension. 2010;55:652-659.)

Key Words: cytokines • cyclooxygenase 2 • perivascular macrophages • clodronate liposomes • myocardial infarction • heart failure • inflammation

There is increasing appreciation for the role of inflammation in cardiovascular and cardiovascular-related diseases, including myocardial infarction (MI), heart failure (HF), hypertension, obesity, and diabetes mellitus. Recent studies have established a causal relationship between inflammation and activation of the sympathetic nervous system, but exactly how inflammatory mediators trigger an increase in sympathetic activity remains unknown. The present study examined one putative mechanism.

The proinflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin 1β (IL-1β), well-recognized markers of inflammation, are too large to cross the blood-brain barrier. In normal animals, they can affect the brain indirectly by inducing cyclooxygenase 2 (COX-2) activity in endothelial cells and perivascular macrophages of the cerebral microvasculature. COX-2 catalyzes the synthesis of prostaglandin E₂ (PGE₂), which can enter the brain to activate neurohumoral systems. This mechanism has been invoked to explain cytokine activation of the hypothalamic-pituitary-adrenal axis. 

Acute studies in normal rats have demonstrated that the perivascular macrophages are more sensitive than endothelial cells to blood-borne proinflammatory cytokines. Moreover, in HF rats that have moderately increased plasma cytokines, COX-2 expression in the paraventricular nucleus (PVN) of the hypothalamus, a key cardiovascular regulatory region of the brain, seems largely confined to the perivascular macrophages. These observations set the stage for the present study to determine whether eliminating the perivascular macrophages of the blood-brain barrier might lower...
sympathetic drive in rats with HF. The overall hypothesis guiding this study is that proinflammatory cytokines activate the sympathetic nervous system in HF rats by inducing COX-2 activity and the synthesis of PGE2 in perivascular macrophages in the brain.

Perivascular macrophages can be selectively eliminated by administering liposomes containing clodronate. The method relies upon phagocytosis of the clodronate-containing liposomes by perivascular macrophages, resulting in intracellular release of clodronate and subsequent cell death. In previous work by others, intracerebroventricular (ICV) injection of clodronate liposomes has been shown to selectively deplete meningeal and perivascular macrophages in the brain while sparing microglial and other nonphagocytic cells. The effect was nearly complete over the 2- to 10-day interval after ICV administration of the clodronate liposomes. An associated transient depletion of some particularly sensitive peripheral macrophages (ie, Kupffer cells) was also observed, with recovery within a week.

The present study used this method to examine the functional significance of brain perivascular macrophages in HF rats that have increased plasma cytokines after MI and in normal rats challenged acutely with a carotid artery injection of TNF-α. The findings suggest that brain perivascular macrophages play a critical role in the relationship between systemic inflammation and sympathetic activation.

**Methods**

**Animals**

Adult male Sprague-Dawley rats weighing 250 to 300 g were obtained from Harlan Sprague-Dawley (Indianapolis, IN). They were housed in temperature- (23±2°C) and light-controlled animal quarters and were provided with rat chow ad libitum. These studies were performed in accordance with the Guiding Principles for Research Involving Animals and Human Beings. The experimental procedures were approved by the institutional animal care and use committee of the University of Iowa and the research and development committee of the Iowa City Department of Veterans’ Affairs Medical Center.

**Drugs Administered**

Clodronate liposomes (CLOD) and control liposomes (LIPO) were kind gifts from Dr. Paul E. Sawchenko (The Salk Institute for Biological Studies, La Jolla, CA). The dose and method for injection of the liposomes and the time point for evaluating the effects of CLOD treatment were derived from a previous study demonstrating that perivascular macrophages in all of the investigated regions of the brain were maximally depleted ~1 week after injection and then gradually recovered.

**Experimental Protocols**

**Study I: Effects of Centrally Administered Clodronate Liposomes on MI and Sham-Operated Rats**

Rats underwent coronary ligation to induce MI or a sham operation (SHAM). Left ventricular function was assessed by echocardiography within 24 hours after recovery from surgery to assign rats to 1 of 4 treatment groups: SHAM rats that received no treatment (SHAM; n=15) and MI rats that received an ICV infusion (1 μL/min over 40 minutes) of CLOD (MI+CLOD; n=16), liposomes alone (MI+LIPO; n=16), or artificial cerebrospinal fluid (MI+aCSF; n=17). The ICV infusion was administered immediately after the echocardiographic assessment. One week later, at the conclusion of the study protocol, a second echocardiogram and hemodynamic measurements were obtained in some rats. Rats were then euthanized with an overdose of urethane to collect blood for the measurement of plasma cytokines and norepinephrine (NE; a marker of sympathetic nerve activity), cerebrospinal fluid (CSF) for measurement of PGE2 level, brain tissues for molecular analysis, and heart and lungs for anatomic analysis, or were perfused with fixative for immunohistochemical studies.

**Study II: Effects of Centrally Administered Clodronate Liposomes on Responses to Intracarotid Artery Injection of TNF-α in Normal Rats**

An acute bolus intracarotid artery (ICA) injection of TNF-α elicits a sympathetically mediated pressor response in normal rats. These studies were performed to determine whether perivascular macrophages in the brain mediate this response. Normal rats received an ICV infusion (1 μL/min over 40 minutes) of CLOD, LIPO, or aCSF, as in study I. A week later they were anesthetized, prepared for recording of renal sympathetic nerve activity (RSNA), blood pressure (BP), and heart rate (HR), and given a bolus ICA injection of TNF-α (0.5 μg/kg). There were 4 treatment groups: (1) aCSF alone (no TNF-α; n=16); (2) aCSF+TNF-α (n=15); (3) CLOD+TNF-α (n=15); and (4) LIPO+TNF-α (n=15). Peak responses of mean BP, HR, and RSNA recorded over a 3-minute interval during ICA infusion were compared with a 3-minute baseline value immediately preceding each intervention. RSNA responses are reported as a percent change from baseline. At the completion of the recording session, 2 hours after the ICA injection, rats were euthanized with an overdose of urethane to collect blood, CSF, and brain tissues for biochemical and molecular analyses or perfused with fixative for immunohistochemical studies.

**Specific Methods**

Please see the online Data Supplement at http://hyper.ahajournals.org for specific methods.

**Statistical Analysis**

All of the data are expressed as mean±SEM. For most studies, the significance of differences among groups was analyzed by 2-way, repeated-measures ANOVA followed by a post hoc Fisher least significant difference test. Echocardiographic parameters were analyzed using 1-way ANOVA followed by the Fisher least significant difference test. Differences were considered significant at P<0.05.

**Results**

**Study I: Effects of Centrally Administered Clodronate Liposomes on MI and Sham-Operated Rats**

**Characteristics of the Study Groups**

Echocardiography performed within 24 hours of coronary ligation revealed that left ventricular (LV) ejection fraction was reduced and LV end-diastolic volume was increased in the MI rats compared with the SHAM rats. Rats assigned to the MI+CLOD, MI+LIPO, and MI+aCSF treatment groups were well matched with regard to echocardiographically defined LV function (Table S1, available in the online Data Supplement at http://hyper.ahajournals.org).

Repeat echocardiography a week later, at the conclusion of the study protocol, revealed that all 3 groups of MI rats still had significant increases in LV end-diastolic volume and decreases in LV ejection fraction compared with SHAM rats. Treatment with ICV CLOD or LIPO, compared with ICV aCSF, had no significant effect on these echocardiographic indicators of LV function (Table S1).

Hemodynamic measurements obtained under anesthesia at the conclusion of the study protocol revealed that systolic BP,
LV peak systolic pressure, and the maximum rate of rise of LV pressure were lower, and LV end-diastolic pressure was higher, in MI rats than in SHAM rats (Table S2). There were no significant differences in diastolic BP or HR across the experimental groups. MI+CLOD rats had higher maximum rates of rise of LV pressure and lower LV end-diastolic pressure than MI+LIPO and MI+aCSF rats, but these values were still significantly different from SHAM rats.

Anatomic assessment after euthanasia revealed that right ventricle:body weight and wet lung:body weight ratios were substantially higher in MI rats compared with SHAM rats (Table S2). These variables were not affected by treatment with CLOD, LIPO, or aCSF.

Effects of CLOD on Perivascular Macrophages
Confocal microscopy revealed ED2-positive cells (perivascular macrophages) in the PVN of MI+LIPO and MI+aCSF rats, as well as in the SHAM rats. There was no ED2-positive cells in the number of ED2-positive cells between MI+aCSF and SHAM rats (data not shown). No ED2-positive cells were found in the PVN in MI+CLOD rats (Figure 1A). In contrast, OX42-positive cells (activated microglia) were present in all 4 of the treatment groups and seemed to be unaffected by treatment with CLOD, LIPO, or aCSF.

As we have shown previously,3,4 COX-2 immunoreactivity in the PVN of MI rats is found primarily in the cytoplasm of perivascular macrophages. In the present study, MI+CLOD rats, lacking perivascular macrophages, had no COX-2 immunoreactivity in the PVN (Figure 1A). MI+LIPO and MI+aCSF rats had the expected COX-2 immunoreactivity in ED2-positive cells.

Effects of CLOD on the Expression of Central Inflammatory Mediators
COX-2 mRNA (Figure 2A) and protein (Figure 2B) were greater in the PVN of MI+LIPO and MI+aCSF rats than in SHAM rats. In MI+CLOD rats, COX-2 mRNA and protein expression were not different from SHAM rats. There were no differences across treatment groups in COX-2 expression in hypothalamic regions immediately adjacent to PVN.

The PGE₂ level in the CSF was increased in all 3 MI groups, compared with SHAM (Figure 3). MI+CLOD rats had less CSF PGE₂ than MI+LIPO and MI+aCSF rats but more than SHAM rats. There was no difference in CSF PGE₂ between the MI+LIPO and the MI+aCSF groups.

IL-1β and TNF-α mRNA (Figure 2A) and protein (Figure 2B) were greater in the PVN of all 3 MI groups than in SHAM rats. Neither CLOD nor LIPO affected IL-1β or TNF-α expression in PVN or adjacent hypothalamic regions.

Effects of CLOD on Neuronal Excitation in PVN
Fra-like activity, an indicator of chronic neuronal excitation, was significantly increased in medial parvocellular, ventrolateral parvocellular, and dorsal parvocellular regions in MI+LIPO and MI+aCSF rats, compared with SHAM rats (Figure 4). MI+CLOD rats had significantly less Fra-like activity in the medial parvocellular and ventrolateral parvo-cellular regions of PVN. Fra-like activity in dorsal parvo-cellular region was not affected. MI+LIPO and MI+aCSF rats had similar Fra-like activity in all 3 parvocellular regions. There were no differences across treatment groups in Fra-like activity in the posterior magnocellular region of PVN.

Effects of CLOD on Plasma Levels of IL-1β, TNF-α, and NE
Plasma levels of IL-1β, TNF-α, and NE were higher (P<0.05) in all 3 of the MI treatment groups as compared with SHAM rats (Figure 3). MI+CLOD rats had significantly lower plasma NE levels than MI+LIPO and MI+aCSF but higher levels than SHAM rats. Plasma levels of IL-1β and TNF-α were similar across all 3 of the MI treatment groups.
Study II: Effects of Centrally Administered Clodronate Liposomes on Responses to ICA Injection of TNF-α in Normal Rats

Effects of CLOD on Perivascular Macrophages

As in study 1, confocal microscopy revealed that central injection of CLOD depleted perivascular macrophages in the PVN but had no apparent effect on activated microglia (data not shown). As expected, TNF-α induced increased COX-2 immunoreactivity in perivascular macrophages of the PVN in aCSF- and LIPO-treated rats, compared with the aCSF (no TNF-α) group, but no COX-2 activity was induced in the CLOD-treated rats in which the perivascular macrophages were depleted (data not shown).

Real-time PCR and Western blot showed significantly increased COX-2 mRNA and protein expression in the PVN of aCSF+TNF-α rats compared with the aCSF (no TNF-α) rats. CLOD+TNF-α rats had less COX-2 mRNA and protein expression than LIPO+TNF-α or aCSF+TNF-α rats (Figure 5). There were no differences in expression of COX-2 mRNA and protein between LIPO+TNF-α and aCSF+TNF-α rats.

Real-time PCR and Western blot showed significantly increased COX-2 mRNA and protein expression in the PVN of aCSF+TNF-α rats compared with the aCSF (no TNF-α) rats. CLOD+TNF-α rats had less COX-2 mRNA and protein expression than LIPO+TNF-α or aCSF+TNF-α rats (Figure 5). There were no differences in expression of COX-2 mRNA and protein between LIPO+TNF-α and aCSF+TNF-α rats.

Figure 2. Quantitative comparison of mRNA expression (A) and protein levels (B) for COX-2, TNF-α, and IL-1β from the PVN and adjacent regions of the hypothalamus of each treatment group. Representative Western blots of COX-2, TNF-α, IL-1β, and β-actin are shown in B. Values were expressed as mean±SEM (n=5 to 8 for each group). *P<0.05 vs SHAM in same region; †P<0.05 MI+ treatment vs MI+aCSF in same region.

Figure 3. Plasma TNF-α (A) and IL-1β (B), CSF PGE2 (C), and plasma NE (D) levels in each group. Values were expressed as mean±SEM (n=6 to 8 for each group). *P<0.05 vs SHAM; †P<0.05 MI+ treatment vs MI+aCSF.
Similarly, as predicted by the COX-2 results, aCSF+TNF-α and LIPO+TNF-α rats had an increased PGE₂ level in CSF as compared with aCSF (no TNF-α) rats. CLOD+TNF-α rats had lower PGE₂ levels in CSF than LIPO+TNF-α or aCSF+TNF-α rats. In LIPO+TNF-α rats, PGE₂ level in CSF was no different from that in aCSF+TNF-α rats.

**Effects of CLOD on Sympathetic Responses to TNF-α**

ICA injection of TNF-α (0.5 μg/kg) in the aCSF-treated rats dramatically (P<0.05) increased integrated RSNA (42.4±6.3%), HR (39.3±4.1 bpm, from baseline of 328.5±16.6 bpm), and MBP (19.0±2.2 mm Hg, from baseline of 95.1±5.8 mm Hg). The onset latency for this response was ≈10 to 15 minutes with duration of ≈120 minutes recorded. The peak responses occurred ≈30 to 40 minutes after TNF-α administration. Compared with aCSF-treated rats, CLOD-treated rats had significantly (P<0.05 versus aCSF+TNF-α) diminished sympatho-excitatory responses to ICA TNF-α, including integrated RSNA (12.8±5.4%), MBP (6.4±2.4 mm Hg), and HR (14.7±4.4 bpm). There were no differences in the responses to TNF-α between LIPO- and aCSF-treated rats (Figure 6).

**Discussion**

This study reports the novel finding that a treatment that selectively eliminates a single cell type, the perivascular macrophage of the blood-brain barrier, normalizes COX-2 expression in the microvessels of the PVN and reduces both PGE₂ levels in CSF and sympathetic nerve activity in rats with increased circulating cytokines after MI. In normal rats, selective depletion of these perivascular macrophages prevents the increases in HR, BP, and sympathetic nerve activity induced by ICA administration of TNF-α. Together, these results demonstrate that perivascular macrophages of the blood-brain barrier contribute to the augmented sympathetic nerve activity in rats with HF after MI and suggest that the proinflammatory cytokines, which increase in the blood and the brain of rats with HF after MI, are a likely driving mechanism. The data also implicate perivascular macrophages as a primary source of cytokine-induced COX-2 activity and PGE₂ production in the PVN, an important presympathetic nucleus, in rats early after MI. A broader interpretation might be that the perivascular macrophages provide a crucial link between systemic inflammation and sympathetic drive.

**Figure 4.** Expression of Fra-like (Fra-LI) activity in the PVN. A, Representative sections from each group showing Fra-LI immunoreactivity in PVN neurons. Dark dots indicate Fra-LI-positive neurons. Squares (dotted lines) indicate the regions in A from which the data in B are derived. Scale bar, 200 μm. B, Quantification of Fra-LI-positive neurons in 4 different regions of the PVN. Values are expressed as mean±SEM (n=4 for each group). *P<0.05 vs SHAM; †P<0.05 MI+ treatment vs MI+aCSF. pm indicates posterior magnocellular; mp, medial parvocellular; vlp, ventrolateral parvocellular; dp, dorsal parvocellular.

**Figure 5.**Effects of bolus ICA injection of TNF-α on COX-2 mRNA (A) and protein (B) expression in PVN and PGE₂ level in CSF (C) in normal rats pretreated a week earlier with ICV CLD, LIPO, or aCSF. aCSF-treated rats not receiving TNF-α injection were used as control. Representative Western blots are aligned with the matching grouped data (B). Values were expressed as mean±SEM (n=5 to 7 for each group). *P<0.05 vs aCSF (no TNF-α); †P<0.05 treatment+TNF-α vs aCSF+TNF-α.
These results complement earlier studies from our laboratory demonstrating that rats with ischemia-induced HF have an increase in COX-2 expression in perivascular macrophages in the microvasculature of the PVN. The increase in COX-2 activity in the perivascular macrophages of the PVN also responds to changes in the proinflammatory cytokine content inside the blood-brain barrier. In those earlier studies, a reduction in COX-2 activity in the perivascular macrophages was uniformly associated with reductions in CSF PGE$_2$ and plasma NE in HF rats, suggesting a tight association between COX-2 activity in perivascular macrophages and sympathetic drive that was confirmed in the present study.

It is surprising that the destruction of the perivascular macrophages normalized COX-2 mRNA and protein in the PVN of the HF rats and eliminated COX-2 immunofluorescence. Microglial cells, which are activated in the HF rats as they are under conditions of chronic COX-2 immunofluorescence, were apparently unaffected by treatment with CLOD. Microglia can produce a wealth of inflammatory mediators, including COX-2, PGE$_2$, and the proinflammatory cytokines TNF-α and IL-1β. Why microglia do not appear to contribute to overall COX-2 activity in the HF rats in these studies remains unexplained. A predominant influence of the perivascular macrophages is suggested, but the incomplete reduction in the PGE$_2$ level in the CSF suggests that COX-2 activity in perivascular macrophages is not the only source of PGE$_2$ production in the HF brain.

In normal rats challenged acutely with a carotid artery injection of TNF-α, there was a direct relationship between the induction of COX-2 and CSF PGE$_2$ levels; pretreatment with CLOD prevented the TNF-α-induced increase in COX-2, PGE$_2$, and sympathetic drive. These new findings are consistent with previous work demonstrating that acutely administered proinflammatory cytokines induce COX-2 activity in perivascular macrophages and activate the sympathetic nervous system via a cyclooxygenase-dependent mechanism. In addition, they implicate the perivascular macrophage as the single cellular element responsible for acute cytokine-induced sympathetic activation. However, they leave open the question of how a sympathetic response dependent on the induction of COX-2 protein can occur so quickly, that is, the induction of a pressor response within 10 to 15 minutes after injection of TNF-α. COX-2 is constitutively expressed in brain tissue, including in PVN, and our data demonstrate some COX-2 present in the perivascular macrophages of the sham-operated animals. It is conceivable that constitutively present COX-2 might somehow contribute to these very early responses. COX-1 seems an unlikely contributor because it does not increase in HF rats that have chronic elevation of proinflammatory cytokines.

Finally, it is notable that CLOD had no effect on the ambient lower levels of COX-2 expression in immediately adjacent hypothalamic tissues. This may simply be a function of the dense vascularity of the PVN, compared with surrounding regions, providing a substrate for higher numbers of perivascular macrophages. Nevertheless, it suggests a heightened responsiveness of this particular region of the brain to inflammatory signals from the periphery. This is consistent with the known influence of cytokines on the hypothalamic-pituitary-adrenal axis and with our previous observation that COX-2 expression in the cortex, where it is more diffusely distributed, does not seem to be affected by HF or by treatments for HF that reduce COX-2 expression in PVN.

An intriguing finding of this study is the absence of any effect of CLOD on the expression of proinflammatory cytokines within the PVN. The mechanism responsible for the increase in the proinflammatory cytokines in cardiovascular
regions of the brain in HF rats has not been elucidated. The present study argues against cytokine signaling across the blood-brain barrier, via PGE2, production, as a potential mechanism. Signaling via the renin-angiotensin-aldosterone system or via cardiovascular afferent fibers remains a viable possibility.

This study focused on the role of COX-2 and PGE2 production by the perivascular macrophages of the blood-brain barrier, a mechanism by which circulating proinflammatory cytokines are thought to activate the hypothalamic-pituitary-adrenal axis. A caveat to be considered is that stimulated macrophages can also produce reactive oxygen species, including superoxide, which may also activate the sympathetic nervous system. Thus, the present study does not exclude the possibility that other products of perivascular macrophages might contribute to cytokine-induced sympathetic discharge in HF.

Central interventions that chronically reduce sympathetic nerve activity in rats with HF after MI can reduce LV remodeling and improve LV function. These beneficial effects likely result from reductions in sympathetically mediated renin release and activation of the renin-angiotensin-aldosterone system, sympathetically mediated renal absorption of sodium and water, and sympathetically mediated direct detrimental effects on cardiac myocytes. Cardiac remodeling is a chronic progressive process evolving over weeks after MI. Significant effects of central intervention on LV remodeling and function in rat models of HF have typically been demonstrated after ≥4 weeks of continuous ICV drug administration. In the present study, in which LV function was reassessed only 1 week after MI and only 1 week after a single ICV injection of CLOD, small but significant improvements in LV systolic function (LV dP/dtmax) and volume regulation (LV end-diastolic pressure) were observed. The short duration of treatment and the reassessment of LV function so early in the course of cardiac remodeling may have minimized our ability to demonstrate greater improvements in LV function. Another factor to consider is that depleting the brain perivascular macrophages reduced but did not normalize plasma NE, the surrogate marker of sympathetic drive used in this study. Thus, the continued influence of other neurohumoral systems on sympathetic drive may also have impaired our ability to demonstrate larger changes in LV function.

Perspectives

The present study used a unique pharmacological tool to target a particular cell type and demonstrate its role as a mediator of increased sympathetic drive in HF. The study unequivocally demonstrates that COX-2 expression and PGE2 production by perivascular macrophages of the blood-brain barrier increase in rats with HF after MI and that selective elimination of these perivascular macrophages reduces cytokine-induced sympathetic drive. Based on what is already known about the effect of proinflammatory cytokines on COX-2 expression by perivascular macrophages in the brain, the effects of cytokine-induced PGE2 production in the brain on sympathetic drive and the present results from both normal and MI rats, it seems reasonable to speculate that the proinflammatory cytokines contribute to the augmented sympathetic drive in rats with HF after MI by inducing COX-2 activity and PGE2 production by the perivascular macrophages. However, a role for other products of perivascular macrophages that might stimulate the sympathetic nervous system cannot be excluded.

A more global implication of these findings might be that the perivascular macrophages signal the brain to increase sympathetic activity in response to systemic inflammation, not only in HF but also in other cardiovascular conditions, such as hypertension, obesity, and diabetes mellitus, in which both proinflammatory cytokines and sympathetic drive are increased. Additional studies are needed to determine whether perivascular macrophages of the blood-brain barrier increase COX-2 expression or the expression of other substances that might activate the sympathetic nervous system in these disease processes. Ultimately, the identification of discrete cellular mechanisms that augment sympathetic drive raises the possibility of novel targeted interventions to disrupt the progression of HF, hypertension, and other devastating cardiovascular diseases.

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Disclosures

None.

References

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SPECIFIC METHODS

Induction of myocardial infarction (MI) or sham operation (SHAM)
Rats were anesthetized (ketamine 90 mg/kg + xylazine 10 mg/kg, i.p.) and underwent coronary ligation (CL) to induce MI (n=75) or sham CL (SHAM, n=15) as described. Twenty-one rats died within 24 hours of CL. All of the surviving rats underwent echocardiography under sedation (ketamine 25 mg/kg, i.p.) within 24 hours of CL to confirm the extent of ischemic injury. Measurements of ischemic zone as a percent of left ventricular (LV) circumference (%IZ), LV ejection fraction (LVEF) and LV end-diastolic volume (LVEDV) were made as previously described. Only animals with large infarctions (IZ ≥ 37%) were used in the study. Five rats with small MI were excluded.

Intracerebroventricular Injection of Liposomes
Rats were anesthetized (ketamine 90 mg/kg + xylazine 10 mg/kg, i.p.) and a small hole was drilled in the skull to permit placement of a 27-gauge needle connected to a Hamilton syringe in the right lateral ventricle (stereotaxic coordinates: 1.5 mm lateral and 1 mm posterior to the bregma, 3.5 mm ventral to dura) as described. Clodronate liposomes, liposome alone or aCSF were then injected at 1 µl/min for 40 minutes.

Hemodynamic and Anatomic Measurements in MI rats
One week after liposomes injection, MI rats were anesthetized (urethane, 1.5 g/kg, IP) for terminal study. A Millar catheter was inserted into the aorta via the right carotid artery, and subsequently advanced across the aortic valve into the heart. Systolic blood pressure (SBP), diastolic blood pressure (DBP), LV end-diastolic pressure (LVEDP), LV peak systolic pressure (LVPSP), maximum rate of rise of LV pressure (dP/dtmax) and heart rate (HR) were measured as described previously.

Wet lung weight and right ventricular (RV) weight, with respect to body weight (BW) of MI rats, were measured as indices of pulmonary congestion and right ventricular remodeling.

Electrophysiological and Hemodynamic Recording in Normal Rats
The general methods for recording and data analysis have been described previously. In brief, rats were anesthetized with urethane (1.5 g/kg, IP), supplemented as needed (0.1g/kg). A femoral arterial catheter was implanted to measure blood pressure (BP). Body temperature was maintained at 37 ± 1°C with a heating pad and heat lamp. A renal nerve was exposed via a left flank incision, and was dissected free from surrounding tissue and placed on bipolar silver wire recording electrodes to record renal sympathetic nerve activity (RSNA). Nerve and electrodes were stabilized with Kwik-Cast silicon sealant (WPI, Inc, Sarasota, FL) and the incisions were closed.

Data were acquired with a Cambridge Electronics Design laboratory interface (CED, model 1401; Cambridge, UK) linked to a personal computer. The RSNA was initially processed with by Paynter filter (20-ms time constant, BAK Electronics; Germantown, MD) to rectify and integrate the raw multifiber signal. The BP signal was passed to the CED 1401 via a Gould TA240S chart recorder (Gould Instruments, Valley View, OH).
Mean blood pressure (MBP) and HR were derived from BP signal. Digitized data were stored for subsequent off-line analysis with Spike2 software (CED).

**Brain Tissue Processing for Molecular Study**

Brain tissue was cut into 500-µm coronal sections. A punch biopsy was obtained from right and left PVN using a 15-gauge needle stub (ID: 1.5 mm) according to a previously described method. Right and left hypothalamic tissues surrounding PVN (adjacent regions; 1.2 mm from midline, between fornix and retrochiasmatic area at the level of PVN) were also punched to obtain control tissues. Tissues were homogenized in TRI Reagent (Molecular Research Center, Inc, Cincinnati, OH) to extract total RNA for gene analysis, or in cell lysis buffer (Cell Signaling Technology Inc, Beverly, MA) to extract protein for Western blotting analysis.

**Quantitative Assessment of mRNA Expression of Inflammatory Mediators**

mRNA expression for COX-2, IL-1β, and TNF-α in PVN and adjacent regions within hypothalamus were measured with real-time PCR following reverse transcription of total RNA. The sequences for primers and probe used were as follows: COX-2: forward primer, 5'-CGCTGTACAAGCAGTGGCAAAG-3'; reverse primer, 5'-GCGTTTGCGGTACTCATTGAGA-3'; probe, 5'-CCTCCATTGAGCCAGAGAGATGAAA-3'; TNF-α: forward primer, 5'-CCAGGAGAAAGTCAGCCTCCTCCT-3'; reverse primer, 5'-TCATACCCAGGCTTGAGCTCA-3'; probe, 5'-AGAGCCCTTGCCCTAAGGCATCCCTGAGCTCA-3'. Primers and probes for IL-1β and GAPDH were purchased from Applied Biosystems (Foster City, CA). Real-time PCR was performed using the ABI prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). The final results of real-time PCR are expressed as the ratio of mRNA of interest to GAPDH.

**Western Blotting Analysis**

Protein levels for inflammatory mediators were analyzed with Western blotting method, as previously described, using polyclonal primary antibodies to COX-2 (Cayman Chemical Co, Ann Arbor, MI), IL-1β and TNF-α (Santa Cruz Biotechnology Inc, Santa Cruz, CA). Band intensities were quantified with NIH ImageJ software and were normalized by β-actin.

**Immunohistochemistry**

Four rats from each group were used for immunohistochemical studies to examine effects of liposomes on depletion of perivascular macrophages and activated microglial cells, and to assess COX-2 immunoreactivity and neuronal activity in the PVN as described before. Perivascular macrophages were identified by immunofluorescent staining with an antibody for ED2 (1:1000, Serotec Inc, Raleigh, NC), an established marker for perivascular macrophages. Activated microglial cells were stained with antibody for OX42 (1:100, Serotec Inc, Raleigh, NC). COX-2 immunoreactivity was determined using antibody for COX-2 (1:1000, Cayman Chemical Co, Ann Arbor, MI). Nuclear stain for To-Pro-3 (1:2000; Molecular Probes, Eugene, OR) was used to visualize cell nuclei. The sections stained with fluorescent dyes were recorded with a confocal laser-
scanning microscope (Zeiss LSM 510, Carl Zeiss Inc, Minneapolis, MN). The ED2 positive immunoreactive cells from 5-10 blood vessels in each subregion of the PVN of MI+aCSF and SHAM rats were counted manually, and the total number of cells from the 4 different regions was used for comparison between the two groups.

Fra-like (Fra-LI; fos family gene) activity, a marker of neuronal activation, was detected using a rabbit polyclonal antibody (c-fos K-25; Santa Cruz Biotechnology). In each animal, Fra-LI–positive neurons within a window superimposed over posterior magnocellular, ventrolateral parvocellular, medial parvocellular and dorsal parvocellular PVN were counted manually in two representative 16-μm transverse sections approximately -1.8 mm from bregma and were averaged to obtain a value for data analysis as described before. The subregions of PVN were defined as described in previous studies.

**Analysis of Blood and CSF**

The levels of IL-1β, TNF-α, NE in plasma and PGE2 in CSF were measured using high sensitivity ELISA kits (Biosource International Inc, Camarillo, CA; Rocky Mountain Diagnostics Inc, Colorado Springs, CO; and R&D Systems Inc, Minneapolis, MN, respectively).
REFERENCES


Table S1. Echocardiographic measurements

<table>
<thead>
<tr>
<th>Variables at Baseline</th>
<th>SHAM (n=15)</th>
<th>MI+aCSF (n=17)</th>
<th>MI+CLOD (n=16)</th>
<th>MI+LIPO (n=16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVEDV (ml)</td>
<td>0.27 ± 0.02</td>
<td>0.52 ± 0.04*</td>
<td>0.56 ± 0.05*</td>
<td>0.53 ± 0.03*</td>
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<tr>
<td>LVEF</td>
<td>0.80 ± 0.02</td>
<td>0.35 ± 0.03*</td>
<td>0.32 ± 0.06*</td>
<td>0.42 ± 0.04*</td>
</tr>
<tr>
<td>%IZ</td>
<td>----</td>
<td>44 ± 3*</td>
<td>46 ± 4*</td>
<td>43 ± 2*</td>
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</table>

<table>
<thead>
<tr>
<th>Variables at 1 week</th>
<th>SHAM (n=6)</th>
<th>MI+aCSF (n=8)</th>
<th>MI+CLOD (n=7)</th>
<th>MI+LIPO (n=7)</th>
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</thead>
<tbody>
<tr>
<td>LVEDV (ml)</td>
<td>0.28 ± 0.02</td>
<td>0.64 ± 0.03*</td>
<td>0.64 ± 0.06*</td>
<td>0.66 ± 0.06*</td>
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<tr>
<td>LVEF</td>
<td>0.84 ± 0.02</td>
<td>0.29 ± 0.04*</td>
<td>0.26 ± 0.03*</td>
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<tr>
<td>% IZ</td>
<td>----</td>
<td>42 ± 2*</td>
<td>41 ± 2*</td>
<td>45 ± 1*</td>
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</table>

SHAM: sham-operated control; MI: myocardial infarction; aCSF, artificial cerebrospinal fluid; MI+aCSF: MI rats treated with aCSF; MI+CLOD: MI treated with clodronate liposomes; MI+LIPO: MI treated with liposomes. LVEDV: left ventricular end-diastolic volume; LVEF: left ventricular ejection fraction; % IZ: ischemic zone as a percent of left ventricular circumference. Values are expressed as mean ± SEM. *P<0.05 vs SHAM at same week.
Table S2. Anatomical and hemodynamic measurements

<table>
<thead>
<tr>
<th>Variables at 1 week</th>
<th>SHAM (n=6)</th>
<th>MI+aCSF (n=8)</th>
<th>MI+CLOD (n=7)</th>
<th>MI+LIPO (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>381 ± 5</td>
<td>366 ± 5</td>
<td>370 ± 4</td>
<td>369 ± 6</td>
</tr>
<tr>
<td>RV/BW (mg/g)</td>
<td>0.50 ± 0.02</td>
<td>0.67 ± 0.03*</td>
<td>0.58 ± 0.03*</td>
<td>0.65 ± 0.05*</td>
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<tr>
<td>Lung/BW (mg/g)</td>
<td>3.46 ± 0.11</td>
<td>4.27 ± 0.20*</td>
<td>4.07 ± 0.15*</td>
<td>4.23 ± 0.25*</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>315 ± 14</td>
<td>334 ± 15</td>
<td>329 ± 11</td>
<td>327 ± 12</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>125 ± 4</td>
<td>106 ± 3*</td>
<td>108 ± 5*</td>
<td>109 ± 4*</td>
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<tr>
<td>DBP (mmHg)</td>
<td>91 ± 3</td>
<td>88 ± 2</td>
<td>89 ± 4</td>
<td>89 ± 3</td>
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<tr>
<td>LVPSP (mmHg)</td>
<td>117 ± 2</td>
<td>100 ± 2*</td>
<td>102 ± 4*</td>
<td>105 ± 2*</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>4 ± 1</td>
<td>17 ± 2*</td>
<td>13 ± 1* †</td>
<td>16 ± 2*</td>
</tr>
<tr>
<td>LV dP/dt_max (mmHg/s)</td>
<td>8812 ± 510</td>
<td>4498 ± 103*</td>
<td>5333 ± 380* †</td>
<td>4516 ± 122*</td>
</tr>
</tbody>
</table>

SHAM: sham-operated control; MI: myocardial infarction; aCSF, artificial cerebrospinal fluid; MI+aCSF: MI rats treated with aCSF; MI+CLOD: MI treated with clodronate liposomes; MI+LIPO: MI treated with liposomes. BW: body weight; RV: right ventricular weight; HR: heart rate; SBP: systolic blood pressure; DBP: diastolic blood pressure; LVPSP: LV peak systolic pressure; LVEDP: LV end-diastolic pressure; dP/dt_max: maximum rate of rise of LV pressure. Values are expressed as mean ± SEM.

*P<0.05 vs SHAM, †P<0.05, MI+Treatment vs MI+aCSF.