Mechanisms of Cardiac Nerve Sprouting After Myocardial Infarction in Dogs

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Abstract—Cardiac nerve sprouting and sympathetic hyperinnervation after myocardial infarction (MI) both contribute to arrhythmogenesis and sudden death. However, the mechanisms responsible for nerve sprouting after MI are unclear. The expression of nerve growth factor (NGF), growth associated protein 43 (GAP43), and other nerve markers were studied at the infarcted site, the noninfarcted left ventricle free wall (LVFW), and the left stellate ganglion (LSG) at several time points (30 minutes to 1 month) after MI. Transcardiac (difference between coronary sinus and aorta) NGF levels were also assayed. Acute MI resulted in the immediate elevation of the transcardiac NGF concentration within 3.5 hours after MI, followed by the upregulation of cardiac NGF and GAP43 expression, which was earlier and more pronounced at the infarcted site than the noninfarcted LVFW. However, cardiac nerve sprouting and sympathetic hyperinnervation were more pronounced in the noninfarcted than the infarcted LVFW site and peaked at 1 week after MI. The NGF and GAP43 protein levels significantly increased in the LSG from 3 days (P<0.01 for all) after MI, without a concomitant increase in mRNA. There was persistent elevation of NGF levels in aorta and coronary sinus within 1 month after MI. We conclude MI results in immediate local NGF release, followed by upregulation of NGF and GAP43 expression at the infarcted site. NGF and GAP43 are transported retrogradely to LSG, which triggers nerve sprouting at the noninfarcted LVFW. A rapid and persistent upregulation of NGF and GAP43 expression at the infarcted site underlies the mechanisms of cardiac nerve sprouting after MI. (Circ Res. 2004;95:000-000.)

Key Words: nerve growth factor ▪ nerve sprouting ▪ sympathetic nerve ▪ ventricular arrhythmia

We previously demonstrated that heterogeneous cardiac nerve sprouting and sympathetic hyperinnervation play important roles in arrhythmogenesis and sudden cardiac death in both human patients and animal models of myocardial infarction (MI). However, the mechanisms and time course of nerve sprouting after MI are unclear. Nerve growth factor (NGF) is a neurotrophin that supports the survival and differentiation of sympathetic neurons and enhances target innervation. NGF also regulates the synthesis of neurofilament and tubulin proteins, promotes Schwann cell migration, modulates synaptic transmission between sympathetic neurons and cardiac myocytes, and increases the half-life of growth associated protein-43 (GAP43). Overexpression of NGF within the heart of transgenic mice causes hyperinnervation. Peripheral nerve injury results in increased local NGF expression, which facilitates nerve regeneration. It is possible that increased NGF expression also underlies the mechanisms of cardiac nerve sprouting after ischemic injury and MI. In the present study, we sampled blood and harvested tissues from the left ventricle and from the left stellate ganglion at different time points after experimental canine MI. NGF expression and the magnitude of cardiac nerve sprouting were studied to test the hypothesis that increased local NGF production underlies the mechanisms of nerve sprouting after MI.

Materials and Methods

The animal experiments were performed with approval of the Institutional Animal Care and Use Committee of the Cedars-Sinai Medical Center. Mongrel dogs (22 to 28 Kg) were obtained from a USDA licensed commercial dog vendor in Missouri. Propofol and isoflurane were used for general anesthesia during all surgical procedures.

Surgical Procedure

MI was created by ligating the left anterior descending coronary artery below its first diagonal branch in 14 open-chest and anesthetized dogs. One concern of open-chest ligation is that the ligation itself might directly result in cardiac nerve injury. To address this issue, we created MI in an additional 7 dogs using continuous intracoronary balloon inflation for 1 to 3 hours to occlude blood flow. ECG was monitored to document ST segment elevation. Blood was drawn from the aorta and coronary sinus (CS) simultaneously at baseline, and then at 0.5, 1.5, 2.5, and 3.5 hours after MI. Dogs were followed up 3.5 hours (n=3), 3 days (n=5), 1 week (n=10), and 1 month (n=3) after MI. The 3-day group included 2 dogs with balloon inflation and 3 dogs with epicardial ligation. The 1-week
group included a subgroup of 5 dogs with intracoronary balloon inflation and another subgroup of 5 dogs with epicardial ligation. We performed a second surgery at the end of the follow-up, and ECG and blood samples were obtained again. Fresh cardiac tissue from the infarcted site, noninfarcted left ventricle free wall (LVFW), and left stellate ganglion (LSG) were sampled using sharp skin biopsy punches (Acu-Punch, Acuderm Inc) and a scalpel. The samples were immediately frozen in liquid nitrogen and stored in \(-80^\circ\text{C}\) for further processing. Two corresponding sites from left ventricle and LSG were sampled from the control group. The hearts were then excised and immersed in 4\% formalin for 1 hour, followed by storage in 70\% alcohol for immunohistological study. Infarct size was determined as previously reported.

**mRNA Analyses**

Total RNA was extracted from the infarcted site, noninfarcted LVFW, and LSG using Trizol (Invitrogen) according to the manufacturer’s protocol, treated with DNAse I (Qiagen) to degrade any trace of DNA, and later cleaned again with Rneasy Kit (Qiagen). Total RNA was extracted from the infarcted site, noninfarcted LVFW, and left stellate ganglion (LSG) were sampled using sharp skin biopsy punches (Acu-Punch, Acuderm Inc) and a scalpel. The samples were immediately frozen in liquid nitrogen and stored in \(-80^\circ\text{C}\) for further processing. Two corresponding sites from left ventricle and LSG were sampled from the control group. The hearts were then excised and immersed in 4\% formalin for 1 hour, followed by storage in 70\% alcohol for immunohistological study. Infarct size was determined as previously reported.

**Western Blotting Studies**

Cardiac tissues and the remaining LSG were homogenized on ice and expressed as the nerve area divided by the total area examined (\(\mu\text{m}^2/\text{mm}^2\)). The investigators were blinded to the specimen’s source. The nerve density of each slide was determined by the average of 3 fields with the highest nerve density.

**Enzyme-Linked Immunosorbent Assay**

Serum NGF concentrations were assayed by sandwich enzyme-linked immunosorbent assay (ELISA) using NGF Emax Immunoassay System (Promega) according to the manufacturer’s instructions. All assays were performed on F-bottom 96-well plates (Nunc). Tertiary antibodies were conjugated to horseradish peroxidase. Wells were developed with tetramethylbenzidine and measured at 450 nm. NGF content was quantified against a standard curve calibrated with known amounts of protein. All samples were assayed in triplicate and expressed as a mean. Transcardiac NGF concentration is defined by the difference of NGF concentrations in the CS and aorta.

**Statistical Analyses**

The Student \(t\) tests were used to compare the means. ANOVA with Newman-Keuls test was used for multiple comparisons. A value of \(P\leq0.05\) was considered significant.

**Results**

**Characterization of the MI Dogs**

During first surgery, ST segment elevation and frequent premature ventricular contractions (PVCs) were observed immediately after the induction of MI in all dogs. Short nonsustained ventricular tachycardia (VT) was observed in 11/21 dogs. Three dogs were euthanized during first surgery, after blood samples were obtained 3.5 hours after MI. All remaining 18 dogs survived during follow-up. During second surgery, we observed ST segment elevation in 18/18, PVC in
Heterogeneous Cardiac NGF Expression After MI

To study the temporal and spatial variation of NGF expression after MI, we analyzed NGF mRNA levels at the infarcted site and noninfarcted LVFW at 3.5 hours, 3 days, 1 week, and 1 month after MI by using real-time qRT-PCR. The specificity of amplified PCR product was verified by agarose gel electrophoresis (Figure 1A, top panel). Nonspecific DNA fragments were not detected. The relative NGF mRNA levels at different time points and locations of heart were shown in Figure 1A. At 3.5 hours after MI, the NGF mRNA levels at both the infarcted site and noninfarcted LVFW were not significantly different from the control group. At 3 days after MI, the infarcted site but not noninfarcted LVFW showed significant increase in NGF mRNA compared with the control group (4.4-fold, \( P < 0.01 \)). At 1 week after MI, NGF mRNA at both the infarcted site (ligation group, 5.6-fold; balloon group, 5.2-fold) and noninfarcted LVFW (ligation group, 1.74-fold; balloon group, 1.96-fold) were significantly increased (\( P < 0.01 \) for both). At 1 month after MI, neither location showed significant changes in NGF expression (\( P = \text{NS} \)). In general, the upregulation of NGF mRNA expression was earlier and more pronounced at the infarcted site than the noninfarcted LVFW.

To confirm qRT-PCR results, we performed Western blotting with an NGF antibody. Figure 1B shows representative bands (26 kDa) from a gel on which NGF protein at the infarcted site was studied. The GAPDH bands used to confirm equal loading were of similar density. Weak NGF protein was expressed in the myocardium of normal hearts. At the infarcted site, the NGF bands in the MI group were consistently denser and wider than those of the control group. Densitometric data showed that NGF protein levels were significantly higher in the MI group than they were in the control at all time points (2.2- to 10-fold; Figure 1C). At the noninfarcted LVFW, NGF increased 3 days after MI and peaked at 1 week (ligation group, 5.2-fold; balloon group, 5.3-fold; Figure 1C). Similar to qRT-PCR results, NGF protein increased earlier and by a greater magnitude at the infarcted site compared with the noninfarcted LVFW. These data suggest that MI induced immediate local NGF release followed by local NGF production. There was a delay in upregulation of NGF mRNA expression at the noninfarcted LVFW compared with the infarcted site.

Heterogeneous Cardiac GAP43 Expression After MI

The membrane phosphoprotein GAP43 expressed in the growth cones of sprouting axons is a marker for nerve sprouting. We studied GAP43 mRNA expression by real-time qRT-PCR. As shown in Figure 2A, GAP43 mRNA at the infarcted site showed significant increase (4.3-fold, \( P < 0.01 \)) at 3.5 hours after MI but not the noninfarcted LVFW. Both the infarcted site (12- to 13-fold, \( P < 0.01 \)) and noninfarcted LVFW (3- to 4-fold, \( P < 0.05 \)) showed signifi-
cant increase in GAP43 mRNA at 3 days and 1 week after MI. Similar to the NGF expression pattern, the GAP43 mRNA levels of both sites returned to the control levels at 1 month after MI, and GAP43 mRNA increased earlier and was more pronounced at the infarcted site than the noninfarcted LVFW.

Gene Expression of Other Nerve Specific Markers
We also assayed the mRNA expression of nerve-specific markers neurofilament (an intermediate filament in soma and neuron processes) and synaptophysin (a marker for synaptogenesis) by using real-time qRT-PCR. At 3.5 hours after MI, there were no significant changes in either neurofilament or synaptophysin expression. At 3 days and 1 week after MI, the mRNA levels of neurofilament showed significant increase at both sites compared with the control group (Figure 2B). The mRNA levels of synaptophysin decreased at the infarcted site at 3 days and 1 week after MI and increased at the noninfarcted LVFW at 1 week after MI in the ligation group (Figure 2C). At 1 month after MI, both neurofilament and synaptophysin mRNA returned to control levels. These data imply that MI induced neurofilament expression and inhibited synaptophysin expression at the infarcted site. Because canine-specific TH gene sequence is not available, we could not evaluate TH mRNA.

Nerve Sprouting and Sympathetic Hyperinnervation After MI
Immunohistochemical staining showed few GAP43-positive nerves in the normal LVFW and the noninfarcted LVFW at 3.5 hours after MI (Figure 3A and 3B), but abundant GAP43-positive nerve fibers were observed at the noninfarcted LVFW at 3 days, 1 week, and 1 month after MI (Figure 3C through 3E, respectively). At 3 days after MI, coagulation necrosis of myocardium distal to ligation was evident, with sarcolemmal disruption, hypereosinophilia of fibers and nucleus dropout. At 1 week after MI, inflammatory cells and fibroblastic proliferation were observed in large numbers at the infarcted site. In addition to GAP43-positive nerves, we also observed GAP43-positive stromal cells. These stromal cells, which contained nuclei and appeared around 3 days after MI, were also observed at the infarcted site (Figure 3F). GAP43-positive nerve numbers reached a peak at 1 week after MI at both the noninfarcted LVFW (Figure 3D) and infarcted site (Figure 3F). Although there was much higher GAP43 mRNA expression at the infarcted site than the noninfarcted LVFW, overall GAP43-positive nerve density was lower at the infarcted site than the noninfarcted LVFW. GAP43-positive nerve density in the noninfarcted LVFW was significantly (P<0.05 for all) higher in the MI group than the control group at 3 days, 1 week, and 1 month after MI (Figure 4A).
These cells are typically around 5 to 10 μm in diameter, and peak at 1 week after MI, reaching a peak at 3 days (3-fold, P<0.01; 1wL indicates ligation group at 1 week; 1wB, balloon occlusion group at 1 week). The NGF and GAP43 protein also increased in the LVFW, without a concomitant increase in mRNA levels. These latter data suggest that the increased NGF and GAP43 in the LVFW is due to retrograde axonal transport from the infarcted site. The increased neurofilament and synaptophysin expression further confirms cardiac nerve growth activity. Cardiac nerve sprouting and sympathetic hyperinnervation persisted beyond the first week after MI, supported in part by increased systemic NGF concentration. Large interindividual differences were observed in systemic serum NGF concentration, suggesting individual differences in genetic control of cardiac nerve density and magnitude of nerve sprouting after ischemic injury.

Time Course of Transcardiac NGF Level After MI
Transcardiac NGF (Figure 7) increased immediately after MI and peaked at 3.5 hours (P<0.01). Serum NGF (both CS and aorta) continued to increase at 3 days (CS by 10±10% and aorta by 10±15%), 1 week (CS by 22±37% and aorta by 20±37% in the ligation group, and CS by 25±35% and aorta by 21±40% in the balloon group), and 1 month (CS by 21±36% and aorta by 18±36%) after MI compared with baseline. However, because both the CS and aortic NGF increased to a similar extent, the transcardiac NGF levels from 3 days to 1 month were not significantly different from baseline. There were significant variations of systemic NGF levels among dogs (range, 200 to 1100 ng/mL).

Discussion
We found that acute MI resulted in the immediate elevation of transcardiac NGF concentration, probably due to release of NGF from damaged cells within the heart. This was followed by upregulation of cardiac NGF and GAP43 expression that was more pronounced at the infarcted site than the noninfarcted LVFW. The NGF and GAP43 protein also increased in the LSG, without a concomitant increase in mRNA levels. These latter data suggest that the increased NGF and GAP43 in the LSG is due to retrograde axonal transport from the infarcted site. The nerve sprouting signal from the LSG triggers a generalized increase in cardiac nerve density throughout the heart, but more so at the noninfarcted LVFW than the infarcted site. The increased neurofilament and synaptophysin expression further confirms cardiac nerve growth activity. Cardiac nerve sprouting and sympathetic hyperinnervation persisted beyond the first week after MI, supported in part by increased systemic NGF concentration. Large interindividual differences were observed in systemic serum NGF concentration, suggesting individual differences in genetic control of cardiac nerve density and magnitude of nerve sprouting after ischemic injury.

NGF Expression After MI
NGF is expressed in heart and other sympathetic targets. NGF was known to enhance reinnervation of surgically denervated canine heart. Hiltunen et al demonstrated in rat hearts that NGF mRNA levels at the border zone increased 2- to 4-fold between 2 hours and 120 hours of reperfusion after MI. In the present study, we found that both NGF protein level and mRNA level increased at the infarcted site and noninfarcted LVFW after MI, but earlier and more pronounced at the infarcted site than the noninfarcted LVFW, and that cardiac NGF mRNA expression was dynamic and temporary. Furthermore, we found that transcardiac NGF concentration increased immediately after MI before detectable elevation of cardiac NGF mRNA, implying that immediate transcardiac NGF increase is the result of cardiac NGF release rather than production. Although there was a signifi-
cant increase in NGF protein levels in the LSG from 3 days to 1 month after MI, no significant mRNA increase was observed throughout the study. This suggests that the higher levels of NGF in the sympathetic ganglia result from retrograde axonal transport rather than local synthesis. The observation was compatible with previous studies,20,21 that demonstrated that superior cervical ganglia contained highest levels of NGF, but its mRNA was barely detectable. We have previously found that nerve sprouting activity in the LSG resulted in generalized nerve sprouting throughout the heart, including both ventricles and atria.2,3 Putting these observations together, we conclude that MI first induces local NGF release, followed by local NGF production and retrograde transport of NGF to the LSG, which subsequently triggers cardiac nerve sprouting. The observation of nerve sprouting at areas local and remote from the site of insult can be explained by the fact that the LSG and thoracic ganglia innervates the heart globally. Although transcardiac NGF levels returned to normal 3.5 hours after MI, systemic NGF levels continued to increase progressively while cardiac NGF mRNA and protein remained elevated. One possible expla-

Figure 5. Examples of TH immunostaining. A through D, Noninfarcted LVFW at different time points. A, Control; B, 3 days after MI; C, 1 week after MI; D, 1 month after MI. There were more TH-positive nerves at 3 days, 1 week, and 1 month after MI compared with control, peaking at 1 week. E, Example of neurofilament staining at the infarcted site at 1 week after MI, showing increased numbers of nerves. F, Example of TH staining at the infarcted site at 1 month, showing hyperinnervation and neovascularization. Positive staining pattern (diffuse and thin) of the infarcted site is different from that of noninfarcted LVFW (B through D). Intrinsic cardiac adrenergic cells were also observed (see inset, 100× objective). Magnification of objective lens: 20×. Scale bar=100 μm.

Figure 6. Protein and mRNA levels of NGF and GAP43 in the LSG at different time points (GAPDH normalized, arbitrary units). A, top, Representative Western blots of NGF (left), GAP43 (right), and GAPDH. Bottom, Both NGF and GAP43 protein levels were significantly higher in the MI group than the control group at 3 days, 1 week, and 1 month (**P<0.01). B, Relative mRNA levels of NGF, GAP43, and neurofilament (NF). There were no significant changes in these three genes in the MI group compared with the control group at any time points.

Figure 7. Time course of transcardiac NGF change after MI. Transcardiac NGF at baseline (n=25), 0.5 hours (n=20), 1.5 hours (n=20), 2.5 hours (n=20), 3.5 hours (n=20), 3 days (n=5), 1wL (n=5), 1wB (n=5), and 1 month (n=3) are shown in this figure. MI results in a progressive increase in transcardiac NGF level in 3.5 hours after MI. 1wL indicates ligation group at 1 week; 1wB, balloon occlusion group at 1 week. **P<0.01 vs baseline.
nation for this discrepancy is that multiple recirculations of NGF minimized the transcardiac difference of NGF levels. A second explanation is that MI triggers NGF production at extracardiac locations, resulting in an elevation of systemic NGF concentration. We also observed that baseline NGF levels were highly variable between dogs, implying large individual differences in constitutive NGF production. These large individual differences of NGF concentration could result in differential cardiac innervation after MI.

GAP43 and Mechanisms of Cardiac Nerve Sprouting After MI

Our previous study showed that NGF infusion to the LSG induces significant increase in GAP43-positive nerves in the heart. In this study, we found a significant temporal and spatial correlation between cardiac NGF protein level and GAP43 expression. Increased GAP43 protein levels were also observed in LSG from 3 days to 1 month after MI with no significant mRNA increase. This implies that the higher levels of GAP43 in the sympathetic ganglia result from retrograde axonal transport rather than local synthesis, consistent with previous finding that GAP43 is bidirectionally transported in neurons of normal sciatic nerves. GAP43 is a major protein kinase C substrate of growth cones and developing nerve terminals. In the growth cone, it accumulates near the plasma membrane, where it associates with the cortical cytoskeleton and membranes. It is expressed not only in sympathetic nerves but also in parasympathetic and sensory fibers, in certain central nervous system glia, Schwann cell precursors, and nonmyelinating Schwann cells. GAP43 overexpression in transgenic mice leads to the spontaneous formation of new synapse and enhanced sprouting after injury. In the absence of GAP43, growth cones adhered poorly, and failed to produce NGF-induced spreading or insulin-like growth factor-1–induced branching. These observations suggest that NGF may increase cardiac nerve sprouting via upregulation of GAP43 expression. We also observed that although NGF and GAP43 expression levels increased more at the infarcted site than the noninfarcted LVFW, there were less GAP43-positive nerves at the infarcted site than the noninfarcted LVFW. This implies that in addition to neurotrophic factors like NGF and GAP43, nerve sprouting requires a permissive local environment in which to occur. The lack of blood supply at the infarcted site, for example, might impede nerve growth.

Source of mRNA

Although MI resulted in increased NGF, GAP43, and neurofilament mRNA at the infarcted site and noninfarcted LVFW, the source of the mRNA is unclear. It is unlikely that the mRNA came from extracardiac sympathetic ganglia, as the mRNA levels in the LSG were not significantly increased. The most likely source of mRNA is nonneuronal cells near the site of ischemic injury. These nonneuronal cells, including but not limited to the cells that ensheath the nerve fibers, express NGF after transection of the sciatic nerve. Schwann cells are known to express GAP43 after nerve transaction. Compatible with these findings, we observed GAP43-positive cells within the infarcted site. Some of these cells are stromal cells that are often found in the infarcted myocardia. These nonneuronal cells are the likely source of mRNA detected in this study. A second possible source of mRNA is intrinsic cardiac adrenergic cells. These cells are small TH-positive cells found mostly around blood vessels, with diameters roughly half that of myocytes. These cells could serve as sources of TH. It is also possible that MI can trigger the expression of other neurotrophic mRNAs in these cells.

Limitations

An association between increased NGF expression and sympathetic hyperinnervation does not directly prove a causal relationship. However, our previous study showed that NGF infusion to the LSG can cause significant cardiac nerve sprouting even in normal dogs. These latter findings support a potential cause and effect between elevated NGF in the LSG and cardiac nerve sprouting.

Conclusions

We conclude that MI results in immediate local NGF release, followed by upregulation of NGF and GAP43 expression from nonneuronal cells at the infarcted site. NGF and GAP43 are transported retrogradely to the LSG, which triggers nerve sprouting at the noninfarcted LVFW and, to a lesser degree, the infarcted site. Individual differences of NGF expression might be partially responsible for differential nerve sprouting and susceptibility to arrhythmia after MI. The timely regulation of NGF and GAP43 expression after MI might provide a novel opportunity for arrhythmia control.

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