Infarction alters both the distribution and noradrenergic properties of cardiac sympathetic neurons

Wei Li, David Knowlton, Donna M. Van Winkle, and Beth A. Habecker

1Departments of Physiology and Pharmacology and 2Anesthesiology, Oregon Health and Science University, and 3Anesthesiology Service, Portland Veterans Affairs Medical Center, Portland, Oregon 97239

Submitted 14 August 2003; accepted in final form 14 January 2004

Loss of noradrenergic function in the infarct zone and in peri-infarct myocardium is retained for weeks or months after infarction. This is revealed by decreased accumulation of metaiodobenzylguanidine (9, 10, 14, 23) and NE (22), the absence of effective refractory period shortening on nerve stimulation (4), and the loss of catecholamine-containing nerve fibers in the left ventricle (LV) apical to the infarction (4). The loss of noradrenergic function, coupled with the absence of catecholamine-containing nerve fibers, is thought to be due to denervation of the infarct and peri-infarct zone. It is accompanied, however, by a paradoxical increase in plasma NE (22). The chronic elevation of extracellular NE and depletion of cellular NE stores are attributed in part to increased activation of the sympathetic nervous system (11).

Recent studies of sympathetic nerve injury revealed that inflammatory cytokines, including leukemia inhibitory factor and cardiotrophin-1 (CT-1), remodel noradrenergic function in sympathetic neurons (8, 28, 36). CT-1, which is elevated in the heart after MI (2), suppresses the production of NE by decreasing the expression of tyrosine hydroxylase (TH) and the uptake of NE by decreasing the expression of NET, which removes NE from the extracellular space (20). This raises the possibility that functional denervation of the peri-infarct zone may reflect suppression of noradrenergic neurotransmission, rather than loss of nerve fibers.

To test the hypothesis that infarction suppresses noradrenergic properties in the peri-infarct innervation, we examined expression of TH, NET, and the panneuronal marker protein gene product (PGP) 9.5/ubiquitin COOH-terminal hydrolase L1 in the cardiac innervation 1 wk after MI. Morphology of the cardiac innervation, relative levels of TH and NET proteins, and TH and NET mRNA expression in the sympathetic stellate ganglia that project to the heart were assessed.

MATERIALS AND METHODS

Induction of infarction. Anesthesia was induced with 5% isoflurane and maintained with 2–3% isoflurane. Rats were then intubated and mechanically ventilated. A left thoracotomy was performed in the fourth intercostal space, the pericardium was opened, and the lung was retracted with sterile gauze. The left anterior descending coronary artery (LAD) was reversibly ligated with a 7-0 suture for 30 min and then reperfused by release of the ligature. Occlusion and reperfusion were confirmed by reversible ST wave elevation. The suture remained within the wound, and the chest and skin were closed in layers. After surgery, the animals were returned to cages and given regular food and water for 7 days before tissue analysis. Buprenorphine (Buprenex) was administered as needed to ensure that the animals were comfortable. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
comfortable after surgery. Infarcted tissue or scar was identified using hematoxylin-and-eosin stain to determine the presence of thin and wavy fibers, pyknotic nuclei, hypereosinophilia, and leukocyte infiltration (Fig. 1). All procedures were approved by the Institutional Animal Care and Use Committee and comply with the Guide for the Care and Use of Laboratory Animals [DHHS Publication No. (NIH) 85-23, revised 1996].

**Experimental groups.** Adult male Sprague-Dawley rats (250–300 g) were used for all experiments, and each group included four to six animals per experiment. Four experimental groups were examined. 1) Control animals did not undergo surgical procedures. 2) Sham rats were subjected to left thoracotomy and pericardiotomy followed by closure of the incision; there were no cardiac manipulations. 3) MI animals underwent the infarction procedure described above. 4) The 90-s group was included to provide an appropriate sham operation to control for nerve injury during the ligation procedure, because cardiac nerve bundles run along major arteries and ligation of the LAD may crush nerve bundles from both stellate ganglia (27). These animals were subjected to thoracotomy and tightening of the LAD ligation for 90 s and then reperfusion and closure of the incision. A 90-s ligation was used, because 90 s is far less time than is required to induce preconditioning or alter cardiac physiology (21). This was our primary control group and was used in all experiments.

**Immunohistochemistry.** Hearts from control, MI, and 90-s rats were excised and cut into 3- to 5-mm transverse cross sections (Fig. 1). The base of the heart was isolated by removing the atria and cutting ~2 mm above the coronary ligature. A corresponding area was isolated in control hearts. Tissue was fixed for 1 h in 4% paraformaldehyde, rinsed in PBS, cryoprotected in 30% sucrose overnight, and frozen in mounting medium for sectioning. Transverse 10-μm sections were cut from different levels of the heart (base to apex) and thaw mounted onto charged slides. Sections were incubated overnight in primary antisera: rabbit anti-PGP 9.5 (diluted 1:300; Accurate), sheep anti-TH (diluted 1:100; Pel-Freeze), and rabbit anti-NET (diluted 1:3,000; a generous gift from Dr. David Gilbey, Vanderbilt University) (34). Some sections were double labeled with rabbit anti-PGP and sheep anti-TH or with rabbit anti-NET and sheep anti-PGP by simultaneous incubation with both antisera overnight. Primary antisera were identified with species-specific Alexa Fluor fluorescent secondary antibodies diluted 1:300 and visualized by fluorescence microscopy. Figure 2H shows a typical section.

**Western blot analysis.** Hearts from control, MI, and 90-s animals were excised and cut into 3- to 5-mm transverse cross sections (Fig. 1A). The base was processed as a single sample that included the top 3 mm of both ventricles. At the level of the LAD occlusion and below, the LV and right ventricle (RV) were separated and processed individually (Fig. 1B). The septum was included with the RV. Only tissue below the ligation (LV “B” and RV “B”) was used for Western blot analysis. Stellate ganglia, which contain the majority of sympathetic neurons projecting to the heart (25), were also collected and frozen on dry ice. Ganglia and heart tissue (base, LV, RV, and apex) were homogenized in ground-glass homogenizers, and protein content was quantified using the Pierce bicinchoninic acid protein assay kit. Five micrograms of neuronal protein from the stellate ganglia or 20 μg of cardiac protein were diluted 1:2 with SDS sample buffer [62.5 mM Tris-HCl (pH 6.8), 2% (wt/vol) SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.01% bromphenol blue], heated at 95°C for 5 min, sized fractionated on 10% SDS-polyacrylamide gels, and transferred to membranes. Membranes were blocked in 5% nonfat dry milk diluted in TBST [100 mM NaCl, 10 mM Tris (pH 7.5), and 0.1% Tween 20], incubated overnight at 4°C with monoclonal mouse anti-TH (diluted 1:1,000 in TBST and 5% nonfat dry milk), washed, and incubated for 1 h at room temperature with goat anti-mouse horseradish peroxidase (diluted 1:10,000 in TBST and 5% nonfat dry milk), and immunoreactive bands were visualized by chemiluminescence. Band intensity was recorded by a −40°C charge coupled device camera and analyzed using LabWorks software (UVP, Upland, CA). Blots were stripped for 1 h at room temperature in stripping solution [62.5 mM Tris (pH 6.8), 2% SDS, and 0.7% (vol/vol) β-mercaptoethanol] and washed extensively in TBST. Blots were then incubated with rabbit anti-PGP in TBST and 5% nonfat dry milk at 4°C overnight and incubated with appropriate secondary antibodies, and immunoreactive bands were visualized by chemiluminescence. TH band density was normalized to PGP from the same sample. Total and mean band density gave similar results. The ratio of TH to PGP in unoperated control animals was set as 100%, and values from 90-s occlusion and MI animals were calculated as percentage of control. Samples from an individual region of the heart (i.e., base) were assayed together on a single blot and compared with the corresponding area of the heart from unoperated rats.

**NE assay.** NE levels in heart tissue were measured by HPLC with electrochemical detection (12, 37). The upper portion of the LV, including ~1–2 mm of tissue above the ligation (LV “A” in Fig. 1A; ≤20% of the total sample), or the corresponding sections from control or 90-s occlusion hearts were used for NE analysis. Frozen heart tissue samples were pulverized with a mortar and pestle, chilled on dry ice, and stored at −80°C until assayed. The frozen, powdered tissue (30–60 mg) was weighed and then homogenized with a microhomogenizer (250 μl of 0.1 M perchloric acid containing 0.1 M sodium standard dihydroxybenzylamine (1.0 μM) to correct for sample recoveries. An aliquot of the supernatant was neutralized with Trizma base, and the catechols in the sample were adsorbed onto acid-washed
INFARCION ALTERS SYMPATHETIC DISTRIBUTION, TH, AND NET

Fig. 2. Distribution of panneuronal marker protein gene product (PGP) in the heart. Sections of rat heart from control animals (A), animals subjected to sham operation to control for nerve injury during ligation procedure (90-s group, B), and animals subjected to MI (C–G) were stained with an antibody directed against PGP (diluted 1:300). H: areas represented by A–G. PGP-immunoreactive fibers were present in the LV of unoperated (A) and 90-s (B) rats (arrowheads). PGP-immunoreactive fibers were not detected in infarct (C) or peri-infarct (D) LV 1 wk after MI, indicating that part of the heart was denervated. Autofluorescence that was independent of the primary antibody was often present in the infarct zone (C). Fibers were present in the base (E) and RV (F) of the same heart after MI (arrowheads). Similar results were obtained from 5 animals for each condition. In 2 animals, regions of the border zone between innervated and denervated LV contained areas of apparent hyperinnervation (G, arrowheads).

alumina. The alumina was washed twice with double distilled H2O, and the catechols were desorbed with 100 μl of 0.1 M perchloric acid. Fifty-microliter aliquots were fractionated by reverse-phase HPLC (C18, 5-μm particle size; Rainin) using a mobile phase containing 75 mM sodium phosphate (pH 3.0), 360 mg/l sodium octane sulfonate, 100 μl/l triethylamine, and 3.0% acetonitrile. A coulometric detector (Coulchem, ESA) was used to detect and quantify NE using a voltage setting of +0.18 V and a sensitivity of 200 or 500 nA. Detection limit for NE was ~0.05 pmol with recoveries >60% (37).

[^3]H]nisoxetine binding. NET expression was identified by saturating[^3]H]nisoxetine binding in membrane homogenates essentially as described previously (13, 39). Hearts were dissected in ice-cold PBS in samples as described previously: base, LV, RV (including septum), and apex. Only tissue below the ligation (LV “B” and RV “B”) was used for binding analysis. Each sample was homogenized in ice-cold homogenization buffer [50 mM Tris (pH 7.4), 120 mM NaCl, and 5 mM KCl] using ground-glass homogenizers. Homogenates were washed twice in ice-cold homogenization buffer after centrifugation at 4°C and 40,000 g for 30 min. Final pellets were resuspended in ice-cold incubation buffer [50 mM Tris (pH 7.4), 300 mM NaCl, and 5 mM KCl] and assayed for total or background binding using 5 nM[^3]H]nisoxetine (250 μl final reaction volume). Total binding was assayed in triplicate, and nonspecific binding, defined by addition of 1 mM desipramine, was assayed in duplicate. Reactions were incubated at 0°C for 4 h, stopped by the addition of 5 ml of ice-cold incubation buffer, and filtered through glass fiber filters presoaked in 0.5% polyethyleneimine, using a Brandel cell harvester. Protein assays were carried out using the Pierce kit.

Real-time PCR. RNA was isolated from individual stellate ganglia using the Qiagen RNAeasy Mini kit. Total RNA was quantified by optical density at 260 nm, and 25 μg of total RNA were treated with DNase and reverse transcribed. Each RT reaction was tested by regular PCR to confirm RT, and an RNA-alone control was included for each sample to test for genomic DNA contamination. Real-time PCR was performed with the LightCycler-FastStart SYBR green I PCR amplification kit in the Roche LightCycler. For the PCR amplification, 2 μl of RT reactions were used in a total volume of 20 μl, and each sample was assayed in duplicate. Controls lacking template were included to determine the level of primer dimer formation and/or contamination. Each 20-μl reaction included 3.0 mM MgCl2, primer at 0.5 μM each, and 2 μl of DNA master. The primers have been described previously (20). Annealing temperatures were 55–60°C for the different primers, and the PCR parameters were as follows: denaturation at 94°C for 10 s, annealing at 94°C for 90 s, 55–60°C for 5 s, and 72°C for 20 s. The temperature transition rate was 20°C/s. One fluorescence reading was taken after each cycle at the end of the 72°C elongation time. Fluorescence was plotted as a function of cycle number to determine when reactions were in the linear phase of amplification. To confirm that only specific PCR products were generated, a melt analysis was carried out to determine the specific melting temperature for each amplification product. Standard curves for TH, NET, and PGP were generated by performing RT reactions with known amounts of RNA (12.5–200 ng) from control stellate ganglia and then carrying out PCR in duplicate with each standard and each set of primers. A slope was generated from the standard-curve PCR amplifications, and unknown samples were compared with the known standard values. Values for TH, NET, and PGP were obtained from the same sample, with TH and NET normalized to PGP as an internal control.

Statistics. Analysis of variance was carried out using GraphPad Prism 3.0. The Newman-Keuls post hoc test was used to compare all conditions in Western blot, NE, and PCR analyses.[^3]H]nisoxetine binding data were compared with the surgical sham control using Dunnett’s post hoc test.

RESULTS

The distribution of nerve fibers in hearts of control, MI, and 90-s rats was examined by immunohistochemistry using the panneuronal marker PGP. Numerous nerve fibers were detected in the LV of unoperated and 90-s occlusion control rats (Fig. 2, A and B). However, no nerve fibers were detected in the infarcted myocardium (Fig. 2C) or in normal myocardium within the peri-infarct zone (Fig. 2D). At the level of the...
occlusion and below, denervation extended several millimeters beyond the infarct (Fig. 2, D and H), but nerve fibers remained in the lateral aspect of the LV (Fig. 2G). Nerve fibers were also present in the base of the LV (Fig. 2E) and the RV (Fig. 2F). The extent of denervation in the apex of the heart varied with the size of the infarct. In two of five animals examined, there were regions of hyperinnervation along the border between innervated and denervated myocardium (Fig. 2G).

Many of the PGP-immunoreactive nerve fibers in control, MI, and 90-s hearts were also immunoreactive for TH (cf. Fig. 2, A, B, and F, with Fig. 3, A, B, and D). The infarct (data not shown) and the normal myocardium of the peri-infarct zone (Fig. 3C) were devoid of TH-immunoreactive nerve fibers. The distribution of TH- and NET-immunoreactive nerve fibers did not differ from the distribution of PGP-immunoreactive fibers. Essentially all the TH-containing nerve fibers in control and MI animals also contained NET (Fig. 4). Many of the nerve fibers in areas of hyperinnervation along the border zone between denervated and innervated myocardium were sympathetic neurons containing TH and NET (Fig. 4).

Although immunohistochemical analysis of TH and NET expression revealed that TH-positive nerve fibers also stained for NET, it did not provide information about the relative expression of TH and NET within those nerve fibers. Because inflammatory cytokines and nerve activity have different effects on the expression and activity of TH and NET in sympathetic neurons (20), we quantified regional changes in the expression of these proteins in the heart after MI. TH content, normalized to PGP to control for changes in neuron density, increased by 75% in the base of the heart 1 wk after MI compared with control and 90-s groups (Fig. 5A). In contrast, TH decreased relative to PGP by 63% in the LV compared with control and 90-s animals (Fig. 5B). This was accompanied by a drop in LV NE content: 5.2 ± 0.3 and 2.0 ± 0.29 (SE) pmol/mg for control and 2 days after MI, respectively (P < 0.001), and 3.6 ± 0.4 pmol/mg for 7 days after MI (P < 0.01, n = 5). The 90-s occlusion caused no change in LV TH-to-PGP ratio compared with unoperated controls [102 ± 29% (SE) of control, n = 4] but decreased cardiac NE content to 3.2 ± 0.3 pmol/ml (P < 0.01, n = 5). TH content normalized to PGP was unchanged in the RV: 99.5 ± 17 and 78 ± 20% of control for 90-s and MI animals, respectively (n = 4 each).

These initial results indicated that TH content per neuron was elevated in the heart above the infarction and decreased in the LV below the coronary ligature. To determine whether the elevation in neuronal TH content observed in the base of the heart reflected increased TH in the stellate ganglia projecting to the heart, the right and left stellate ganglia were collected from control, 90-s, and MI animals 1 wk after surgery. Both ganglia were pooled before Western blot analysis of TH and PGP content. TH levels, normalized to PGP, were elevated in the ganglia of animals that had undergone MI compared with 90-s and control groups (Fig. 5, C and D), consistent with increased TH in the base of the heart. There was a trend toward decreased

---

Fig. 3. Tyrosine hydroxylase (TH) immunoreactivity in cardiac innervation. Sections of rat heart from control (A), 90-s (B), and MI (C and D) animals were stained with an antibody directed against TH to identify sympathetic neurons. TH immunoreactivity was robust in neurons innervating LV of unoperated and 90-s rats (A and B, arrowheads). TH was not detected in peri-infarct LV 1 wk after MI (C) but was present in RV (D). Many nerve fibers that were PGP immunoreactive were also TH immunoreactive (cf. A and B with Fig. 2, A and B, and cf. D with Fig. 2F). Similar results were obtained from ≥5 animals for each condition.

Fig. 4. TH and NE transporter (NET) expression in control and hyperinnervated myocardium. Sections of rat heart from control and MI animals were double labeled with antibodies directed against TH and NET. All TH-immunoreactive fibers were also immunoreactive for NET (arrowheads). Border zone between denervated and innervated myocardium (schematic at bottom) contained areas of disorganized hyperinnervation. Many nerve fibers in these regions of hyperinnervation were immunoreactive for TH and NET, indicating that they were sympathetic neurons.
binding in the heart 1 wk after MI. NET was identified in membrane homogenates by radioligand binding with subsaturating levels of [3H]nisoxetine and normalized to total cardiac protein (Table 1). In contrast to the increased TH expression, NET binding in the base of the heart was unchanged after MI. Surprisingly, [3H]nisoxetine binding was similar in the LV of 90-s and MI animals, even though the LV is fully innervated after 90 s of occlusion but partially denervated after MI. To determine whether nerve damage due to the ligation procedure altered NET in the 90-s animals, binding was assayed after sham surgery without LAD ligation (sham). The level of binding throughout the heart was unaltered in the sham animals compared with unoperated controls (Table 1), and [3H]nisoxetine binding was decreased significantly in the LV of MI and 90-s ligation animals compared with the surgical sham group ($P < 0.05$). A trend toward decreased NET binding was also observed in the apex after 90 s of ligation. These results are consistent with the possibility that nerve damage and inflammation from the ligation procedure lowered [3H]nisoxetine binding in the LV in 90-s occlusion animals.

Nerve activity and inflammatory cytokines regulate TH and NET by altering their activity (41, 42), distribution (32), or gene expression (15, 20). Therefore, we used real-time PCR to determine whether MI altered the expression of TH or NET mRNA in the stellate ganglia. The left and right stellate ganglia of each animal were isolated individually, and cDNAs encoding TH, NET, and PGP were amplified. TH and NET were normalized to PGP as an internal control. TH mRNA was increased significantly in the left and right stellate ganglia of MI animals 1 wk after infarction (Fig. 6, A and B). Thus the increased TH protein in the stellate ganglion and in the base of the heart after MI results at least in part from elevated expression of TH mRNA. In contrast, NET mRNA remained unchanged in the left and right stellate ganglia of infarcted animals (Fig. 6, C and D) but was decreased in the ganglia of 90-s occlusion animals compared with unoperated controls. TH mRNA was not decreased significantly in the 90-s occlusion animals, but there was a trend toward lower TH mRNA.

**DISCUSSION**

The principal findings of the present study were that large portions of the LV became denervated below the coronary ligature, but sympathetic nerve fibers were retained in the base of the heart. TH expression (normalized to a panneuronal marker) was increased significantly in the base of the heart and in the stellate ganglia but decreased in the LV below the coronary occlusion. TH mRNA was increased significantly in the left and right stellate ganglia after MI, whereas NET mRNA was not.

**Table 1.** [3H]nisoxetine binding

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 6)</th>
<th>Sham (n = 6)</th>
<th>90 s (n = 6)</th>
<th>MI (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV</td>
<td>20.7 ± 1.9</td>
<td>19.8 ± 2.3</td>
<td>28.1 ± 6.7</td>
<td>20.2 ± 4.4</td>
</tr>
<tr>
<td>Base</td>
<td>25.2 ± 2.2</td>
<td>20.2 ± 2.2</td>
<td>25.7 ± 3.3</td>
<td>21.4 ± 5.8</td>
</tr>
<tr>
<td>LV</td>
<td>19.6 ± 3.0</td>
<td>24.2 ± 3.6</td>
<td>13.0 ± 2.4*</td>
<td>12.6 ± 2.0*</td>
</tr>
<tr>
<td>Apex</td>
<td>23.3 ± 3.8</td>
<td>20.6 ± 1.6</td>
<td>12.8 ± 2.6</td>
<td>21.2 ± 5.0</td>
</tr>
</tbody>
</table>

Values are means ± SE in fmol/mg. Control, unoperated; Sham, surgical sham without occlusion; 90 s, 90-s occlusion; MI, myocardial infarction; RV, right ventricle; LV, left ventricle. *$P < 0.05$. 

TH content in the stellate ganglia after 90 s of coronary occlusion, but it was not significant.

Because cellular and extracellular NE levels reflect a balance between synthesis and release, on the one hand, and reuptake through the NET, on the other hand, we also examined NET content in the stellate ganglia after MI. TH content in the stellate ganglia was increased significantly in the LV at 90 s of occlusion but partially denervated after MI. To determine whether nerve damage due to the ligation procedure altered NET in the 90-s animals, binding was assayed after sham surgery without LAD ligation (sham). The level of binding throughout the heart was unaltered in the sham animals compared with unoperated controls (Table 1), and [3H]nisoxetine binding was decreased significantly in the LV of MI and 90-s ligation animals compared with the surgical sham group ($P < 0.05$). A trend toward decreased NET binding was also observed in the apex after 90 s of ligation. These results are consistent with the possibility that nerve damage and inflammation from the ligation procedure lowered [3H]nisoxetine binding in the LV in 90-s occlusion animals.

Nerve activity and inflammatory cytokines regulate TH and NET by altering their activity (41, 42), distribution (32), or gene expression (15, 20). Therefore, we used real-time PCR to determine whether MI altered the expression of TH or NET mRNA in the stellate ganglia. The left and right stellate ganglia of each animal were isolated individually, and cDNAs encoding TH, NET, and PGP were amplified. TH and NET were normalized to PGP as an internal control. TH mRNA was increased significantly in the left and right stellate ganglia of MI animals 1 wk after infarction (Fig. 6, A and B). Thus the increased TH protein in the stellate ganglion and in the base of the heart after MI results at least in part from elevated expression of TH mRNA. In contrast, NET mRNA remained unchanged in the left and right stellate ganglia of infarcted animals (Fig. 6, C and D) but was decreased in the ganglia of 90-s occlusion animals compared with unoperated controls. TH mRNA was not decreased significantly in the 90-s occlusion animals, but there was a trend toward lower TH mRNA.

**DISCUSSION**

The principal findings of the present study were that large portions of the LV became denervated below the coronary ligature, but sympathetic nerve fibers were retained in the base of the heart. TH expression (normalized to a panneuronal marker) was increased significantly in the base of the heart and in the stellate ganglia but decreased in the LV below the coronary occlusion. TH mRNA was increased significantly in the left and right stellate ganglia after MI, whereas NET mRNA was not.
Previous studies showing the loss of noradrenergic functional parameters (4, 9, 17, 22), coupled with histological studies revealing the loss of catecholamine-containing neurons in the peri-infarct zone (4), suggested that sympathetic nerve fibers were absent or dysfunctional in the peri-infarct myocardium. However, there are difficulties with using these functional parameters to assess the presence or absence of sympathetic innervation, because the neurochemical properties of postganglionic sympathetic neurons are altered by axon damage (40). These changes include decreased production of NE, diminished expression of TH and neuropeptide Y, and induction of neuropeptides not normally produced by sympathetic neurons (28, 35, 36, 43). Many of the phenotypic changes that occur in sympathetic neurons after injury are due to the induction of inflammatory cytokines related to CT-1, which is induced throughout the heart after MI (2).

The change in sympathetic function induced by inflammatory cytokines raised the possibility that the functional sympathetic denervation observed in the peri-infarct zone might represent, in part, an injury-induced change in neurochemical properties, rather than the loss of nerve fibers. To test this possibility, we used the panneuronal marker PGP, which is not regulated by inflammatory cytokines (31), as well as the sympathetic markers TH and NET. We found that substantial denervation occurred in the LV after MI and that the loss of sympathetic function in the peri-infarct zone resulted from the absence of sympathetic nerve fibers, as previously believed. No histological changes were observed in innervation of the heart after 90-s LAD ligation, indicating that denervation was the result of MI, rather than a crush injury to the nerve fibers. In one animal, nerve fibers were detected in areas of otherwise denervated myocardium (data not shown), whereas several areas of hyperinnervation were observed along the border zone with peri-infarct denervated myocardium. Although obvious hyperinnervation was noted in only two of five rats, this may be more common, because we examined only a subset of sections from each level of the heart. Likewise, nerve bundles may extend into otherwise denervated tissue with greater frequency than the one example we identified.

Immunohistochemistry provides direct evidence for distribution of TH and NET within the heart but does not address whether there are changes in their expression or activity. Studies using Western blot and radioligand binding assays to quantify TH and NET levels revealed regional differences in expression. The relative amount of TH was increased in the base of the heart, whereas NET levels were unchanged in the base, and TH and NET were decreased in the LV below the coronary ligature. Consistent with loss of TH and NET, NE content was also decreased in the LV. The lowest levels of cardiac NE and the neuronal marker PGP were detected 2 days after MI, and by 1 wk NE and PGP had recovered to levels similar to those observed in the 90-s animals. The change between days 2 and 7 may reflect reinnervation and growing areas of hyperinnervation in the distal aspect of the LV. The relatively high level of NE observed after MI compared with other studies (22) may reflect the fact that a small portion of the tissue used for HPLC was above the ligation (Fig. 1B, LV “A”) and, therefore, in the border zone between an area with elevated TH (base) and an area with decreased TH (Fig. 1B, LV “B”). The decreased NE content after 90 s of occlusion was surprising and may result from a combination of decreased TH activity and loss of NE uptake.

The increased TH in the base of the heart, coupled with the loss of TH below the ligation, suggests subtle regional changes in sympathetic neurotransmission in addition to the more obvious alterations in innervation density. It is likely that the increased TH, which is apparent only in the base of the heart, would have been missed had we examined the entire heart together in one sample. It is not clear what accounts for this discrepancy in TH expression from the base to the apex, but it is not simply the higher innervation density in the base of the heart, because normalizing to PGP accounts for changes in innervation density. Although we cannot exclude the possibility that there is selective retention of sympathetic neurons, rather than sensory or parasympathetic neurons, in the base of the heart compared with the ventricle, this seems less likely than regulation of TH expression by cytokines, hypoxia, or nerve activity. All these parameters can regulate TH expression.
and activity (5, 7, 20, 26, 30) and are present to different degrees throughout the heart after MI, providing distinct microenvironments within the heart that could differentially regulate TH expression and activity.

The same environmental changes may have a different set of effects on NE uptake and NET expression. In the context of the cardiac innervation after MI, this could result in high levels of NE release that are not fully compensated for by increased NE removal through NET. NET binding sites decreased only in the LV of the heart, which included denervated and innervated myocardium. The loss of NET was due at least in part to denervation, because NET binding was normalized to total protein, rather than a neuron-specific protein. Western blots would have allowed us to normalize NET to a neuron-specific protein, such as PGP, but all the NET antibodies that are available exhibited variability and high nonspecific binding in Western analysis of cardiac tissue. A high degree of background binding was also observed in the radioligand binding assays, and 1 mM desipramine was required to identify nonspecific binding. Although this is an unusually high concentration of desipramine, similar concentrations of NET antagonists have been used in other studies to identify nonspecific binding in the heart (3). The single concentration of nisoxetine used in the binding assays did not allow calculation of the total number of binding sites, but it did identify significant changes in NET binding after 90 s or 30 min of LAD occlusion.

Although denervation may account for the loss of NET after MI, it cannot explain the decreased binding after 90 s of ligation. One explanation for the loss of binding sites and cardiac NE in the LV after 90 s of ligation is damage to the nerve during the coronary artery occlusion. The sympathetic axons innervating the heart follow the coronary arteries, and the process of occluding the artery may crush nerve branches from the right and left stellate ganglia (see Fig. 5 in Ref. 27). Consistent with this possibility, NET binding sites were not decreased in the LV of sham animals, in which the LAD was not occluded. Decreased NET mRNA after 90 s of occlusion is consistent with the ligation procedure causing nerve damage. Although TH was not decreased significantly in the stellate ganglia after 90 s of ligation, there was a trend toward lower TH protein and mRNA in the stellate ganglia of these animals. All these changes are consistent with the effects of nerve injury and underscore the importance of using this procedure as a control.

If the decreased NET binding and mRNA after 90 s of occlusion reflect nerve injury caused by LAD ligation, then one must question why NET and TH mRNAs are elevated in MI animals compared with the 90-s ligation controls. Coronary ligation could induce opposing effects: one mediated by nerve crush and inflammatory cytokine release and the other by increased sympathetic nerve activity secondary to hemodynamic compromise. Cardiac NE spillover, which is often used as an indirect measure of sympathetic nerve activity (11, 38), is increased after MI (22, 24), and the induction of TH mRNA and protein in the stellate ganglia is consistent with elevated nerve activity (5, 7, 41). Studies with cultured sympathetic neurons indicate that chronic nerve activation simulates TH expression, activity, and catecholamine production and over-rides the inhibitory effects of inflammatory cytokines on these parameters (15, 29, 20). Depolarization also increases NET mRNA and NE uptake in CT-1-treated cultured sympathetic neurons (20), suggesting that the increase in NET mRNA in MI animals compared with 90-s occlusion animals is due to increased nerve activity after MI. Culture studies suggest that TH expression and catecholamine production are stimulated to a greater degree by nerve activity than are NE uptake and NET (20). This may explain why TH mRNA and protein are elevated by MI to levels higher than in unoperated control animals, whereas NET mRNA returns to control levels.

In conclusion, these studies examined sympathetic innervation of the heart after MI and identified a number of changes, including substantial denervation of the LV and areas of apparent hyperinnervation at the border of innervated and denervated myocardium. In addition, TH levels were altered in a region-specific manner, increasing in the ganglia and base of the heart but decreasing apical to the infarct. Fewer changes were noted in NET binding sites, which were decreased in the infarct and peri-infarct zone but otherwise unchanged. Analysis of gene expression revealed that NET mRNA was decreased by 90 s of LAD occlusion but not by MI, whereas TH mRNA was elevated significantly after MI. These changes in gene expression and protein content may be due to the actions of inflammatory cytokines in the heart coupled with increased central stimulation of postganglionic sympathetic neurons. The increase in TH, but not NET, in the cardiac innervation may help explain the increased NE spillover observed after infarction. These regional differences in sympathetic function may contribute to the onset of arrhythmias after infarction.

ACKNOWLEDGMENTS

The authors thank Dr. Kent Thornburg for use of the LightCycler and Michelle Barney, Kun Grimm, Dr. Koh Kuzume, and Dr. Bill Woodward for assistance with the experiments.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grant HL-68231 and American Heart Association Grant 0151349Z (B. A. Habecker).

REFERENCES


H2236

INFARCTION ALTERS SYMPATHETIC DISTRIBUTION, TH, AND NET


