Cardiac sympathetic innervation and PGP9.5 expression by cardiomyocytes after myocardial infarction: effects of central MR blockade

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Central mechanisms involving mineralocorticoid receptor (MR) activation contribute to an increase in sympathetic tone after myocardial infarction (MI). We hypothesized that this central mechanism also contributes to cardiac sympathetic axonal sprouting and that central MR blockade reduces cardiac sympathetic hyperinnervation post-MI. Post-MI, tyrosine hydroxylase (TH) and norepinephrine transporter protein content in the noninfarcted base of the heart remained unaltered. In contrast, protein gene product (PGP)9.5 protein was increased twofold in the base of the heart and sixfold in the peri-infarct area at 1 wk post-MI and was associated with increased ubiquitin expression. These changes persisted to a lesser extent at 4 wk post-MI and were no longer present at 12 wk. Cardiac myocytes rather than sympathetic axons were the main source of this elevated PGP9.5 expression. At 7–10 days post-MI, in the peri-infarct area, sympathetic hyperinnervation was observed with a fourfold increase in growth-associated protein 43, a twofold increase in TH, and a 50% increase in PGP9.5-positive fibers compared with the epicardial side of the left ventricle in sham rats. Central infusion of the MR blocker eplerenone markedly attenuated these increases in nerve densities but did not affect overall cardiac PGP9.5 and ubiquitin protein overexpression. We conclude that central MR activation contributes to sympathetic hyperinnervation, possibly by decreasing cardiac sympathetic activity post-MI, or by affecting other mechanisms, such as the expression of nerve growth factor. Marked PGP9.5 expression occurs in cardiomyocytes early post-MI, which may contribute to the increase in ubiquitin.

myocardial infarction; cardiac sympathetic hyperinnervation; brain mineralocorticoid receptors; eplerenone; ubiquitin; protein gene product 9.5

Increased CSNA may also contribute to the development of cardiac sympathetic hyperinnervation after MI. In normal dogs, long-term subthreshold electrical left stellate ganglia (SG) stimulation increases nerve fiber density in the heart (45). In rodents, sympathetic hyperinnervation occurs in the peri-infarct area, particularly within the first week post-MI, and to a lesser extent in remote cardiac areas chronically (8, 25, 28, 51, 52).

A chronic increase in CSNA and norepinephrine (NE) release can also lead to an increase in NE synthesis and changes in neuronal gene expression. The conversion of tyrosine to DOPA by tyrosine hydroxylase (TH) is a rate-limiting step in NE synthesis, and the regulation of TH molecule numbers and enzyme activity is the main mechanism for controlling NE synthesis in the heart (3, 7, 18). NE transporter (NET) protein shifts NE from the synapse back into its vesicles for storage and later use (26). A chronic increase in CSNA post-MI may increase TH and NET expression in the heart and SG, since membrane depolarization upregulates TH and NET gene expression in sympathetic neurons in vitro and in vivo (39, 42, 53). A two- to threefold increase in NET and TH mRNA in the SG and in NET and TH protein in the noninfarcted base of the heart were observed in rats 1 wk after ischemia-reperfusion (33). TH and NET expression were normalized to the neuronal marker protein gene product (PGP)9.5 to control for variations in innervation. However, extraneuronal expression of PGP9.5 might take place in the heart. Fibroblasts in human wounds express PGP9.5 during the healing process (29), and in patients with dilated cardiomyopathy, cardiomyocytes exhibit strong PGP9.5 immunoreactivity (48). PGP9.5 removes ubiquitin from attached proteins before proteolysis, and PGP9.5 overexpression is associated with increased levels of free ubiquitin (30).

Central mechanisms involving aldosterone and mineralocorticoid receptor (MR) activation play an important role in the increase of sympathetic tone post-MI. In rats, intracerebroventricular infusion of spironolactone for 4–6 wk post-MI prevents general sympathetic hyperactivity and impairment of arterial baroreflex control (12). Central effects of aldosterone and MR activation are likely mediated via increased release of endogenous ouabain in the hypothalamus, which chronically activates angiotensinergic sympathoexcitatory pathways (13). Intracerebroventricular infusion of losartan for 5 h normalized elevated CSNA in CHF induced by rapid ventricular pacing (37). Whether central MR activation influences parameters of cardiac sympathetic activity has not yet been studied.

We hypothesized that central mechanisms contribute to upregulation of TH and NET expression in the SG and heart and facilitate cardiac sympathetic hyperinnervation post-MI and that these increases can be attenuated by central MR blockade. The main objectives of the present study were to

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assess the time course of TH, NET, and PGP9.5 expression in the SG and heart post-MI and to study effects of central MR blockade on cardiac sympathetic innervation and possible extraneuronal PGP9.5 expression post-MI.

METHODS

Male Wistar rats (body weight: 200–250 g, Charles River Breeding Laboratories, Montreal, QC, Canada) were housed on a 12:12-h light-dark cycle at 24°C and given free access to regular rat chow and tap water. All experiments were approved by the Animal Care Committee of the University of Ottawa and complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (8th ed., 2011). After a 7-day acclimatization period, open-chest MI surgery was performed as previously described (34). Rats with MI < 20% were excluded from the study.

Experimental Protocols

Protocol 1: time course of TH, NET, and PGP9.5 expression. Animals were randomly divided into two groups for permanent coronary artery ligation (n = 10) or sham operation (n = 8). At 1, 4, and 12 wk after the MI surgery, left ventricular (LV) dimensions and function were assessed by echocardiography and Millar catheter. Under pentobarbital anesthesia (100 mg/kg ip), rats were then euthanized by transcardiac PBS (pH 7.4) perfusion. The left and right SG were collected under a surgical microscope. The LV and right ventricle (RV) were separated on ice and weighed. The infarct size was measured by planimetry from the endocardial surface and expressed as a percentage of the total LV area. Tissues from the base (area above the ligation) of the LV and RV were snap frozen in liquid nitrogen and transferred to the −80°C freezer. TH, NET, and PGP9.5 protein expression was assessed by Western blot analysis, and gene expression was assessed by quantitative RT-PCR.

Protocol 2: cardiac sympathetic innervation and extraneuronal PGP9.5 expression. Animals were randomly divided into two groups for permanent coronary artery ligation (n = 8) or sham operation (n = 6). One week after MI surgery, LV function was assessed by Millar catheter, and rats were euthanized by transcardiac PBS (pH 7.4) perfusion under pentobarbital anesthesia. Hearts were removed immediately, and the infarct size was measured by planimetry. The LV was divided into two halves. One half of the LV was fixed for 24 h at 4°C in 4% paraformaldehyde and cryoprotected in 30% sucrose for 48 h at 4°C before being frozen for immunohistochemistry. The second half of the LV was used for Western blot analysis. The LV for Western blot analysis was further separated on ice into the noninfarcted base and peri-infarct area (3-mm zone adjacent to the MI border).

PGP9.5, growth-associated protein (GAP)43, and TH fluorescent double staining was performed to visualize sympathetic axons in the heart post-MI. Extraneuronal PGP9.5 expression was evaluated by PGP9.5 immunoreactivity in the LV and fluorescent double staining with vimentin (marker for fibroblasts and macrophages) and α-sarcosmeric actin (marker for cardiomyocytes). PGP9.5, ubiquitin, and ubiquitinated protein expression were assessed by Western blot analysis.

Protocol 3: central MR blockade and cardiac sympathetic hyperinnervation. At 1 day post-MI, an intracerebroventricular cannula was placed into the left cerebral ventricle, and the upper end of

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense Sequence</th>
<th>Antisense Sequence</th>
</tr>
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<tbody>
<tr>
<td>TH</td>
<td>5’-CGGCGTGCAGTCTCTCTCTACTAC-3’</td>
<td>5’-GGGCTGTTAGCTGAAATTCCTAT-3’</td>
</tr>
<tr>
<td>NET</td>
<td>5’-GGGCGCCAGCACACAGACACAGACAC-3’</td>
<td>5’-GGGCGCCACAGCACAGACACAGACAC-3’</td>
</tr>
<tr>
<td>PGP9.5</td>
<td>5’-GGGCGCGAGACTCTGAATCTCTCTACTAC-3’</td>
<td>5’-GGGCGCGACTCTGAATCTCTCTACTAC-3’</td>
</tr>
<tr>
<td>PGK1</td>
<td>5’-GGGCGTGCAGTCTCTCTCTACTAC-3’</td>
<td>5’-GGGCGTGCAGTCTCTCTCTACTAC-3’</td>
</tr>
</tbody>
</table>

Table 1. Primers sequences used for analysis of expression of TH, NET, PGP9.5, and PGK1

Table 2. Changes in LV function and cardiac weight post-MI

<table>
<thead>
<tr>
<th>Time Course</th>
<th>1 wk post-MI</th>
<th>4 wk post-MI</th>
<th>12 wk post-MI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham group</td>
<td>MI group</td>
<td>Sham group</td>
</tr>
<tr>
<td>n</td>
<td>8</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>MI size, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW, g</td>
<td>307 ± 5</td>
<td>286 ± 7</td>
<td>419 ± 6</td>
</tr>
<tr>
<td>LV, mg/100 g BW</td>
<td>242 ± 6</td>
<td>263 ± 8</td>
<td>220 ± 6</td>
</tr>
<tr>
<td>RV, mg/100 g BW</td>
<td>74 ± 2</td>
<td>74 ± 4</td>
<td>69 ± 3</td>
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<tr>
<td>EF, %</td>
<td>84 ± 3</td>
<td>60 ± 3*</td>
<td>80 ± 2</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>5 ± 0.5</td>
<td>15 ± 2*</td>
<td>4 ± 0.2</td>
</tr>
<tr>
<td>LVSP, mmHg</td>
<td>125 ± 2</td>
<td>113 ± 2*</td>
<td>120 ± 3</td>
</tr>
</tbody>
</table>

Effects of Central MR Blockade for 10 Days Post-MI

<table>
<thead>
<tr>
<th>Time Course</th>
<th>10 days post-MI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
</tr>
<tr>
<td>n</td>
<td>8</td>
</tr>
<tr>
<td>MI size, %</td>
<td>83 ± 2</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>4 ± 0.5</td>
</tr>
<tr>
<td>LVSP, mmHg</td>
<td>118 ± 3</td>
</tr>
<tr>
<td>dP/dt max</td>
<td>7.76 ± 200</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of animals/group. MI, myocardial infarction; BW, body weight; LV, left ventricle; RV, right ventricle; EF, ejection fraction; LVEDP, left ventricular (LV) end-diastolic pressure; LVSP, LV peak systolic pressure; MR, mineralocorticoid receptor. *P < 0.05 vs. the sham-operated (sham) group.

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the cannula was connected to an Alzet osmotic minipump (model 2002, Alza, Palo Alto, CA) for a 9-day infusion at a flow rate of 0.5 μL/h (24). The pumps were implanted subcutaneously on the back of the neck. Animals were randomly divided into the following groups (n = 8 animals/group) for treatment with eplerenone or vehicle:

1. MI group with an intracerebroventricular infusion of the MR blocker eplerenone (5 μg/day) dissolved in artificial cerebrospinal fluid (aCSF) with 4% acetonitrile.
2. MI group with an intracerebroventricular infusion of vehicle (aCSF with 4% acetonitrile).
3. Sham-operated group with intracerebroventricular cannulation surgery only.

The rate of intracerebroventricular infusion of eplerenone was based on a previous study (11). After 9 days of infusion, LV dimensions and function were assessed by echocardiography and Millar catheter. Rats were then euthanized, and LVs were collected for immunohistochemistry as described above for protocol 2.

Assessment of LV Dimensions and Function

Echocardiography. Echocardiography was performed under mild isoflurane anesthesia with a Vevo 770 echocardiography system (VisualSonics) with a 25-MHz transducer. Simultaneous M-mode recording of intraventricular septum thickness, posterior wall thickness, and chamber size was performed for four cardiac cycles. LV ejection fraction and fractional shortening were calculated.

Assessment of LV hemodynamics. Under mild isoflurane anesthesia, a 2-Fr high-fidelity micromanometer catheter (SPR-407, Millar Institute, Houston, TX) was inserted into the LV via the right carotid artery. The Millar catheter was connected to a Harvard data-acquisition system for measurements of LV end-diastolic pressure, LV peak systolic pressure, \( \frac{dP}{dt_{\text{max}}} \), and heart rate.

Real-Time Quantitative RT-PCR

RNA isolation and cDNA synthesis. SG were homogenized using a pestle (Bel-Art Products) driven by a Pellet Pestle Motor (Grainger). Total RNA was isolated using TRIzol reagent buffer (Invitrogen) according to the manufacturer’s instruction. To avoid potential genomic DNA contamination, the total amount of RNA from the SG was treated with DNase I using a DNA-free kit (Ambion). cDNA was then synthesized by incubation of 1 μg DNase I-treated RNA with oligo-dT primers and 200 units of SuperScript II RNase H (Invitrogen) at 42°C for 50 min.

Real-time RT-PCR. Real-time PCR amplifications were performed with a Roche Light Cycler using Fast Start DNA Master SYBR green I (Roche Diagnostics). One microliter of 1:10 diluted reverse transcription product from the SG was used as a template. The TH, NET, PGP9.5, and phosphoglycerate kinase (PGK1) primer sequences used are listed in Table 1.

Real-time PCR conditions were optimized as follows: an initial step of 95°C for 10 min followed by 45 cycles of denaturation at 95°C for 5 s and annealing of primers to the target for 5 s at 62°C for the PGP9.5 and PGK1 primers and 62°C for 7 s for the TH and NET primers. The extension step was performed at 72°C. The specificity of real-time PCR products was determined with a melting curve analysis and agarose gel electrophoreses. TH, NET, PGP9.5, and PGK1 PCR products were subcloned into the pCRII-TA vector (Invitrogen) individually, and the vector containing the cDNA insert of each gene was verified by EcoRI restriction enzyme digestion and sequencing analysis. DNA plasmids containing cDNA of the gene of interest were quantified by an ND-1000 (NanoDrop Technologies). A series of 10-fold dilutions from 100 to 0.001 pg/μL of each plasmid was used to generate a standard curve. The real-time PCR efficiencies for TH, NET, PGP9.5, and PGK1 amplification were 1.98, 1.99, 1.89, and 1.92, respectively. Absolute quantification was used to establish TH, NET, PGP9.5, and PGK1 cDNA concentrations, and normalization was accomplished using the constitutively expressed reference gene (PGK1) as an internal control.

Western Blot Analysis

Tissue from the base of the LV, the peri-infarct area, and the RV was homogenized in lysis buffer (20 mM Tris-HCl, 137 mM NaCl, 2 mM EDTA, 10 mM NaF, 0.5% Igepal, and 10% glycerol; pH 8) with protease inhibitor cocktail (Sigma) using a Polytron. SG were homogenized in 100 μL of lysis buffer using a pestle. Protein concentration in the samples was quantified by the Bradford method. The optimal loading concentration was established to be 5 μg of total neuronal protein from the SG for TH, 15 μg of total neuronal protein for NET, and 20 μg of total cardiac protein for TH, NET, PGP9.5, and ubiquitin. TH, NET, and ubiquitin proteins were fractionated on separate gels and transferred to different membranes. After transfer, membranes were blocked with filtered TBS with Tween 20 (TBST) containing 5% nonfat milk powder for 1 h at room temperature. Membranes for TH, NET, and PGP9.5 immunoblot analysis were cut below 37 kDa. The upper parts of the membranes (250–37 kDa) containing TH and NET proteins were incubated overnight at 4°C with antibodies raised against TH, NET, and PGP9.5.
with polyclonal rabbit anti-TH (1:500, AB152, Chemicon) or polyclonal rabbit anti-NET (1:1,000, AB5066P, Chemicon). The lower parts of both membranes containing PGP9.5 protein were incubated separately overnight at 4°C with mouse monoclonal anti-PGP9.5 (1:40, VP-P983, Vector). Membranes containing ubiquitin and ubiquitin-bound proteins were incubated overnight at 4°C with rabbit polyclonal anti-ubiquitin (1:1,000, ab7780, Abcam). Membranes were then washed with TBST buffer and incubated for 1 h at room temperature with goat anti-rabbit horseradish peroxidase conjugate for NET, TH, and ubiquitin (1:5,000, sc 2004, Santa Cruz Biotechnology) and sheep anti-mouse horseradish peroxidase conjugate for PGP9.5 (1:10,000, NXA93, Amersham). Immunoreactive bands were then visualized by chemiluminescence (Western Lighting Plus ECL, Perkin-Elmer). Membranes containing NET and TH proteins (250- to 37-kDa area) and ubiquitin were reblocked overnight at 4°C with filtered 5% nonfat dry milk diluted in TBST and then incubated for 1 h at room temperature with monoclonal mouse anti-β-actin (1:10,000, A2228, Sigma). After being washed with TBST buffer, membranes were incubated with sheep anti-mouse horseradish peroxidase conjugate (1:10,000, NXA93, Amersham). Immunoreactive bands of β-actin were then visualized by chemiluminescence. An Alpha Innotech Fluorochem HD camera system was used to expose and take images of the membranes. The intensity of the bands was quantified with AlphaEaseFC image-analysis software. Normalization of TH, NET, PGP9.5, ubiquitin, and ubiquitin-bound proteins was accomplished using the constitutively expressed reference gene (β-actin) as an internal control.

**Immunohistochemistry**

LVs were cut longitudinally into 10-μm cryosections that were thaw mounted onto charged slides. Sections were air dried, washed in PBS, and incubated with 1% sodium borohydride for 20 min to reduce fixative-induced autofluorescence. After three washes for 5 min in PBS, sections were blocked with 10% normal donkey serum in PBS (Jackson ImmunoResearch) at room temperature for 1 h.

**Protocol A: TH, GAP43, or PGP9.5 double staining.** Sections were incubated with anti-TH sheep polyclonal antibody (1:1,000, ab113, Abcam) overnight at 4°C. All further steps were performed in a darkened room, and incubations were carried out protected from light. Sections were rinsed three times for 5 min in PBS and incubated for 1 h at room temperature with Dylight 594-conjugated AffiniPure donkey anti-sheep IgG (1:500, 713-515-147, Jackson ImmunoResearch). Sections were rinsed three times for 5 min in PBS and then incubated with anti-PGP9.5 rabbit polyclonal antibody (1:400, CL95101, Ultraclone) or with anti-GAP43 rabbit polyclonal antibody (1:500, ab16053, Abcam) overnight at 4°C. After incubation, sections were rinsed three times for 5 min in PBS and incubated for 1 h at room temperature with Alexa fluor488-conjugated AffiniPure donkey anti-rabbit IgG (1:800, 711-545-152, Jackson ImmunoResearch).

The specificity of the GAP43, TH, and PGP9.5 primary antibodies was confirmed by preabsorption with a 5-fold (TH) or 10-fold (GAP43 and PGP9.5) excess of the immunizing peptide or full-length protein (ab16379, Abcam; NBP1-42461, Novus Biologicals; and 30R-1093, Fitzgerald Industries, respectively). After an overnight

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Fig. 2. TH, NET, and PGP9.5 proteins in the left SG at 1, 4, and 12 wk post-MI normalized to β-actin. Various forms of NET protein are shown separately: 46-kDa native protein, active fully glycosylated 80-kDa protein, and total NET. Data are shown as percentages of the control group (100%) and are presented as means ± SE; n = 4–6 animals/group. *P < 0.05 vs. the sham group.
Fig. 3. TH, NET, and PGP9.5 proteins in the left ventricle (LV; left) and right ventricle (RV; right) at 1, 4, and 12 wk post-MI normalized to β-actin. PGP9.5 protein expression was significantly increased twofold at 1 wk post-MI and by ~60% at 4 wk post-MI. Data are shown as percentages of the control group (100%) and are presented as means ± SE; n = 5–8 animals/group. *P < 0.05 vs. the sham group.

Table 3. Effects of central MR blockade for 10 days post-MI on TH, GAP43, and PGP9.5 nerve fiber densities

<table>
<thead>
<tr>
<th></th>
<th>Percent Area of TH-Positive Fibers</th>
<th>Percent Area of GAP43-Positive Fibers</th>
<th>Percent Area of PGP9.5-Positive Fibers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endocardial side</td>
<td>0.11 ± 0.01</td>
<td>0.23 ± 0.02</td>
<td>0.27 ± 0.02</td>
</tr>
<tr>
<td>Epicardial side</td>
<td>0.82 ± 0.08</td>
<td>0.51 ± 0.06</td>
<td>1.10 ± 0.07</td>
</tr>
<tr>
<td>MI group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epicardial side (LV base)</td>
<td>0.90 ± 0.09</td>
<td>0.55 ± 0.06</td>
<td>1.22 ± 0.11</td>
</tr>
<tr>
<td>MI + vehicle group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peri-infarct area</td>
<td>1.63 ± 0.11ab</td>
<td>2.88 ± 0.23abcde</td>
<td>1.54 ± 0.13abc</td>
</tr>
<tr>
<td>MI + MI blocker group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peri-infarct area</td>
<td>0.45 ± 0.10cde</td>
<td>0.89 ± 0.13abcde</td>
<td>0.75 ± 0.15abcde</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6–7 animals/group. Tyrosine hydroxylase (TH), growth-associated protein (GAP43), and protein gene product (PGP)9.5 immunoreactivities in the LVs of rats treated post-MI with intracerebroventricular vehicle showed hyperinnervation in the peri-infarct area, with the most marked increase in GAP43. Central MR blockade significantly attenuated the increase in TH, GAP43, and PGP9.5 nerve densities. In the peri-infarct area, the hyperinnervation was fairly homogeneous and therefore not divided by endocardial versus epicardial location. *P < 0.05 vs. the sham group (endocardial side); bP < 0.05 vs. the sham group (epicardial side); cP < 0.05 vs. the MI + vehicle group; dP < 0.05 vs. TH and PGP9.5; eP < 0.05 vs. TH; fP < 0.05 vs. PGP9.5.
incubation at 4°C, with gentle mixing, the mixtures were centrifuged at >16,000 g for 30 min, and the clear supernatants used for immunostaining in parallel with primary antibodies that had not been preabsorbed.

Protocol B: PGP9.5, vimentin, and α-sarcomeric actin double staining. Sections were incubated with anti-PGP9.5 rabbit polyclonal antibody (1:400, CL95101, Ultraclone) overnight at 4°C. Sections were rinsed three times for 5 min in PBS and incubated for 1 h at room temperature with Dylight 594-conjugated AffiniPure donkey antirabbit IgG (1:500, 713-515-147, Jackson ImmunoResearch). Sections were rinsed three times for 5 min in PBS and then incubated with anti-α-sarcomeric actin mouse monoclonal antibody (1:200, ab28052, Abcam) or anti-vimentin mouse monoclonal antibody (1:1,000, ab20346, Abcam) overnight at 4°C. Sections were then rinsed three times for 5 min in PBS and incubated for 1 h at room temperature with Dylight 594-conjugated AffiniPure donkey anti-mouse IgG (1:500, 715-515-151, Jackson ImmunoResearch).

Sections for both protocols were coverslipped with Dako Fluorescent mounting medium and examined using an Olympus BX fluorescent microscope with a ×10 objective, and images were then captured with a SPOT camera system (RT color) and Application software (Diagnostic Instruments, Sterling Heights, MI). Noninfarcted areas of the LV and peri-infarct areas were assessed and compared with corresponding areas in the control group. Six sections from each
animal located at least 200 μm apart were analyzed from each heart, and data were averaged together. TH, GAP43, and PGP9.5 innervation density were determined by threshold discrimination using ImageJ. Innervation density was expressed as a percentage of the area above the threshold. Overlay of images was performed using Adobe Photoshop.

Statistical Analysis

Data are means ± SE. Differences between two groups were compared by an unpaired t-test. Differences between three groups were compared by two-way ANOVA. When the F values were significant for a main effect, Duncan’s method was applied for multiple comparisons as a post hoc analysis. The level of statistical significance was set at \( P \leq 0.05 \).

RESULTS

General

Average infarct sizes were 25–35% at 1, 4, and 12 wk post-MI. Mean body weight was similar in MI and sham-operated rats. Hypertrophy of the RV and LV was evident in MI rats at 4 and 12 wk but not yet at 1 wk post-MI. MI groups had a significant decrease in LV peak systolic pressure and ejection fraction and a significant increase in LV end-diastolic pressure (Table 2). Treatment with the MR blocker eplerenone for 9 days did not affect these parameters.

Time Course of TH, NET, and PGP9.5 Expression

Stellate ganglia. No significant changes were found in TH and NET mRNA (Fig. 1) and TH protein (Fig. 2) in the left SG post-MI. No significant changes were observed in NET protein at 1 and 4 wk post-MI, but at 12 wk post-MI, NET 80-kDa protein content was increased approximately twofold, with no significant changes in NET 46-kDa protein and total NET (Fig. 2). No changes in PGP9.5 mRNA content were noted at 1 and 4 wk post-MI, whereas at 12 wk post-MI, PGP9.5 mRNA was significantly decreased approximately twofold but with no parallel changes in the protein level of PGP9.5 (Figs. 1 and 2). In the right SG, the pattern of TH, NET, and PGP9.5 gene and protein expression was similar, except that at 12 wk post-MI, there were no changes in NET 80-kDa protein content (data not shown).

Left ventricle (area above ligation) and right ventricle. No changes in TH and NET proteins were found. In contrast, PGP9.5 protein was significantly increased twofold at 1 wk post-MI and by ~60% at 4 wk post-MI but no longer at 12 wk post-MI (Fig. 3). In the RV, TH, NET, and PGP9.5 protein expression showed a similar pattern (Fig. 3).

Cardiac Sympathetic Innervation

The sympathetic innervation pattern of ventricles is heterogeneous, with the epicardial side of the myocardium having
more notable innervation density than the endocardial side (Table 3). The myocardium adjacent to the epicardial surface was selected for quantification in the base of the LV. In sham rats, nearly all PGP9.5-positive fibers were also positive for TH (Fig. 4). The percent area occupied by PGP9.5 protein was slightly (nonsignificantly) higher, 1.10% for PGP9.5 versus 0.82% for TH (Table 3). All GAP43-positive nerve fibers were positive for TH as well, but the area occupied by GAP43 was significantly less, 0.51% (Fig. 4 and Table 3), reflecting its lower expression in the case of stable cardiac innervation.

MI and sham rats showed similar densities of TH-, GAP43-, and PGP9.5-positive fibers in the base of the LV, indicating that there were no notable changes in cardiac sympathetic innervation in this region of the heart (Table 3). In contrast, in the peri-infarct area, TH-positive fibers comprised 1.63% and PGP9.5-positive fibers comprised 1.54% of the area, which were similar (Table 3), and most of the fibers demonstrated colocalization (Figs. 4 and 5). GAP43-positive fibers comprised 2.88% of the area, which was significantly higher (Table 3 and Figs. 4 and 5). Although many GAP43-positive fibers colocalized with TH, ~50% of the fibers were only GAP43 positive (Fig. 4). This likely reflects immaturity of the new sprouting axons and more pronounced upregulation of GAP43 expression at the nerve growth cones.

Treatment with the MR blocker significantly attenuated the increase in TH, GAP43, and PGP9.5 nerve densities in the peri-infarct area (Figs. 5 and 6 and Table 3). Compared with innervation at the epicardial side of the LV in sham rats, TH and PGP9.5 nerve densities at the MI border of eplerenone-treated rats were normalized, but GAP43 was still 75% higher (Table 3).

Preabsorption of the primary antibodies completely abolished the immunostaining for GAP43 and PGP9.5 and greatly reduced immunostaining for TH (Fig. 7). The latter is likely due to a lower protein-to-antibody ratio for TH preabsorption, due to a limited amount of the neutralizing protein and a wide concentration range provided by the supplier for the stock antibody.

EXTRANEURONAL PGP9.5 EXPRESSION

Fluorescent immunohistochemistry was used to further study the location of the increased PGP9.5 protein in the heart after MI. In the base of the LV, PGP9.5 immunoreactivity was noted predominantly within cardiac nerves but was also present in a small number of individual spindle-shaped cells. In the peri-infarct and adjacent area, large numbers of spindle-shaped cells were found, which resembled fibroblasts and had strong PGP9.5 immunoreactivity but not TH immunoreactivity (Fig. 8). However, no colocalization between PGP9.5-positive cells and the fibroblast marker vimentin was present. Vimentin-positive cells surrounded PGP9.5-positive cells, confirming...
their peri-infarct location (Fig. 8). In contrast, PGP9.5 protein and α-sarcomeric actin protein showed positive colocalization, indicating that cardiomyocytes in this area express PGP9.5 (Fig. 9).

Since upregulation of PGP9.5 expression may cause changes in ubiquitin and in ubiquitinated proteins, we proceeded with the assessment of protein expression of PGP9.5, ubiquitin, and ubiquitinated proteins as well as neuronal protein GAP43 in the areas where PGP9.5-positive cardiomyocytes were found. No significant differences in GAP43 protein expression were present between the groups. In contrast, PGP9.5 protein expression was significantly elevated approximately sixfold. The contents of ubiquitin and of ubiquitinated proteins with molecular weights of 60 and 250 kDa were significantly increased approximately twofold, but the content of ubiquitinated proteins with molecular weights of 30, 40, and 100 kDa was significantly decreased approximately twofold in the MI group (Fig. 10). Treatment with the MR blocker eplerenone for 9 days post-MI had no effect on the content of PGP9.5, ubiquitin, and ubiquitinated proteins (data not shown).

**DISCUSSION**

The present study shows that in rats post-MI, central treatment with eplerenone markedly attenuates cardiac sympathetic hyperinnervation in the peri-infarct area. The time course of TH, NET, and PGP9.5 expression in the noninfarcted base of the heart showed a selective increase in PGP9.5 protein content, twofold at 1 wk post-MI and 60% at 4 wk post-MI, but no more at 12 wk. TH and NET were located only within cardiac sympathetic nerves. PGP9.5 was expressed in both sympathetic axons and cardiomyocytes in the base of the heart and the peri-infarct area after the MI and was associated with increased ubiquitin expression.

**Time Course of TH, NET, and PGP9.5 Expression**

No changes in TH and NET mRNA and protein in the SG were found at 1, 4, and 12 wk in rats post-MI. At 12 wk post-MI, in the left SG, the content of highly glycosylated NET protein with a molecular mass of 80 kDa was increased by twofold. Highly glycosylated NET with molecular masses of 80 and 54 kDa showed increased protein stability, surface trafficking, and transport activity compared with unglycosylated NET with a molecular mass of 46 kDa (27). No notable changes in TH and NET proteins were found either in the noninfarcted base of the LV or in the RV. However, the absence of changes in TH expression does not exclude increased TH activity mediated by other mechanisms, such as TH phosphorylation (2).

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Fig. 8. Representative images of cells in the peri-infarct area that had strong PGP9.5 immunoreactivity but not TH and vimentin immunoreactivity. Vimentin-positive cells surrounded PGP9.5-positive cells, confirming their peri-infarct location. Pictures were taken with a ×10 objective.
Parrish et al. (33) reported a significant twofold increase of TH and NET mRNA in the SG and a twofold increase of TH and NET proteins in the base of the heart in rats at 1 wk after ischemia-reperfusion. One explanation for these different findings is that ischemia-reperfusion may affect TH and NET expression in a different fashion than the permanent ligation MI model. The choice of normalization control could also affect results. PGP9.5 was used as a control by Parrish et al. (33) to normalize TH and NET expression for variations in innervation. When GAPDH was used as a control, no changes in TH and NET mRNA were found in the SG of mice 1 wk after ischemia-reperfusion (32). PGP9.5 gene expression can be downregulated by oxidative stress (41), and ROS generation is increased during ischemia-reperfusion injury (36) and can be induced by ANG II (49). The latter mechanism may contribute to the observed twofold decrease of PGP9.5 mRNA in the SG at 12 wk post-MI, since circulating plasma levels of ANG II are persistently elevated in rats post-MI (23).

Cardiac Sympathetic Innervation

In the present study, cardiac sympathetic hyperinnervation was apparent in the peri-infarct area with a fourfold increase in GAP43, a twofold increase in TH, and a 50% increase in PGP9.5 immunoreactivities compared with the epicardial side of the LV in sham rats. The absolute percent area occupied by TH- and PGP9.5-positive fibers was similar (∼1.6%) but was significantly larger for GAP43 (2.9%). Approximately 50% of GAP43-positive fibers did not demonstrate colocalization with TH, suggesting that these fibers are immature new sprouting axons. In the noninfarcted base of the LV, no significant changes in nerve densities were observed. This pattern is consistent with several studies (10, 25) that also described sympathetic hyperinnervation predominantly in the peri-infarct area at this time point post-MI. Sympathetic nerve density in the noninfarcted LV may increase later post-MI in rats (20, 22, 51).

Central treatment with the MR blocker eplerenone markedly attenuated the increase in nerve densities in the peri-infarct area for TH, GAP43, and PGP9.5. Several mechanisms may contribute to this effect of central MR blockade. First, central MR blockade decreases various parameters of sympathetic hyperactivity in rats post-MI (12, 19). Effects on CSNA post-MI have not yet been evaluated, but it is reasonable to expect that central MR blockade also inhibits CSNA. In normal dogs, subthreshold electrical stimulation of the left SG induces nerve sprouting and increases nerve density in the heart. In dogs post-MI, similar stimulation for 1 mo promotes sympathetic hyperinnervation and increases nerve density to a larger

Fig. 9. PGP9.5 and α-sarcomeric actin fluorescent double staining in the peri-infarct area. A: α-sarco- meric actin immunoreactivity. B: PGP9.5 immuno- reactivity. C: PGP9.5 and α-sarcomeric actin colocalization. Pictures were taken with a ×20 objective. D: magnified image of PGP9.5 and α-sarcomeric actin colocalization. Cells that stained both green and red on this merged micrograph demonstrated coexpression of PGP9.5 and α-sarcomeric actin, as indicated with arrows. Pictures were taken with a ×40 objective.
extent than infusion of nerve growth factor (NGF) in the left SG (45).

Second, central MR blockade may decrease cardiac sympathetic hyperinnervation post-MI by affecting NGF expression/function by decreasing the level of inflammation or oxidative stress (17). Pronounced NGF release and overexpression occurs within hours post-MI at the infarct site and in the peri-infarct area and, to a lesser extent, in the non-infarcted LV (28, 52). Macrophages and myofibroblasts in the peri-infarct area express NGF at 7–14 days post-MI (10), and macrophage depletions with liposomes containing clodronate markedly reduces cardiac sympathetic hyperinnervation (50). Superoxide and nitric oxide can trigger NGF expression and thereby promote hyperinnervation (21, 47). ANG II can induce oxidative stress in the heart by increasing NAD(P)H-dependent superoxide anion production and intracellular generation of ROS (49). Blockade of the brain renin-angiotensin-aldosterone system by central MR blockade inhibits the activation of the circulatory and cardiac renin-angiotensin-aldosterone system post-MI (19, 46). Further studies are needed to assess the effects of central MR blockade on CSNA and cardiac inflammation, ROS formation and NGF, and their contribution to the decrease in cardiac sympathetic hyperinnervation post-MI by central MR blockade.

Extraneuronal PGP9.5 Expression

Western blot analysis showed a selective sixfold increase in PGP9.5 protein in the peri-infarct and adjacent area and a twofold increase in the noninfarcted base of the heart. These increases appear to take place early post-MI, are less by 4 wk, and are no longer apparent at 12 wk post-MI. At 10 days post-MI, large clusters of PGP9.5-positive cardiomyocytes were found in the peri-infarct area and individual PGP9.5-positive cells in the noninfarcted LV. These findings indicate that after MI, cardiomyocytes upregulate PGP9.5 expression. Weckes et al. (48) reported that in patients with dilated cardiomyopathy, cardiomyocytes also exhibit strong PGP9.5 immunoreactivity. At 1 wk post-MI, myofibroblasts synthesizing NGF and brain-derived neurotrophic factor, along with neural stem cells, astrocytes, and oligodendrocytes, have been described in the scar (6, 10). Local secretion of neurotrophic proteins by these cells may be a potential trigger for PGP9.5 expression by cardiomyocytes post-MI. It is unlikely that
epicardial ganglia contribute to the LV PGP9.5 levels, since the vast majority of the intrinsic ganglia are located in the atria (1).

PGP9.5 is also referred to as ubiquitin carboxy-terminal hydrolase L1. By hydrolyzing a peptide bond between ubiquitin and target protein, ubiquitin carboxy-terminal hydrolase L1 generates a free mono-ubiquitin molecule available for the next catalytic cycle (9). It also stabilizes mono-ubiquitin, which elongates its half-life (30). Consistent with enhanced activity of PGP9.5, in the peri-infarct area, expression of ubiquitin and ubiquitinated proteins with molecular masses of 60 and 250 kDa was increased approximately twofold, but expression of ubiquitinated proteins with molecular masses of 30, 40, and 100 kDa was decreased approximately twofold in the MI group. Further studies are needed to identify the actual ubiquitinated proteins and the cellular processes involved, which likely represent the extensive remodeling early post-MI.

In summary, the present study demonstrates that in rats post-MI, central MR blockade markedly attenuates cardiac sympathetic hyperinnervation. Cardiac sympathetic hyperactivity and sympathetic hyperinnervation have been implicated in ventricular arrhythmias and sudden cardiac death post-MI (4, 15), and one may speculate that central MR blockade early post-MI can improve these adverse outcomes (35).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: A.D. and F.H.L. conception and design of research; A.D., M.A., and F.H.L. interpreted results of experiments; A.D. drafted manuscript; M.A., F.H.L., and A.D. edited and revised manuscript; F.H.L. approved final version of manuscript.

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