Hibernating myocardium results in partial sympathetic denervation and nerve sprouting

Stanley F. Fernandez,2,3 Vladislav Ovchinnikov,2,3 John M. Canty, Jr.,1,2,3,4,5 and James A. Fallavollita1,2,3

1VA Western New York Health Care System at Buffalo, University at Buffalo, Buffalo, New York; 2Center for Research in Cardiovascular Medicine, University at Buffalo, Buffalo, New York; 3Department of Medicine, University at Buffalo, Buffalo, New York; 4Department of Physiology and Biophysics, University at Buffalo, Buffalo, New York; and 5Department of Biomedical Engineering, University at Buffalo, Buffalo, New York

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Fernandez SF, Ovchinnikov V, Canty JM Jr, Fallavollita JA. Hibernating myocardium results in partial sympathetic denervation and nerve sprouting. Am J Physiol Heart Circ Physiol 304: H318–H327, 2013. First published November 2, 2012; doi:10.1152/ajpheart.00810.2011.—Hibernating myocardium due to chronic repetitive ischemia is associated with regional sympathetic nerve dysfunction and spontaneous arrhythmia, which is the mechanism responsible for sudden death. Although inhomogeneity has been the subject of intense investigation, but remains unclear. The mechanism(s) responsible for these abnormalities in viable, dysfunctional myocardium (i.e., neural stunning vs. sympathetic denervation) and their association with nerve sprouting are unknown. Accordingly, markers of sympathetic nerve function and nerve sprouting were assessed in subendocardial tissue collected from chronically instrumented pigs with hibernating myocardium (n = 18) as well as sham-instrumented controls (n = 7). Hibernating myocardium exhibited evidence of partial sympathetic denervation compared with the normally perfused region and sham controls, with corresponding regional reductions in tyrosine hydroxylase protein (−32%, P < 0.001), norepinephrine uptake transport protein (−25%, P = 0.01), and tissue norepinephrine content (−45%, P < 0.001). Partial denervation induced nerve sprouting with regional increases in nerve growth factor precursor protein (31%, P = 0.01) and growth associated protein-43 (38%, P < 0.05). All of the changes in sympathetic nerve markers were similar in animals that developed sudden death (n = 9) compared with electively terminated pigs with hibernating myocardium (n = 9). Conclusion: Sympathetic nerve dysfunction in hibernating myocardium is most consistent with partial sympathetic denervation and is associated with regional nerve sprouting. The extent of sympathetic remodeling is similar in animals that develop sudden death compared with survivors; this suggests that sympathetic remodeling in hibernating myocardium is not an independent trigger for sudden death. Nevertheless, sympathetic remodeling likely contributes to electrical instability in combination with other factors.

Address for reprint requests and other correspondence: J. A. Fallavollita, CTRC Ste. 7030 Dept. of Medicine/Cardiology, Univ. at Buffalo, 875 Ellicott St., Buffalo, NY 14203-1034 (e-mail: jaf7@buffalo.edu).

Sudden cardiac death (SCD) due to ventricular tachyarrhythmias is an important complication of chronic ischemic heart disease, accounting for approximately one-third of mortality in these patients (15, 34, 43). The substrate responsible for SCD has been the subject of intense investigation, but remains unclear and is likely multifactorial (40, 46). One very popular and well-supported hypothesis invokes regional inhomogeneity in sympathetic innervation as a substrate that promotes lethal arrhythmias during sympathetic activation (40, 46). This has classically been described following transmural myocardial infarction where the area of sympathetic denervation exceeds the extent of necrosis, reflecting viable but denervated myocardium in the subepicardium or peri-infarct regions (3). As reinnervation proceeds in the postinfarction period, it is accompanied by sympathetic nerve sprouting and areas of hyperinnervation (11). The resultant hypersensitivity to catecholamine stimulation along with marked heterogeneity in sympathetic innervation results in regional dispersion in action potential duration during sympathetic activation that potentially initiates ventricular tachyarrhythmias that result in SCD.

Inhomogeneity in myocardial sympathetic innervation can also occur in viable dysfunctional myocardium associated with chronic ischemic heart disease when infarction is absent (1, 16). Retrospective clinical studies have shown that the presence and possibly the volume of viable dysfunctional, or hibernating myocardium predict cardiovascular mortality (1, 16). Although only limited cause-specific mortality data are available, the increased event rate appears to be due to an increased risk of SCD (16). The importance of SCD is further supported by previous studies in pigs with hibernating myocardium, where there is a ~50% rate of spontaneous SCD due to ventricular tachycardia degenerating into ventricular fibrillation without evidence of acute infarction (7). Episodes of ventricular tachycardia/ventricular fibrillation are always preceded by sinus tachycardia reflecting transient sympathetic activation (38). In addition, these hearts have regionally reduced cardiac sympathetic nerve function as evidenced by regionally reduced uptake of the norepinephrine analogs 131I-meta-iodo-benzylguanidine (MIBG) (29) and 11C-meta-hydroxyephedrine (HED)(30). The functional significance of these changes has been confirmed by demonstrating reduced myocardial thickening during presynaptic sympathetic stimulation (36). Collectively, the similarity of these observations with those associated with infarction supports the possibility that remodeling of myocardial sympathetic innervation may be a common substrate factor responsible for SCD.

In this previous work we have shown that hibernating myocardium was associated with abnormal sympathetic nerve function, but whether this was due to an anatomic loss of sympathetic nerves as opposed to a reversible (functional) abnormality [i.e., neural stunning (12)] remained unclear. Although the presence of either abnormality in the setting of viable myocardium may have prognostic implications (40, 46), neural stunning would be reversible with amelioration of ischemia and would be less likely to induce secondary alterations such as nerve sprouting (18). To address this issue, we hypothesized that abnormalities in sympathetic nerve function seen in hibernating myocardium are reversible, reflecting primarily...
functional changes, and would be associated with preserved levels of sympathetic markers. Alternatively, a coordinated reduction in these markers relative to controls and the normally perfused remote myocardium would be consistent with anatomic denervation. Because our subsequent results were consistent with the alternate hypothesis, we performed additional analyses to determine whether the partial denervation in hibernating myocardium was a stimulus for nerve sprouting as an additional mechanism for arrhythmogenesis (11). Finally, we determined whether the regional expression of any of these markers differed between animals with hibernating myocardium that experienced SCD as compared with those that survived.

MATERIAL AND METHODS

All animal experimental procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were approved by the University at Buffalo Institutional Animal Care and Use Committee.

Porcine model of hibernating myocardium and tissue sampling. Protein analyses were performed on frozen tissue samples (–80°C) previously obtained from pigs with established hibernating myocardium, the details of which have been previously published (21). Briefly, juvenile pigs underwent a limited thoracotomy under isoflurane anesthesia (2–4% inhaled; monitored with heart rate, blood pressure, jaw tone, and palpebral reflex) and the proximal left anterior descending coronary artery (LAD) was instrumented with a 1.5-mm Delrin (DuPont) stenosis. Postoperative analgesia was achieved with an intercostal nerve block (marcaine 0.5%), butorphanol (0.1–0.3 mg/kg IM once), and flunixin (1–4 mg/kg IM twice a day for 2 to 3 days). Because one of the objectives of this study was to identify sympathetic nerve abnormalities that may promote SCD, an equal number of samples were obtained from animals that were selectively euthanized (survivor group, n = 9) (36) as those who developed spontaneous SCD (sudden death group, n = 9) (7). Sham-instrumented control animals (n = 7) underwent a similar procedure, including dissection of the LAD to control for potential injury to sympathetic nerves coursing with the artery (29, 36). Animals that were selectively euthanized (survivors and controls) were anesthetized (isoflurane 2–4% inhaled) and the heart arrested with potassium chloride. Subendocardial samples were obtained from the central part of the LAD risk region (hibernating myocardium) as well as the center of the remote, normally perfused region (remote myocardium), taking care to avoid sampling border regions. For the sudden death group, myocardial samples were obtained as soon as feasible with the median care to avoid sampling border regions. For the sudden death group, including dissection of the LAD to control for potential injury to a medium that experienced SCD as compared with those that determined whether the regional expression of any of these additional mechanism for arrhythmogenesis (11). Finally, we analyses to determine whether the partial denervation in hibernating myocardium would be consistent with ana- reduce in these markers relative to controls and the normally levels of sympathetic markers. Alternatively, a coordinated functional changes, and would be associated with preserved myocardium, the norepinephrine concentration was determined from 10 μl of homogenate using a norepinephrine EIA immunosassay and the manufacturers recommended procedure (MP Biomedicals). Immunochemistry. Protein colocalization was performed with immunofluorescence microscopy (Bio-Rad MRC 1024), as previously described (45). Frozen tissues sections (5 μm) were incubated with protein blocking agent (Dako protein block; Dako) followed by incubation with rabbit anti-NGF (1:200; Chemicon) and mouse anti-noradrenalin to total protein (NET and GAP-43) or 1:10,000 dilution (TH and NGF) and visualized with the Super Signal PICO Chemiluscent substrate (Pierce). Chemiluminescent signals were captured using a Fujifilm LAS-1000 imager coupled to a cooled charge-coupled device camera and analyzed using Quantity One software (Bio-Rad). Analyses were performed in duplicate. Variability in the number of studies for each analysis was due to limited tissue samples in a few animals.

Norepinephrine analysis. Myocardial samples were homogenized in a volume 10x the tissue weight with 0.1N HCl/5 mM reduced glutathione. After centrifuging at 30,000 g for 30 min at 4°C, the supernatants were stored at –80°C until assayed. Tissue norepinephrine concentration was determined from 10 μl of homogenate using a norepinephrine EIA immunosassay and the manufacturers recommended procedure (MP Biomedicals). Regional myocardial function. Regional myocardial function at rest and during sympathetic stimulation was assessed with segment shortening (SS), as previously described and reported (36). Animals were intubated and mechanically ventilated, and anesthesia was maintained with a continuous intravenous infusion of propofol (20–75 μg·kg⁻¹·min⁻¹) and ketamine (30–125 μg·kg⁻¹·min⁻¹) with continuous monitoring of heart rate and blood pressure. The heart was exposed through a midline sternotomy, and pairs of piezoelectric crystal were placed ~1 cm apart in the subendocardium of the midanterior wall. Regional SS in response to direct stellate ganglion stimulation (5-ms pulses at 10 Hz for 10 s) or intracoronary tyramine infusion (10 μg/kg) were quantified as: [(end-diastolic segment length – end-systolic segment length)/end-diastolic segment length]. Stellate stimulation was performed for 10 s based on preliminary data that showed maximal and stable responses from 5 to 10 s of stimulation.

Data analysis. Data are presented as means ± SE. For all candidate sympathetic markers there were no regional differences in the sham-instrumented control animals; therefore, these samples were combined into a single control group. Optical density values from the hibernating and remote samples were compared with paired t-tests (Microsoft Excel). Each group was also compared with the control group using an unpaired t-test, with the Bonferroni correction for multiple compar-isons. To clarify presentation of the figures, values from hibernating and remote regions are shown relative to the mean of the control group. To compare sympathetic markers between animals with SCD and survivors, values from the hibernating region were normalized to the remote region for each animal, and the ratios compared with unpaired t-tests. Correlation studies were analyzed using linear re-gression analysis. All t-tests were two-tailed, and P < 0.05 was considered statistically significant.
Differential expression of sympathetic nerve markers

<table>
<thead>
<tr>
<th></th>
<th>TH, o.u.</th>
<th>NET, o.u.</th>
<th>NE, ng/gm</th>
<th>NGF, o.u.</th>
<th>GAP-43, o.u.</th>
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<tr>
<td>Control</td>
<td>318 ± 32</td>
<td>680 ± 78</td>
<td>1,843 ± 135</td>
<td>216 ± 29</td>
<td>163 ± 22</td>
</tr>
<tr>
<td>Remote</td>
<td>297 ± 27</td>
<td>486 ± 75</td>
<td>1,559 ± 135</td>
<td>293 ± 48</td>
<td>189 ± 28</td>
</tr>
<tr>
<td>Hibernating</td>
<td>202 ± 17†</td>
<td>364 ± 60‡</td>
<td>860 ± 119‡</td>
<td>385 ± 62‡</td>
<td>261 ± 31‡</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05 vs. control; †P < 0.05 vs. remote. TH, tyrosine hydroxylase; NET, norepinephrine transporter; NE, norepinephrine content; NGF, nerve growth factor precursor protein; GAP-43, growth associated protein-43.

RESULTS

All animals were in good health at the time of study. Detailed physiological characteristics have been separately reported for each of the experimental groups: hibernating myocardium survivors (36), hibernating myocardium with SCD (7), and sham-instrumented controls (29, 36) with the salient features compiled here. Approximately 3 mo after initial instrumentation chronically instrumented pigs developed high-grade stenoses or complete occlusion of the LAD, with hibernating myocardium characterized by regional dysfunction with reduced resting perfusion, and no infarction by triphenyltetrazolium chloride staining (7, 36). In electively terminated animals (survivors), quantitative echocardiography demonstrated severe anterior wall dysfunction (LAD systolic wall thickening 2.5 ± 0.2 mm vs. 6.0 ± 0.4 mm in remote, \( P < 0.01 \)), with reduced resting subendocardial perfusion using microspheres (0.90 ± 0.07 vs. 1.12 ± 0.06 ml·min\(^{-1}\)·g\(^{-1}\) in remote, \( P = 0.05 \)) (7, 36).

**TH.** TH is a rate-limiting enzyme catalyzing norepinephrine synthesis. In pigs with hibernating myocardium we found a 32% regional reduction in TH expression in hibernating compared with remote myocardium (\( P < 0.001, n = 17 \); Table 1). TH expression in hibernating regions was also reduced relative to controls (hibernating/control = 0.64 ± 0.05, \( P < 0.01 \) for hibernating vs. control; Figure 1 and Table 1), but normal in the remote region. This partial reduction was confirmed using immunohistochemistry, with a proportionately similar regional reduction in the number of TH-positive fibers in hibernating compared with remote myocardium (Fig. 2). Concurrent staining for TH and PGP9.5 (a general neuronal marker) also demonstrated significant reductions in both markers in hibernating compared with remote myocardium (Figs. 3 and 4). However, when TH staining was normalized to PGP9.5, there was no significant difference between hibernating and remote regions. This suggests that there is a generalized decrease in the sympathetic nerve fiber innervations of hibernating myocardium.

**Norepinephrine transporter.** NET facilitates the cellular re-uptake of norepinephrine released into the synaptic cleft. Several immune-reactive bands of NET were identified (48, 54, and 81 kDa), which is consistent with previously published findings and felt to represent different glycosylation states (32, 33). These data represent the average of all identifiable NET isoforms. We found a 25% regional reduction in total NET expression (all 3 bands) in hibernating compared with remote myocardium (\( P = 0.01, n = 12 \)). NET expression in hibernating myocardium was also reduced relative to controls (hibernating/control = 0.53 ± 0.08, \( P < 0.01 \) for hibernating vs. control; Fig. 1 and Table 1). There were no changes in the remote region compared with sham controls.

**Tissue norepinephrine content.** Tissue norepinephrine is nearly undetectable in surgical or chemically induced myocardial denervation. We found a 45% regional reduction in subendocardial tissue norepinephrine content in hibernating myo-
cardium \((P < 0.001, n = 16)\). Norepinephrine levels in hibernating myocardium were also reduced relative to controls \((\text{hibernating/control} = 0.47 \pm 0.06, P < 0.001\) for hibernating vs. control; Fig. 1 and Table 1) but were normal in remote myocardium. Thus the concordant reductions in TH, NET, and norepinephrine levels in hibernating myocardium were consistent with regional ischemia-induced sympathetic remodeling and partial denervation.

**NGF.** NGF supports sympathetic nerve survival and differentiation, can produce myocardial nerve sprouting, and is increased after neuronal injury and regeneration \((42)\). Our Western blot analysis using anti-NGF antibody identified a

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**Fig. 2.** Immunohistochemical staining of TH in an animal with hibernating myocardium. Paired samples from a representative animal show TH containing sympathetic nerves as darkly stained structures in both the hibernating (top) and remote (bottom) region. The relative difference in TH between the 2 regions is further emphasized in the background subtracted images of the same photomicrographs. The number of TH-positive cells was reduced in hibernating compared with remote myocardium, consistent with partial sympathetic denervation.

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**Fig. 3.** Immunofluorescence staining of TH and PGP9.5 in an animal with hibernating myocardium. Paired samples from a representative animal show PGP9.5-containing nerve structures (green; left) and TH-containing sympathetic nerves (red; middle). There is significant overlap in fluorescence on the merged images (yellow; right). There is generalized reduction in overall TH and PGP9.5 staining in hibernating myocardium (top row) compared with the remote normally perfused region (bottom row).
weak and inconsistent signal at the 13.5-kDa molecular weight range (corresponding to the mature NGF), but detected a strong and robust signal at the 35-kDa range (corresponding to the higher molecular weight NGF precursor protein). Competitive binding using NGF blocking peptide showed inhibition of the 35-kDa band in a dose-response pattern. Subsequent quantitation was based on this band. We found a 31% increase in NGF precursor protein expression in hibernating compared with remote myocardium (*P < 0.01, n = 17). The increase in NGF precursor protein in hibernating myocardium was also evident relative to controls (hibernating/control = 1.78 ± 0.29, *P < 0.05 for hibernating vs. control; Fig. 1 and Table 1), with a nonsignificant increase in remote regions (remote/control = 1.36 ± 0.22, *P = 0.37).

Consistent with the Western analysis results, NGF immuno-histochemical staining was accentuated in hibernating compared with the remote normally perfused myocardium (Fig. 5). Cardiomyocytes were identified as the major source of increased NGF expression, with predominant colocalization of anti-NGF and anti-troponin T staining (Fig. 5). Consistent with previous reports (39, 41), NGF staining was primarily cytoplasmic.

**GAP-43**. GAP-43 is concentrated in the axonal growth cone and is associated with nerve sprouting (31, 47). There was a
38% increase in GAP-43 expression in hibernating compared with remote myocardium ($P < 0.05, n = 18$). The increase of GAP-43 in hibernating myocardium was also evident compared with sham controls (hibernating/control = 1.60 ± 0.19, $P < 0.05$ for hibernating vs. control; Fig. 1 and Table 1), with normal levels in remote regions (remote/control = 1.16 ± 0.17, $P = 1.0$). The colocalization of GAP-43 with TH (Fig. 6) supports its utility as a marker of sympathetic nerve sprouting.

In chronic myocardial infarction models, it has been demonstrated that sympathetic remodeling appears to be most critical at the border zone of infarction. To determine whether the area adjacent to hibernating myocardium manifests a similar physiology we quantified expression of GAP-43 and TH in the border zone and compared them with the remote normally perfused region ($n = 3$ each). We found no significant change in either GAP-43 (border: 54 ± 10 o.u. vs. 81 ± 14 o.u. in remote, $P = 0.38$) or TH (border: 63 ± 7 o.u. vs. 55 ± 24 o.u. in remote, $P = 0.81$).

**Correlation of TH expression and functional responses to sympathetic stimulation.** To further test the hypothesis that sympathetic nerve protein changes in hibernating myocardium were physiologically relevant, we correlated previously reported functional responses to sympathetic stimulation (36) with their corresponding TH protein levels from individual animals (Fig. 7). Tyramine- and stellate stimulation-mediated changes in anterior segment shortening (LAD perfusion territory) correlated with LAD TH expression (expressed as LAD-to-remote ratio). This correlation suggests that sympathetic marker downregulation in hibernating myocardium has physiologic consequences.

**Sympathetic markers in sudden death versus survivors.** We hypothesized that increases in molecular markers of sympathetic nerve sprouting and/or the degree of sympathetic denervation would be more accentuated in animals developing SCD. Accordingly, the relative expression of these markers (hibernating/remote) in animals that developed SCD was compared with those that survived. The results in Fig. 8 and Table 2 illustrate that there were no significant differences in the magnitude of denervation or nerve sprouting between survivors and SCD animals. Specifically, there were similar regional reductions in TH (survivors: $-32 \pm 4\%$ vs. $-28 \pm 5\%$ in SCD, $P = 0.65$), NET (survivors: $-15 \pm 6\%$ vs. $-23 \pm 10\%$ in SCD, $P = 0.50$), and norepinephrine content (survivors: $-46 \pm 10\%$ vs. $-40 \pm 13\%$ in SCD, $P = 0.74$). In addition, the regional increases in NGF precursor protein (survivors: $38 \pm 18\%$ vs. $58 \pm 32\%$ in SCD, $P = 0.58$) and GAP-43 (survivors: $67 \pm 33\%$ vs. $45 \pm 9\%$ in SCD, $P = 0.56$) expression in hibernating myocardium were comparable in the two groups of animals (Fig. 8).

**DISCUSSION**

There are several novel findings from the present investigation that have significant implications regarding our understanding of the heterogeneity and plasticity of sympathetic nerve function in ischemic heart disease and their potential causal relation to SCD. Although it is widely recognized that sympathetic nerve dysfunction can occur in viable, chronically dysfunctional myocardium (18), the present study demonstrates that this is not the result of reversible dysfunction.
neural stunning) but rather the partial anatomic loss of sympathetic nerves. Furthermore, the partial sympathetic denervation arising in response to repetitive ischemia in the absence of infarction can stimulate nerve sprouting, which may play a role in the SCD associated with this model (7). Nevertheless, the fact that the magnitude of alterations in sympathetic nerve protein expression as well as markers of nerve sprouting were similar in animals developing SCD compared with survivors supports the conclusion that this is not a specific cause for the development of ventricular tachycardia/ventricular fibrillation and SCD in this model (18, 40, 46).

Partial sympathetic denervation in hibernating myocardium. In the present study, all three candidate markers of sympathetic nerve function (TH, NET, and tissue norepinephrine) were regionally downregulated to approximately the same extent in pigs with hibernating myocardium. This extends our previous in vivo observations regarding the physiological effects of cardiac sympathetic nerve function in this model. We previously found that the deposition of the norepinephrine analog 131I-MIBG was regionally reduced in hibernating myocardium (hibernating/remote = 0.75 ± 0.03) (29). The regional reduction in norepinephrine tracer uptake was even more evident using HED and positron emission tomography, with a 48% reduction in hibernating myocardium compared with the remote normally perfused region (30). Thus the concordant reductions in the three markers (TH, NET, and tissue norepinephrine) coupled with the regional reduction in histological sympathetic nerve density is consistent with partial loss of sympathetic nerves in response to repetitive reversible ischemia.

Nevertheless, Kawai and associates, using a rabbit model of pacing-induced heart failure, have demonstrated that down-regulation of sympathetic markers may still be a reversible phenomenon (25). Within 2–4 wk after cessation of rapid pacing, they noted recovery of myocardial function and sympathetic markers and concluded that the mechanism of sympathetic dysfunction was secondary to neural stunning (25). In contrast, we have previously shown that norepinephrine uptake does not improve in pigs with hibernating myocardium despite therapies that are associated with improvement in regional myocardial function (17). Both percutaneous coronary revascularization (2) and pravastatin therapy (44) improved regional function within 1 mo in pigs with hibernating myocardium; however, neither intervention was associated with any significant change in HED uptake, retention, defect size, or defect severity (17). Therefore, our results support the contention for partial sympathetic denervation in ischemically mediated hibernating myocardium, with no significant reversible sympathetic nerve dysfunction.

Partial sympathetic denervation induces nerve sprouting. Neuronal sprouting is purported to be a compensatory response to nerve degeneration or injury and has been well documented in both animal infarct models (9, 28, 35, 47) and in the border zone around fibrosis in explanted human hearts (10). It is hypothesized that nerve sprouting leads to hyperinnervation that exacerbates the underlying inhomogeneity in sympathetic nerve dysfunction that develops after infarction. As a result, the dispersion in action potential duration following sympathetic activation is increased, which accentuates the risks of ventricular arrhythmias and SCD in the healing phase of myocardial infarction (40, 46). The available clinical data support this contention, with an association between spontaneous ventricular arrhythmias and increased sympathetic nerve density when assessed at the periphery of infarction or in perivascular regions (10). Furthermore, in canine studies nerve sprouting and sympathetic hyperinnervation were evident within 1 wk after myocardial infarction (47), and exogenous infusion of NGF into the stellate ganglion increased myocardial sympathetic nerve density, nerve sprouting (GAP-43), and spontaneous

### Table 2. Expression of sympathetic nerve markers in survivors vs. SCD

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<tr>
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<th>TH, o.u.</th>
<th>NET, o.u.</th>
<th>NE, ng/gm</th>
<th>NGF, o.u.</th>
<th>GAP-43, o.u.</th>
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<tr>
<td>Survivors</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Hibernating</td>
<td>191 ± 23</td>
<td>252 ± 48</td>
<td>781 ± 129</td>
<td>364 ± 86</td>
<td>241 ± 42</td>
</tr>
<tr>
<td>Remote</td>
<td>281 ± 28</td>
<td>324 ± 59</td>
<td>1,482 ± 125</td>
<td>258 ± 53</td>
<td>176 ± 39</td>
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<tr>
<td>SCD</td>
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<tr>
<td>Hibernating</td>
<td>182 ± 33</td>
<td>520 ± 93</td>
<td>939 ± 205</td>
<td>409 ± 96</td>
<td>285 ± 47</td>
</tr>
<tr>
<td>Remote</td>
<td>315 ± 50</td>
<td>714 ± 89</td>
<td>1,636 ± 247</td>
<td>333 ± 86</td>
<td>207 ± 40</td>
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Values are means ± SE. Comparison of sympathetic marker expressions by using normalized values (hibernating/remote) between survivors and sudden cardiac death (SCD) animals showed no significant differences.
ventricular arrhythmias (9). In the present study GAP-43 was regionally increased in hibernating myocardium and was associated with upregulation of NGF precursor protein that was primarily derived from cardiac myocytes. We also found a trend toward increased NGF precursor protein in the remote, normally perfused region of pigs with hibernating myocardium, which would be analogous to the increased expression found in the remote region after myocardial infarction in dogs (47). Thus our results extend previous studies that were focused on myocardial infarction, by confirming that a similar process of NGF precursor protein upregulation and nerve sprouting can occur as a result of partial sympathetic denervation associated with viable myocardium.

GAP-43 is a marker for general neuronal sprouting (31), and our findings demonstrating colocalization of GAP-43 and TH are consistent with other animal models of myocardial ischemia (27, 28, 35). The increase in GAP-43 is presumably in response to the neurotrophic factor, NGF. Although our Western blot analysis failed to consistently identify the mature 13.5-kDa molecular weight NGF, it is generally expected that the fate of the NGF precursor protein will yield the mature NGF to stimulate neuronal survival and differentiation (13). Nevertheless, recent reports have suggested that NGF precursor protein may also possess proapoptotic activity, a function that appears independent of the mature NGF (14, 22, 26). Thus the precise role of this protein in our model of hibernating myocardium is somewhat unclear and will require further investigation.

Partial sympathetic denervation as a substrate for sudden death. The results of the present study also extend the potential relevance of the inhomogeneity in sympathetic innervation after infarction (40, 46) to viable dysfunctional myocardium without infarction. In some (3), but not all studies (24), of transmural infarction, sympathetic denervation extends beyond the scar due to ischemically mediated disruption of nerves that course through the subepicardium. Thus denervated but viable myocardium after infarction can contribute to heterogeneity of sympathetic innervation. This is hypothesized to be an important substrate for arrhythmogenesis and SCD (40, 46). The subsequent heterogeneity in action potential duration is further exacerbated by hypersensitivity of denervated myocardium to catecholamines, ischemically mediated electrical remodeling, nerve sprouting at the periphery of denervated myocardium, and episodes of sympathetic activation (40, 46). Our results suggest that this same arrhythmogenic substrate occurs in pigs with hibernating myocardium, with heterogeneity in sympathetic innervation exacerbated by both partial denervation and nerve sprouting. This mechanism of arrhythmia is further supported by the fact that spontaneous SCD in pigs with hibernating myocardium is the result of ventricular tachycardia degenerating into ventricular fibrillation (7), which is always preceded by sinus tachycardia indicative of transient sympathetic activation (38). Thus neither infarction nor complete denervation appears to be required to result in an arrhythmogenic substrate.

The specific substrate that results in SCD associated with hibernating myocardium remains elusive. Our previous study (7) demonstrated that physiology (e.g., coronary angiographic anatomy, myocardial perfusion, and left ventricular systolic dysfunction) was similar in animals with hibernating myocardium before SCD compared with survivors. In addition, regional sympathetic nerve dysfunction has been present in all animals with hibernating myocardium that have been assessed as evidenced by impaired norepinephrine uptake (17, 29, 30) or blunted functional responses to sympathetic stimulation (36). We originally hypothesized that the extent of sympathetic denervation and/or nerve sprouting would differ in animals that developed SCD and pursued a molecular approach since it would be impractical to image myocardial sympathetic innervation before SCD. Nevertheless, the similarity of reductions in functional markers of sympathetic innervation coupled with the similar upregulation in NGF precursor protein and GAP-43 suggest that the alterations in sympathetic innervation and nerve sprouting were probably similar. Thus, although partial regional sympathetic denervation is present in animals that develop SCD, the extent of denervation and nerve sprouting is no different from survivors. This further suggests that sympathetic remodeling in hibernating myocardium is not an independent mediator of SCD. However, this does not discount the possibility that sympathetic remodeling may, in combination with other factors, contribute to electrical instability.

Although the present study was performed in growing pigs with a chronic extrinsic coronary stenosis, there are ample data to suggest that our results are applicable to clinical coronary artery disease. First, the physiological sequelae of a chronic stenosis in pigs, i.e., regional myocardial dysfunction with reduced resting perfusion and a critical limitation in flow reserve, is identical to the physiology of patients with hibernating myocardium (6, 8, 21). Furthermore, we have documented that the mechanism of spontaneous SCD in these animals is ventricular tachycardia degenerating into ventricular fibrillation without acute coronary occlusion, which mirrors the most common mechanism of SCD in patients with ischemic cardiomyopathy (7). Finally, clinical investigations have clearly shown regional reductions in norepinephrine tracer uptake with both MIBG (23) and HED (4, 20) in the presence of ischemic heart disease without infarction, as we have shown in pigs with hibernating myocardium. However, further studies will be necessary to determine whether similar molecular alterations are also present from clinically derived samples.

Methodological limitations. Although we did not find any differences in regional expression of sympathetic nerve or nerve sprouting proteins between animals with SCD compared with survivors, our analysis was obviously limited to five candidate neural markers, and more extensive proteomic approaches (i.e., 2-dimensional differential in-gel electrophoresis) might yield additional insights into differential substrate factors (37). By design our analysis included tissue samples from animals that experienced SCD, which could have been susceptible to postmortem changes. Furthermore, samples were harvested at different time-points and stored for variable periods of time, which could have differentially affected degradation and protein integrity. Nevertheless, our methodology also included sampling of the remote myocardium, which was subject to the same delays in sampling and storage as hibernating myocardium, and our primary analysis was on these paired samples. Furthermore, our results showed that values from the remote regions were similar to control samples (Fig. 1), and we have previously shown that histological analysis of myocardium from these animals was similar to electively terminated pigs (7). Therefore, we do not believe that this approach has significantly influenced our results. Finally, we also have not
evaluated potential alterations in parasympathetic function nor the intriguing possibility of the arrhythmogenic potential of a mismatch between presynaptic and postsynaptic sympathetic function (i.e., mismatch between the β-adrenergic receptor antagonist 11C-CGP-12177 and HED imaging) (5). Further studies will be required to identify whether these factors differ in animals with SCD compared with survivors.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: S.F.F., V.O., and J.A.F. performed experiments; S.F.F., V.O., and J.A.F. analyzed data; S.F.F., V.O., J.M.C., and J.A.F. interpreted results of experiments; S.F.F., V.O., and J.A.F. prepared figures; S.F.F., V.O., and J.A.F. drafted manuscript; S.F.F., J.M.C., and J.A.F. edited and revised manuscript; S.F.F., V.O., J.M.C., and J.A.F. approved final version of manuscript; J.M.C. and J.A.F. conception and design of research.

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