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Original Article

Reduced epicardial vagal nerve density and impaired vagal control in a rat myocardial infarction–heart failure model☆,☆☆

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ABSTRACT

Background: Autonomic remodeling, characterized by sympathetic activation and vagal withdrawal, contributes to heart failure (HF) progression. However, the exact mechanism(s) responsible for vagal withdrawal in HF remain(s) unclear, and whether HF causes epicardial autonomic nerve remodeling is unknown.
Methods and results: Myocardial infarction (MI) was produced in 14 Sprague–Dawley rats, and 10 sham surgery rats served as the control. MI–HF was confirmed 2 months after the surgery by echocardiography and hemodynamic measurement. Cervical vagal nerve stimulation was delivered to examine the heart rate slowing effect. Whole heart acetylcholinesterase histochemistry was used to examine the epicardial autonomic nerve remodeling at dorsal ventricles (remote from the infarcted area). Compared with the control animals, the same vagal nerve stimulation had less heart rate slowing effect in MI–HF group. Both epicardial nerve bundle length-density (2.56 ± 0.60 μm/mm^2 versus 1.68 ± 0.46 μm/mm^2, $P=.001$) and branching point-density (1.24 ± 0.25 points/mm^2 versus 0.66 ± 0.18 points/mm^2, $P<.001$) were lower in MI–HF rats. The chemically stained epicardial nerve bundles contain both sympathetic (tyrosine hydroxylase positive) and vagal (choline acetyltransferase positive) fibers. However, within the stained nerve bundle, the chemical color corresponds mainly with the vagal fibers.
Conclusions: Whole heart acetylcholinesterase histochemistry revealed a decreased ventricular epicardial vagal nerve density in MI–HF rats, which may contribute to impaired cardiac vagal control in HF.

1. Introduction

Heart failure (HF) is a leading cause of morbidity and mortality in the United States, with approximately 5.7 million Americans ≥20 years of age having HF [1]. The autonomic nervous system (sympathetic and vagal) plays an important role in regulating normal cardiac function as well as in promoting pathological cardiac remodeling in various conditions, especially in HF. It is known that HF leads to autonomic dysfunction, shifting the balance from normal vagal dominance to sympathetic dominance in HF (i.e., sympathetic activation and vagal withdrawal), which in turn contributes to HF progression [2]. The role of sympathetic excitation in HF is highlighted by the beneficial effects of sympathetic inhibition with β-blocker therapy [3,4]. The role of cardiac vagal tone in HF has also been recognized. Decreased cardiac vagal tone is correlated with increased mortality in HF [5,6]. Recent studies from our group [7] as well as others [8,9] have demonstrated that enhancing vagal tone by vagus nerve stimulation is beneficial in improving autonomic balance and in treating HF.

While it is known that vagal activity is reduced in HF, the mechanisms responsible for such abnormal cardiac vagal activity in HF remain unclear. There are early reports that identified potential defect in vagal postganglionic transmission in HF [10], while others found reduced postganglionic neuron excitability in HF [11]. A recent report indicated altered neurotransmission response of cardiac vagal neurons in the brain stem [12]. However, contradicting data exist regarding cardiac vagal innervation in HF. There are reports of epicardial neuron hypertrophy and increased vagal nerve density in HF [13,14]. In contrast, reduced vagal nerve density in the ventricles was also reported [15], and there is evidence of increased heterogeneity of vagal innervation in HF [15]. The increased heterogeneity could be one of the possible reasons leading to inconsistent findings when vagal nerve density was examined microscopically.

Thus, it would be desirable if cardiac vagal nerves could be grossly examined in whole hearts. Acetylcholinesterase (AChE) histochemical staining has been used to label cholinergic nerve fibers [16], and whole heart chemical staining of autonomic nerves that express AChE has been described in various animals and human hearts [17,18]. Whole heart histochemical labeling of epicardial vagal nerve network would reveal a more complete picture of cardiac autonomic remodeling

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in HF. Accordingly, in this study, we utilized whole heart AChE histochemical staining to examine the epicardial vagal nerve remodeling in HF, and we found reduced vagal nerve density associated with impaired vagal control in a rat myocardial infarction (MI)–HF model.

2. Materials and methods

The study was approved by the Institutional Animal Care and Use Committee at New York Institute of Technology College of Osteopathic Medicine and is in compliance with the "Guide for the Care and Use of Laboratory Animals."

2.1. Animal model and study design

A rat MI–HF model was used in this study. Adult (12-week-old) Sprague–Dawley rats (Harlan Laboratories, Indianapolis, IN, USA) of both sexes were enrolled. MI was produced by ligation of the left descending coronary artery (n=14), as described in our previous reports [19]. Sham operated animals served as the control (n=10). Two months after the surgery, echocardiography was performed to determine left ventricular (LV) chamber dimension and cardiac function as well as MI size just before terminal study. At terminal study, LV function was assessed by LV catheterization. Heart rate response to cervical vagal nerve stimulation was examined. Finally, whole hearts were processed for histochemical staining and subsequently immunohistochemical staining.

2.2. Echocardiographic measurements

As previously described [19], echocardiographic data were acquired from all animals using a GE Vivid 7 Dimension System (GE Vingmed Ultrasound, Horten, Norway). In brief, rats were anesthetized with 1.5% isofluorane and placed on an isothermal pad maintained at 40°C. Two-dimensional echocardiograms were obtained from LV short-axis and long-axis views. MI size was determined from the short-axis view by measuring the length of the MI as a percentage of the LV circumference. Two dimensionally targeted M-mode echocardiograms were used to measure the LV dimensions in systole and diastole. The following parameters were measured: anterior wall thickness in end-diastole and end-systole, LV diastolic and systolic internal diameters, posterior wall thickness in end-diastole and end-systole, and LV fractional shortening.

2.3. Cardiac hemodynamic measurements

LV hemodynamics were obtained from all 10 control animals and 11 of the 14 MI–HF animals with a pressure catheter (Transonic Scisense, London, Ontario, Canada) as previously described [19]. Briefly, the right carotid artery was isolated and cannulated with a 1.9-F Scisense pressure catheter under isofluorane anesthesia. The tip of the catheter was advanced through the aorta into the LV. The following parameters were measured: heart rate, LV peak systolic pressure, LV end-diastolic pressure, and positive/negative change in pressure over time ($\pm$dP/dt). The data were acquired and analyzed with the use of Labscribe software (iWorx Systems, Dover, NH, USA).

2.4. Cervical vagus nerve stimulation

The right cervical vagus nerve was isolated, and a bipolar hook electrode was placed around the vagus nerve. The electrode was connected to a current isolator (model A385; WPI, Sarasota, FL, USA), coupled with a programmable eight-channel stimulator (Master-8; AMPI, Jerusalem, Israel). The following parameters were used for vagal nerve stimulation: frequency 30 Hz, pulse width 0.1 ms, and intensity 1 mA. Standard surface electrocardiogram (ECG) lead II was recorded before and during the nerve stimulation to monitor the cardiac cycle length changes. The ECG signals were recorded using Powerlab data acquisition systems (ADInstruments, Colorado Springs, CO, USA). Vagus nerve stimulation was tested in all 10 control rats and 9 MI–HF animals.

2.5. Whole heart preparation and AChE histochemical staining

Under deep anesthesia with 5% isoflurane and oxygen inhalation, all rats underwent a median laparotomy and thoracotomy. To prevent blood clots formation inside the heart, 1000 U of heparin were injected into the inferior vena cava. The whole heart was then removed, perfused with cold phosphate-buffered saline (PBS; Sigma, St. Louis, MO, USA) supplemented with high potassium (~30 mM) to arrest the heart in diastole, and rinsed in PBS at 4°C.

The hearts were prepared as previously reported [17,18], with minor modifications. To keep the heart in a natural filling status, the chambers were filled with warm 2% agarose (Research Products International Corp., Mount Prospect, IL, USA) solution in PBS. After the gel was solidified in 4°C PBS, the heart was trimmed to remove pericardium, lungs, and other noncardiac tissue (Fig. 1A). The prepared hearts were prefixed for 30 min in 4% paraformaldehyde (PFA) solution in PBS at 4°C. Then hearts were rinsed for 30 min at 4°C in washing media, which consist of PBS, hyaluronidase (0.5 mg/100 ml), and tetraisopropylphosphoramide (0.5 mmol/L, which inhibits pseudocholinesterase).

Whole heart histochemical staining for AChE was performed as described previously [17,18]. The hearts were incubated in the AChE staining medium at 4°C overnight (~12 h). The composition of AChE staining medium was as follows (in mmol/L): Na acetate (60), acetylthiocholine iodide (2), Na citrate (15), $CuSO_4$ (3), $K_3Fe(CN)_6$ (0.5), and tetraisopropylphosphoramide (0.5). The staining medium was supplemented by Triton-X 100 (1%) and hyaluronidase (0.5 mg/100 ml). All chemicals were from Sigma, St. Louis, MO, USA. The epicardial nerve bundles were stained in brown (Fig. 1B). After staining, hearts were preserved in 4% PFA for further analysis.

2.6. Analysis of epicardial nerve density

AChE-stained hearts were examined using a Motic K-400L stereo microscope (Motic Instruments, Inc., Richmond, BC, Canada) equipped with an Olympus DP70 digital camera (Olympus America, Inc., Center Valley, MA, USA). Digital images for quantitative assessment of the epicardial nerve bundles were obtained under the medium-power magnification (×25). To avoid MI scar area, the pattern of epicardial autonomic innervation was digitized only on the dorsal ventricles (remote from the infarct region). A standardized field in the same well-recognized anatomical area of the heart (shown later in "Results") was then morphometrically analyzed in all hearts using Image-Pro Analyzer 7.0 software (Media Cybernetics, Bethesda, MD, USA). The network of all AChE-stained nerves within that field was digitally traced. The total nerve length and number of branch points were determined, and the nerve length density and the branch points density were calculated.

2.7. Immunofluorescence staining for neurofilament M (NF-M), tyrosine hydroxylase (TH) and choline acetyltransferase (ChAT)

After the whole heart analysis, eight hearts from each group were transversely cut into parallel slices with a multiblade guillotine, and one midventricular slice per heart (at the level of the papillary muscles) was cryopreserved and embedded in tissue-freezing medium (TFM-5; Triangle Biomedical, Durham, NC, USA) for cryosectioning. Eight-micrometer-thick serial sections were cut from each midventricular slice using a Leica CM1900 cryostat (Leica Microsystems, Nussloch. Germany) and placed on histological slides for immunofluorescence staining.

In one group of slides, three adjacent serial cryosections from the same heart were separately immunostained with antibodies against either NF-M (an axonal maker), TH (a sympathetic marker), or ChAT (a cholinergic marker), with the purpose to identify the sympathetic and vagal components of the AChE-stained epicardial autonomic