Low-level vagus nerve stimulation upregulates small conductance calcium-activated potassium channels in the stellate ganglion

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BACKGROUND Small conductance calcium-activated potassium (SK) channels are responsible for afterhyperpolarization that suppresses nerve discharges.

OBJECTIVES To test the hypothesis that low-level vagus nerve stimulation (LL-VNS) leads to the upregulation of SK2 proteins in the left stellate ganglion.

METHODS Six dogs (group 1) underwent 1-week LL-VNS of the left cervical vagus nerve. Five normal dogs (group 2) were used as controls. SK2 protein levels were examined by using Western blotting. The ratio between SK2 and glyceraldehydes-3-phosphate-dehydrogenase levels was used as an arbitrary unit (AU).

RESULTS We found higher SK2 expression in group 1 (0.124 ± 0.049 AU) than in group 2 (0.085 ± 0.031 AU; $P < .05$). Immunostaining showed that the density of nerve structures stained with SK2 antibody was also higher in group 1 ($11{,}546 \pm 7{,}271$ $\mu m^2/mm^2$) than in group 2 (5321 ± 3164 $\mu m^2/mm^2$; $P < .05$). There were significantly more ganglion cells without immunoreactivity to tyrosine hydroxylase (TH) in group 1 ($11.4\% \pm 2.3\%$) than in group 2 ($4.9\% \pm 0.7\%$; $P < .05$). The TH-negative ganglion cells mostly stained positive for choline acetyltransferase ($95.9\% \pm 2.8\%$ in group 1 and $86.1\% \pm 4.4\%$ in group 2; $P = .10$). Immunofluorescence confocal microscopy revealed a significant decrease in the SK2 staining in the cytosol but an increase in the SK2 staining on the membrane of the ganglion cells in group 1 compared to group 2.

CONCLUSIONS Left LL-VNS results in the upregulation of SK2 proteins, increased SK2 protein expression in the cell membrane, and increased TH-negative (mostly choline acetyltransferase-positive) ganglion cells in the left stellate ganglion. These changes may underlie the antiarrhythmic efficacy of LL-VNS in ambulatory dogs.

KEYWORDS Autonomic nervous system; Vagus nerve stimulation; Stellate ganglion; Small conductance calcium-activated potassium channel; Western blot

ABBREVIATIONS **AU** = arbitrary unit; **ChAT** = choline acetyltransferase; **LL-VNS** = low-level vagus nerve stimulation; **LSG** = left stellate ganglion; **SK** = small conductance calcium-activated K^+; **TBST** = Tris buffered saline with Tween 20; **TH** = tyrosine hydroxylase

(Heart Rhythm 2013;10:910–915)

Introduction

Small conductance calcium-activated K^+ (SK) channels are widely expressed in nerve structures.[1] The SK channels are known to be sensitive to intracellular calcium and are primarily responsible for the slow afterhyperpolarization that hyperpolarizes neurons and reduces the frequency of neuronal discharges.[1,2] Apamin, a Western honeybee toxin that specifically inhibits SK channels, reduces the slow afterhyperpolarization, increases neuronal discharges, and breaks down the burst-termination patterns.[2,3] Because similar burst-termination patterns are observed in the left stellate ganglion (LSG) of ambulatory dogs,[4] it is possible that SK channels also play a role in regulating sympathetic outflow.

This manuscript was processed by a guest editor. This study was supported in part by National Institutes of Health grants P01HL78931, R01HL78932, R01HL71140, and R21HL106554; a Heart Rhythm Society Fellowship in Cardiac Pacing and Electrophysiology (to Dr Shen); R01HL85844, R01HL85727; VA Merit Review Grant I01BX000576 (to Dr Chiamvimonvat); and a Medtronic-Zipes Endowment (Dr Chen) and an Indiana University Health-Indiana University School of Medicine Strategic Research Initiative. Cyberonics Inc donated the neurostimulator used in the present study. Dr Chen is a consultant to Cyberonics. **Address reprint requests and correspondence:** Dr Peng-Sheng Chen, Krannert Institute of Cardiology and the Division of Cardiology, Department of Medicine, 1800 North Capitol Avenue, E475, Indianapolis, IN 46202. E-mail address: chenpp@iupui.edu.

1547-5271/$-see front matter http://dx.doi.org/10.1016/j.hrthm.2013.01.029

Manipulating SK channel expression may be a novel approach to regulating sympathetic outflow and controlling cardiac arrhythmias. We[5] recently showed that continuous left low-level vagus nerve stimulation (LL-VNS) suppressed LSG nerve activity and increased tyrosine hydroxylase (TH)-negative nerve structures in the LSG. These changes are associated with a reduced incidence of paroxysmal atrial tachyarrhythmias, including atrial fibrillation. The increase in TH-negative cells suggests that LL-VNS can cause adrenergic neurons to lose their ability to produce catecholamines. However, whether or not these TH-negative cells undergo phenotypic switching to cholinergic ones, that is, choline acetyltransferase (ChAT) positivity, remains unknown. The purpose of the present study was to test the hypothesis that LL-VNS induces significant LSG neural remodeling, including upregulation of SK proteins, increased SK protein trafficking to the cell membrane, and increased percentage of TH-negative cells. Immunostaining was used to test the hypothesis that most of the TH-negative ganglion cells stain positive for ChAT.

Methods

Chronic LL-VNS in ambulatory dogs

The study protocol was approved by the Institutional Animal Use and Care Committee and conforms to the *Guide for the Care and Use of Laboratory Animals*. Six male adult mongrel dogs (group 1) were used for LL-VNS. Under isoflurane inhalation general anesthesia, an incision was made on the left anterior side of the neck. A bipolar pacing lead and an anchor lead were placed around the left cervical vagus nerve (Figure 1) and connected to a subcutaneously positioned Cyberonics Demipulse neurostimulator (Cyberonics Inc, Houston, TX). The wound was closed, and the dog was allowed to recover for 1 week. We then defined the cardiac threshold by stimulating the left cervical vagus nerve at 15 Hz and 500-μs pulse duration. The stimulus amplitude (mA) that elicited an abrupt decrease in heart rate by $>20\%$ from baseline was defined as the stimulation threshold. We then programmed the neurostimulator output to 1 mA below the stimulation threshold[5,6] and confirmed that this stimulus strength (1.1 ± 0.4 mA; range 0.5–3 mA) did not cause any heart rate changes. The stimulator was programmed to 60-second ON and 12-second OFF for 1 week while the dogs were ambulatory. The dogs were then euthanized, and the LSG was harvested for protein analyses and histological examinations. An additional 5 normal dogs (group 2) were used as controls. We did not perform any nerve recordings in these dogs because surgical implantation of recording wires may cause irritation and potentially change the underlying histology and protein expression.

Western blotting

The rostral half of the LSG was used for the Western blot to quantify the amount of protein. We loaded 10 μg of homogenates of the LSG on a sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The blot was probed with an anti-subtype 2 SK (SK2) polyclonal antibody (1:600, Abcam, Cambridge, MA). The SK channels have 3 subtypes.[1,7] Among them, SK1 is insensitive to apamin.[8] SK3 is intermediately sensitive to apamin and is expressed in very low levels in atria and ventricles.[9] In the sensory ganglia, SK3 is expressed only in satellite glial cells and not in ganglion neurons.[10] On the other hand, SK2 is known to play an important role in regulating nerve activities of sympathetic ganglions.[11] Therefore, we chose to focus our efforts in studying SK2 expression in stellate ganglia. Antibody-binding protein bands were visualized by ^{125}I-protein A and quantified with a Bio-Rad Personal FX phosphorimager, not by densitometry of the film. We adjusted the exposure to

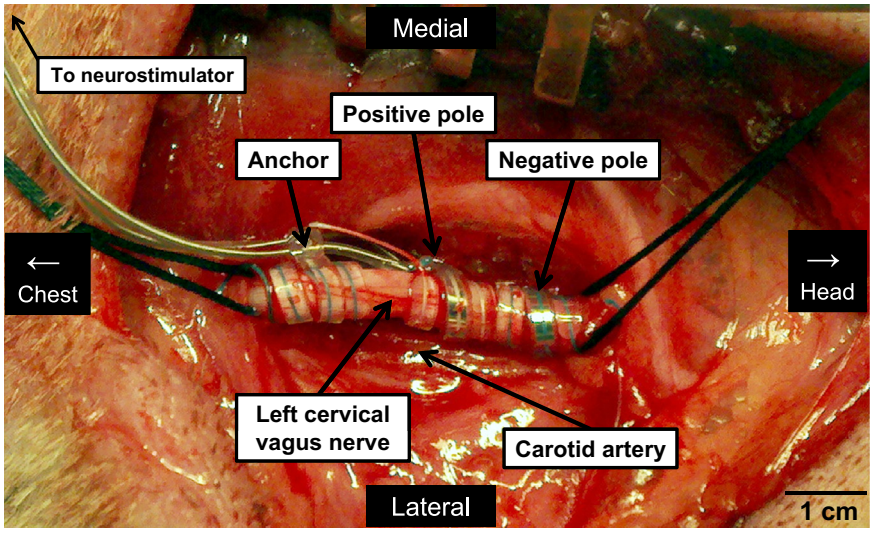

Figure 1 Implantation of electrodes on left cervical vagus nerve. A bipolar pacing lead and an anchor lead were placed around the left cervical vagus nerve and connected to a subcutaneously positioned Cyberonics Demipulse neurostimulator. The electrode orientation ensures that the negative pole is cranial to the positive pole, which is the configuration used clinically for the implanted Cyberonics stimulators.