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Decreased adrenoceptor stimulation in heart failure rats reduces NGF expression by cardiac parasympathetic neurons

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ABSTRACT

Postganglionic cardiac parasympathetic and sympathetic nerves are physically proximate in atrial cardiac tissue allowing reciprocal inhibition of neurotransmitter release, depending on demands from central cardiovascular centers or reflex pathways. Parasympathetic cardiac ganglion (CG) neurons synthesize and release the sympathetic neurotrophin nerve growth factor (NGF), which may serve to maintain these close connections. In this study we investigated whether NGF synthesis by CG neurons is altered in heart failure, and whether norepinephrine from sympathetic neurons promotes NGF synthesis. NGF and proNGF immunoreactivity in CG neurons in heart failure rats following chronic coronary artery ligation was investigated. NGF immunoreactivity was decreased significantly in heart failure rats compared to sham-operated animals, whereas proNGF expression was unchanged. Changes in neurochemistry of CG neurons included attenuated expression of the cholinergic marker vesicular acetylcholine transporter, and increased expression of the neuropeptide vasoactive intestinal polypeptide. To further investigate norepinephrine's role in promoting NGF synthesis, we cultured CG neurons treated with adrenergic receptor (AR) agonists. An 82% increase in NGF mRNA levels was detected after 1 h of isoproterenol (β-AR agonist) treatment, which increased an additional 22% at 24 h. Antagonist treatment blocked isoproterenol-induced increases in NGF transcripts. In contrast, the α-AR agonist phenylephrine did not alter NGF mRNA expression. These results are consistent with β-AR mediated maintenance of NGF synthesis in CG neurons. In heart failure, a decrease in NGF synthesis by CG neurons may potentially contribute to reduced connections with adjacent sympathetic nerves.

1. Introduction

Postganglionic cardiac parasympathetic and sympathetic nerves normally interact reciprocally in modulating heart rate, and presynaptic norepinephrine (NE) release by sympathetic nerves, or acetylcholine (ACh) release by parasympathetic nerves, are important regulatory mechanisms for heart rate control in the effector pacemaker and conduction regions (Vanhoutte and Levy, 1980; Wetzel and Brown, 1985; Loiacono and Story, 1986; Levy, 1990; Yang and Levy, 1992). With the progression of congestive heart failure (CHF), however, autonomic disturbances occur including sympathetic overactivity and reduced vagal tone (Eckberg et al., 1971; Porter et al., 1990; Binkley et al., 1991; Kinugawa and Dibner-Dunlap, 1995; Dunlap et al., 2003; Ramchandra et al., 2009; Ondicova and Mravec, 2010; Bibevski and Dunlap, 2011). Functionally, sympathetic nerves in CHF convert from a balanced NE synthesis, release, re-uptake system to one that predominantly releases NE, resulting in excessive myocardial stimulation (and eventual fatigue) and catecholamine toxicity (Bohm et al., 1995; Backs et al., 2001; Kreusser et al., 2008). Reduced vagal activity in CHF has been correlated with attenuated heart rate variability and therefore elevated risk of sudden cardiac death (La Rovere et al., 1998; Lombardi and Mortara, 1998; Lechat et al., 2001). Attenuated vagal tone may also manifest as reduced cardiac postjunctional innervation that will result in fewer inhibitory connections on co-projecting sympathetic fibers (Du et al., 1990; Azevedo and Parker, 1999; Dunlap et al., 2003; Nihei et al., 2005; Bibevski and Dunlap, 2011; Deneke et al., 2011).

The mechanism for altered autonomic axo-axonal communication in heart failure is unclear. However in control rats, parasympathetic cardiac ganglion (CG) neurons synthesize the neurotrophin nerve growth factor (NGF) (Hasan and Smith, 2000, 2009); since the mature NGFβ moiety exerts a powerful trophic effect on sympathetic neurons, its release from CG neurons may be critical for maintaining axo-axonal appositions and hence for maintaining parasympathetic inhibition of sympathetic function in CHF. In turn, impulse activity from sympathetic neurons promotes NGF synthesis by CG neurons (Hasan and Smith, 2009). In contrast to mature NGFβ, the precursor pro-form of NGF

Abbreviations: CG, Cardiac ganglion; NGF, Nerve growth factor; AR, Adrenergic agonist; NE, Norepinephrine; ACh, Acetylcholine; CHF, Congestive heart failure; CAL, Coronary artery ligation; HR, Heart rate; VAChT, Vesicular acetylcholine transporter; VMAT, Vesicular monoamine transporter-2; VIP, Vasoactive intestinal polypeptide; GAPDH, Glyceraldehyde 3-phosphate; IsoP, Isoproterenol; Prop, Propranolol; PhE, Phenylephrine; Phent, Phentolamine.

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(proNGF) is involved in the promotion of nerve pruning, degeneration and apoptosis of sympathetic neurons (Lobos et al., 2005; Bierl and Isaacson, 2007; Al-Shawi et al., 2008), and proNGF expression by CG neurons is not directly regulated by adjacent sympathetic nerve impulse activity (Hasan and Smith, 2009). The relative expression of NGFβ to proNGF may determine the extent of sympathetic–parasympathetic axo-axonal associations in CHF; evaluating NGFβ and proNGF protein expression in CG neurons from CHF rats is therefore a central focus of this study.

Although we have previously shown that impulse activity, and not the physical presence of sympathetic nerves, is necessary for regulating NGF synthesis by parasympathetic nerves (Hasan and Smith, 2009), whether NE is directly responsible has not been determined. In several cell types, activation of adrenergic receptors (ARs), primarily β-ARs, has been shown to augment NGF synthesis (Dal Toso et al., 1988; Carswell et al., 1992; Hayes et al., 1995; Semkova et al., 1996; Colangelo et al., 1998; Culmsee et al., 1999; Samina Riaz and Tomlinson, 2000; Colangelo et al., 2004). We hypothesize that adrenergic heteroreceptors on CG neurons will similarly promote NGF synthesis. We therefore evaluate, in an *in vitro* CG dissociated neuronal system, the effects of adrenergic agonists on NGF synthesis by CG neurons.

In addition to regulating NGF expression, we have previously shown that another consequence of cardiac sympathectomy is a decrease in the cholinergic phenotype of rat CG neurons (Hasan and Smith, 2009). We propose that reduced cross-talk between autonomic neurons in CHF will similarly promote alterations in the neurochemistry of CG neurons from CHF animals. We examine therefore cholinergic, peptidergic and adrenergic markers in CG from CHF animals.

Together these studies attempt to identify alterations in NGF expression and neurochemistry within CG neurons from heart failure rats. The role of AR-mediated mechanisms in regulating NGF synthesis by CG neurons is also evaluated. Understanding the mechanisms involved in disruption of cardiac autonomic nerve interactions is crucial for future development of targeted therapies to reverse dysfunctional autonomic activity in the progression of CHF.

2. Materials and methods

2.1. Coronary artery ligation

Adult male Sprague–Dawley rats (60–70 days postnatal, ~225 g, Harlan Breeding Laboratories, Indianapolis, IN) were anesthetized by intraperitoneal injection of 60 mg/kg ketamine, 8 mg/kg xylazine, and 0.4 mg/kg atropine. Rats were intubated, respired mechanically, and a left lateral thoracotomy performed as in our previous studies (Hasan et al., 2006; Wernli et al., 2009). The left anterior descending coronary artery was ligated (6–0 silk suture with an atraumatic needle) approximately 8 mm distal to its emergence beneath the left atrium (n = 20) (Hasan et al., 2006; Wernli et al., 2009). This elicited a visible infarct corresponding to the ischemic region of the myocardium in the coronary artery ligation (CAL) group. Sham surgery (SHAM, n = 21) involved similarly passing a suture around the artery but leaving it untied for a comparable period. The incision was closed with 4–0 suture and the animals were allowed to recover. After 15 ± 2 weeks, a subgroup of rats (n = 7) underwent *in vivo* hemodynamic measurements while the rest of the animals were sacrificed for tissue harvest under pentobarbital (60 mg/kg, i.p.) anesthesia. All experimental manipulations were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center and conformed with the **Guide for the Care and Use of Laboratory Animals** published by the US National Institutes of Health (8th edition, revised 2011).

2.2. In vivo hemodynamics

A subset of rats (see Table 1) were anesthetized (1.5 g/kg urethane, i.p) and the cervical right carotid artery exposed prior to its rostral bifurcation. An intraventricular conductance catheter (SPR-838, Millar Instruments, Houston, TX), sensitive to pressure and volume, was inserted from the right carotid artery into the left ventricle and pressure-volume recordings were made using PowerLab with LabChart software (ADInstruments, Colorado Springs, CO). A femoral artery was cannulated for measuring blood pressure and heart rate (HR) using a blood pressure transducer (MLT0699, ADInstruments). Body temperature was monitored using a rectal probe and digital monitor (BAT-12, Physitemp, Clifton, NJ), and maintained at 37 °C using a heating pad.

2.3. Immunohistochemistry

The base of the heart including atria and major cardiac vessels, which includes the cardiac ganglia, was removed, and embedded in tissue freezing medium (Triangle Biomedical Sciences, NC, USA), frozen on dry ice and stored at −80 °C. Sets of 15 μm sections perpendicular to the basal–apical axis of the atria were collected as consecutive sections onto 5 slides as a stepped series. Sections were collected beginning ~2 mm superior from the point of entry of the aorta into the heart and continued until at least two cardiac ganglia had been identified under differential interference microscopy and completely sectioned. Ganglia were generally identified in epicardial fat pads or embedded within the atrial walls. Sections were incubated with primary antibody overnight at 4 °C, followed by 90 min incubation with cy3-conjugated secondary antibodies as previously (Hasan and Smith, 2000, 2009). Combinations of primary and secondary antibodies were evaluated to exclude the possibility of antibody cross-reactivity. Antibodies were raised against the cholinergic marker vesicular acetylcholine transporter (VAChT, goat IgG, 1:100, Chemicon), the adrenergic marker vesicular monoamine transporter-2 (VMAT, rabbit IgG, 1:100, Chemicon), vasoactive intestinal polypeptide (VIP, rabbit IgG, 1:250, Immunostar), proNGF (rabbit IgG, 1:100, Chemicon) and mature NGFβ (rabbit IgG, 1:100, Santa Cruz). Antibody omission, pre-adsorption to the relevant antigen, and substitution of naïve immunoglobulin for the primary antibody served as negative controls.

2.3.1. Quantitation of immunoreactive neurons

Within every 2nd section in one stepped series through the entire ganglion, all neurons displaying immunofluorescence and a nucleus whose uppermost membrane boundary was contained within the section, were counted and divided by the total number of neuronal nuclei in that section. Total neuron counts per ganglion within sampled sections ranged from 50 to 250 neurons. Percentages of stained neurons were averaged to obtain a mean value per ganglion (Hasan and Smith,

Table 1
Hemodynamic measurements from rats following coronary artery ligation (CAL) or sham surgery.

	BL HR (bpm)	MAP (mm Hg)	SP (mm Hg)	PP (mm Hg)	EF (%)	Ea mm Hg/μl
SHAM (n = 10)	278 ± 15 (10)	82 ± 1 (10)	113 ± 2 (10)	47 ± 3 (10)	100 ± 1 (n = 5)	1.21 ± 0.16 (n = 4)
CAL (n = 10)	274 ± 11 (10)	71 ± 6 (10)*	93 ± 7 (10)*	34 ± 3 (10)*	69 ± 2 (n = 3)*	2.51 ± 0.41 (n = 3)*

Resting heart rate (RHR), mean arterial pressure (MAP); systolic pressure (SP); pulse pressure (PP); ejection fraction (EF); arterial elastance (Ea). Data are mean ± SEM, n in parenthesis; *$p < 0.05$.