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Changes in parasympathetic system in medulla oblongata in male pigs in the course of tachycardia-induced cardiomyopathy

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ABSTRACT

Background: Autonomic imbalance constituting a fundamental feature of heart failure (HF) has been assessed mainly at the periphery. Changes in the functioning of autonomic centers in the brain remain unclear. We investigated the molecular elements of parasympathetic system, i.e. α7 nicotinic acetylcholine receptor (α7nAChR) and enzymes metabolizing acetylcholine (acetylcholinesterase, AChE, choline acetyltransferase, ChAT) in medulla oblongata (MO) of male pigs with chronic tachycardia-induced cardiomyopathy.
Methods: The mRNA levels of AChE, ChAT, α7nAChR and X-box binding protein 1 (spliced form, XBP1s) in MO were analyzed using qPCR, AChE and ChAT activities using spectrophotometry, proteasome activity using fluorometry, and the protein level of α7nAChR using Western blotting.
Results: The development of systolic HF was accompanied by an increase in circulating catecholamines, a decrease in the AChE and α7nAChR mRNA in MO, an increase in AChE activity (all $p < 0.05$), and no change in either the mRNA or activity of ChAT. Both circulating catecholamine levels and AChE activity were inversely related to systolic function of left myocardial ventricle ($p < 0.05$). The level of α7nAChR protein in MO and its cytoplasmatic fraction were higher in pigs with moderate and severe HF as compared to the other animals ($p < 0.01$). There was no difference in proteasome activity in MO between diseased and healthy animals, whereas the XBP1s mRNA decreased during HF progression ($p < 0.05$).
Conclusions: Molecular elements of parasympathetic system are changed within the medulla oblongata during the progression of systolic non-ischemic heart failure in male pigs, indicating a functional link between MO and heart in HF.

1. Introduction

Autonomic imbalance constitutes a fundamental pathophysiological feature of heart failure (HF) (Jankowska et al., 2006; Olshansky et al., 2008). The role of an augmented sympathetic drive in the progression of HF has been established (Benedict et al., 1994), and β-blockers have become a milestone in standard HF therapy (Packer et al., 1996; MERIT-HF Study Group, 1999). Diminished parasympathetic drive has been recognized as an integral component of autonomic imbalance both in a canine model of HF (Binkley et al., 1991; Ishise et al., 1998) and in patients with systolic HF (Binkley et al., 1991).

Clinical evaluation of parasympathetic activity in humans with HF is limited to the assessment of peripheral autonomic system (Binkley et al., 1991; Kinugawa and Dibner-Dunlap, 1995), and the majority of available measures assess the parasympathetic modulation of the myocardial sinoatrial node, e.g. measuring heart rate variability (HRV) (Standards of heart rate variability, 1996; Bauer et al., 2008). Importantly, most of these parameters are indirect measures, and do not provide an insight into the parasympathetic system within central nervous system (CNS). Although it has been postulated that the autonomic imbalance within CNS orchestrates the changes seen in the periphery in patients with HF (Bibevski and Dunlap, 1999; Dunlap et al., 2003; Jankowska et al., 2006), the available evidence

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on the derangements of the parasympathetic system within CNS occurring during the natural history of HF remains scarce.

Preganglionic parasympathetic cardiac neurons playing a critical role in the autonomic control of cardiac function are located mainly in medulla oblongata (MO) (Wang et al., 2001). Acetylcholine (ACh) is the major neurotransmitter in parasympathetic preganglionic neurons (Wang et al., 2001), being synthesized from choline and acetylcoenzyme A by choline acetyltransferase (ChAT), and rapidly hydrolyzed by acetylcholinesterase (AChE) both in neural synapses (Taly et al., 2009) and also intracellularly (Zhang et al., 2002). Enzymatic activity of brain AChE can modulate the outflow of the vagus nerve (Gotoh et al., 1989; Pavlov et al., 2006). ACh binds nicotinic and muscarinic acetylcholine receptors, and nicotinic acetylcholine receptor alpha 7 (α7nAChR) is the most frequent subtype of nicotinic receptors in the brain (Millar and Gotti, 2009). Experimental evidence coming from α7-nicotinic-subunit-null mice indicates that α7nAChR plays a crucial role in the autonomic control of cardiovascular system (Franceschini et al., 2000). Also, the deficiency in brain α7nAChR (Miwa et al., 2011) has been shown to be accompanied by a depleted parasympathetic drive in patients with schizophrenia (Chang et al., 2009; Henry et al., 2010).

In this paper, we aimed to investigate the selected molecular elements of parasympathetic system in MO in male pigs in subsequent stages of chronic tachycardia-induced cardiomyopathy. Our results show changes in the activity and levels of various elements in the parasympathetic system of MO, providing a functional link between MO and heart during the progression of HF.

2. Methods

2.1. Porcine model of chronic non-ischemic systolic HF

In order to investigate the changes in the parasympathetic system in MO during the natural history of HF, we used the experimental porcine model of chronic systolic non-ischemic HF, i.e. tachycardia-induced cardiomyopathy (TIC), which was previously established by our group (Paslawska et al., 2011). The study was performed among 24 adult homogenous male sibling pigs of Polish Large White breed swine. All animals received animal care in compliance with the *Guide for the Care and Use of Laboratory Animals* as published by the National Institutes of Health (NIH Publication No. 85-23, revised in 1996). All experiments were performed in compliance with the guidelines of the Bioethical Committee of the Wroclaw University of Environmental and Life Sciences for experimenting on animals (approval numbers: 03/2008; 40/2011).

Briefly, single-chamber pacemakers (SENSIA SESR01, Medtronic, Poland) were implanted in all 24 pigs, with a bipolar screw-in pacing transvenous lead (CAPSUREFIX NOVUS 58 cm, Medtronic, Poland) positioned at the apex of right ventricle (RV) apex of myocardium. The pacemakers were programmed for sequential RV pacing at 170 bpm in 18 randomly chosen animals, whereas 6 remaining pigs served as sham-operated controls. Clinical examinations and echocardiography evaluation confirmed that the RV-paced animals developed the symptoms of HF, left ventricle (LV) dilatation, impaired LV systolic function along with the neurohormonal activation (e.g. an increase in plasma brain natriuretic peptide [BNP]). These changes were not seen in sham-operated animals (Kiczak et al., 2013).

2.2. Plasma catecholamine assessments

Venous blood samples were drawn from each animal directly before euthanasia, immediately centrifuged, further processed and stored as serum and plasma samples at −80 °C until the further analyses.

Plasma adrenalin (ADR, nmol/L) and noradrenalin (NOR, nmol/L) were assayed using a CatCombi ELISA (IBL International, Immuniq, Poland) according to the manufacturer's instructions. All tests were performed in duplicate.

2.3. Quantitative RT-PCR

Tissue sections from porcine MO were taken during autopsy, and were immediately frozen in liquid nitrogen. Total RNA was prepared from 30 mg samples of porcine MO using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Poland) according to the manufacturer's instructions. The protocol included an on-column DNAse digestion to remove the genomic DNA. Next, the RNA was reverse-transcribed using a SuperScript III First-Strand Synthesis System with an oligo(dT)20 primer (Invitrogen, Poland).

We analyzed the mRNA expression in MO of the following genes: ChAT, AChE, α7nAChR, X-box binding protein 1 (spliced form, XBP1s). Based on the genomic and cDNA sequences, the primers for porcine ChAT, AChE, α7nAChR and XBP1s were designed using a Molecular Beacon Software (Bio-Rad, Poland) (Table 1). All primers spanned exon junctions to prevent the amplification of genomic DNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was chosen as a reference to normalize the differences in the amount of RNA and the efficiency of reverse transcription.

The relative transcript levels of genes were analyzed by quantitative Real-time PCR using the iQ5 Optical System (Bio-Rad, Poland) with the EvaGreen SuperMix (Bio-Rad, Poland). The reactions were performed under the following conditions: an initial denaturation of 94 °C for 30 s, 35 cycles of 94 °C for 10 s, 58 °C (ChAT, XBP1s), and 62 °C (α7nAChR, AChE) for 15 s (EvaGreen SuperMix, Bio-Rad, Poland). All measurements were performed in triplicate. The specificity of PCR was determined using a melt-curve analysis for each reaction.

The amplification efficiency was established by running a template at successive dilutions. Successive dilutions were plotted against the appropriate Ct values to generate a standard curve. The slope calculated from the standard curve was used to determine the amplification efficiency (E) according to the formula: $E = 10^{1/slope}$. Since the amplification efficiencies for the target amplicons and GAPDH were not comparable, the Pfaffl method was used to determine the relative expression (Pfaffl et al., 2002). mRNA expression was presented in arbitrary units (AU), where the MO sample from one of the control pigs was chosen as the calibrator, and its mRNA expression was considered as 1.

2.4. AChE activity

AChE activity was determined in porcine MO homogenates spectrophotometrically, according to Jamal et al. (2010). This assay for the enzymatic activity of AChE has been shown to be sensitive and specific, providing the reliable quantitative data (Massoulie et al., 2008). Briefly, MO samples were homogenized in the solution of 20 mM ice-cold Tris–HCl (pH 7.4) and 0.32 mM sucrose at a proportion

Table 1
Oligonucleotide primers used in the study.

Gene	Primer sequence 5′-3′	GenBank accession no.
GAPDH	TCACTGCCACCCAGAAGA TACCAGGAAATGAGCTTGAC	ABO38240
AChE	AGCGACTGATGAGATACTGG TGAGCAATTTGGGTAGGAAGC	DY427986.1 partial
ChAT	GTACGACAAGTCCCTACAGTTTG AACACTTCCACCTCAGCCTTC	NM_001001541
α7nAChR	TTTATTGTCCCTCACCGTCTTC GGGTCCACTTGGGCATCTTG	JN982966
XBP1s	CTTGTAGTTGAGAACCAGGAGTT CTGCACCTGCTGCGGAC	NM_001142836

GAPDH — glyceraldehyde-3-phosphate dehydrogenase; AChE — acetylcholinesterase; ChAT — choline acetyltransferase; α7nAChR — α7 nicotinic acetylcholine receptor; XBP1s — X-box binding protein 1 (spliced form).