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δ-Opioid receptors: Pivotal role in intermittent hypoxia-augmentation of cardiac parasympathetic control and plasticity

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ABSTRACT

Background: Intermittent hypoxia training (IHT) produces robust myocardial protection against ischemia-reperfusion induced infarction and arrhythmias. Blockade of this cardioprotection by antagonism of either β_1-adrenergic or δ-opioid receptors (δ-OR) suggests autonomic and/or opioidergic adaptations.
Purpose: To test the hypothesis that IHT shifts cardiac autonomic balance toward greater cholinergic and opioidergic influence.
Methods: Mongrel dogs completed 20 d IHT, non-hypoxic sham training, or IHT with the δ-OR antagonist naltrindole (200 μg/kg *sc*). The vagolytic effect of the δ-OR agonist met-enkephalin-arg-phe delivered by sinoatrial microdialysis was evaluated following IHT. Sinoatrial, atrial and left ventricular biopsies were analyzed for changes in δ-OR, the neurotrophic monosialoganglioside, GM-1, and cholinergic and adrenergic markers.
Results: IHT enhanced vagal bradycardia vs. sham dogs ($P < 0.05$), and blunted the δ_2-OR mediated vagolytic effect of met-enkephalin-arg-phe. The GM-1 labeled fibers overlapped strongly with cholinergic markers, and IHT increased the intensity of both signals ($P < 0.05$). IHT increased low and high intensity vesicular acetylcholine transporter labeling of sinoatrial nodal fibers ($P < 0.05$) suggesting an increase in parasympathetic arborization. IHT reduced select δ-OR labeled fibers in both the atria and sinoatrial node ($P < 0.05$) consistent with moderation of the vagolytic δ_2-OR signaling described above. Furthermore, blockade of δ-OR signaling with naltrindole during IHT increased the protein content of δ-OR (atria and ventricle) and vesicular acetylcholine transporter (atria) vs. sham and untreated IHT groups. IHT also reduced the sympathetic marker, tyrosine hydroxylase in ventricle ($P < 0.05$).
Summary: IHT shifts cardiac autonomic balance in favor of parasympathetic control via adaptations in opioidergic, ganglioside, and adrenergic systems.

1. Introduction

Controlled IHT reduces cardiac arrhythmias and improves the clinical efficacy of pharmacological agents [Manukhina et al. 2006]. IHT also dramatically increases myocardial resistance to ischemia-reperfusion injury and arrythmogenesis in dogs [Zong et al. 2004; Mallet et al. 2006; Estrada et al. 2016] and rats [Manukhina et al. 2013], and preserves post-infarct vascular endothelial responses and coronary blood flow in rats [Manukhina et al. 2013]. This gradually evolving, IHT mediated cardioprotection is abrogated by antioxidants or antagonists of β_1-adrenergic (β_1-AR) or δ-opioid (δ-OR) receptors [Mallet et al. 2006; Estrada et al. 2016]. Collectively, these findings indicate that IHT mobilizes adrenergic, opioid and reactive oxygen species (ROS) signaling systems, to induce a unique ischemia resistant phenotype. Consistent with these observations, hypoxic training elicits enkephalin accumulation in myocardium [Maslov et al. 2013].

Acute hypoxia appears to improve cholinergic transmission in both adrenergic and opioid environments [Levy and Zieske 1969; Farias et al. 2003]. For example, when enkephalins accumulate locally during repeated coronary occlusions, vagal transmission improves [Jackson et al. 2001]. This improved vagal function appears to result from a shift in the local receptor environment that favors the vagotonic δ_1-OR [Farias et al. 2001] at the expense of vagolytic δ_2-ORs. The environmental shift may result from local changes in the vagotonic neurotrophin monosialoganglioside-1 (GM-1) [Davis et al. 2006].

The δ-OR is widely expressed on parasympathetic fibers innervating atrial and sinoatrial tissues [Deo et al. 2008], and stimulation of δ-ORs induces GM-1 synthesis and nerve growth [Narita et al. 2006,

Abbreviations: AR, adrenergic receptor; ChT-1, choline transporter-1; IHT, intermittent hypoxia training; OR, opioid receptor; ROS, reactive oxygen species; TH, tyrosine hydroxylase; VAChT, vesicular acetylcholine transporter.

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Kappagantula et al. 2014]. The net result is more efficient vagotonic δ_1-OR signaling and parasympathetic transmission [Ledeen and Wu 2015]. The resulting hypothesis suggests that IHT should improve parasympathetic influence by producing greater arborization and selected increases in GM-1, δ-OR, cholinergic markers and reciprocal declines in adrenergic markers. This study was designed to evaluate functional, biochemical and structural evidence in support of this hypothesis.

2. Materials and methods

2.1. Animals

All animal experimentation was approved by the Institutional Animal Care and Use Committee of the University of North Texas Health Science Center and conducted in accordance with the *Guide to the Care and Use of Laboratory Animals* (U.S. National Research Council Publication 85-23, revised 2011). Mongrel dogs were assigned to 3 groups: non-hypoxic sham ($n = 6$), IHT ($n = 11$), or IHT + daily *sc* naltrindole (IHT + N; $n = 2$). Dogs completed a previously described IHT program [Zong et al. 2004; Estrada et al. 2016] consisting of 20 consecutive days of 5–8 daily cycles of 5–10 min hypoxia (9.5–10% FIO_2) and 4 min intervening room air exposures in a 270 l acrylic chamber [Zong et al. 2004]. The IHT + N dogs received the δ-OR antagonist naltrindole 15 min before each hypoxia session. Naltrindole hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) was dissolved (2 mg/ml) in sterile 0.9% NaCl, filtered and injected (200 μg/kg, *sc*) as described previously [Estrada et al. 2016]. Sham dogs were exposed to 21% O_2 for 20 consecutive days [Zong et al. 2004; Mallet et al. 2006]. On day 21 animals were physiologically evaluated and cardiac tissue was sampled for biochemical and histochemical analyses.

2.1.1. Surgical preparation and instrumentation

Surgical preparation was performed as previously described [Farias et al. 2003]. Briefly, sodium pentobarbital (32.5 mg/kg, *iv*) anesthetized dogs were intubated and ventilated mechanically with room air at approximately 225 ml/kg/min. Supplemental pentobarbital was administered as required. An infusion port Millar Mikro-Tip transducer was inserted into the right femoral artery and advanced into the aorta above the L5 region to measure heart rate and sample arterial blood, and to perfuse the target L5 sympathetic ganglion with experimental agents. Lead II electrocardiogram was continuously monitored via surface electrodes. An electro-magnetic flow probe (10 mm) was installed around the left femoral artery to measure hindlimb blood flow. Blood gases, bicarbonate concentration and pH in arterial samples were measured with an Instrumentation Laboratory Gem Premier 3000 blood gas analyzer (Lexington, MA, USA). The pO_2 (90–120 mm Hg), pH (7.35–7.45), and pCO_2 (30–40 mm Hg) were kept within the respective normal limits by administering supplemental O_2 or bicarbonate, or by adjusting the minute ventilation. The right and left cervical vagus nerves were isolated through a ventral midline incision and double ligated with umbilical tape to abort afferent nerve traffic. The nerves were then returned to the prevertebral compartment for later retrieval. The heart was exposed by right lateral thoracotomy and pericardiotomy. Fig. 1 summarizes experimental and analytical procedures applied to the IHT and sham dogs, which are described in the following sections.

2.1.2. Sinoatrial nodal microdialysis

Dialysis probes were made from 1 cm lengths of dialysis fiber (200 μm i.d. × 220 μm o.d.) obtained from a Clirans TAF 08 artificial kidney (Asahi Medical, Northbrook, IL, USA) as previously described [Farias et al. 2003]. Each dialysis probe, with its glass fiber inflow and outflow lines, was inserted into the sinoatrial node using a 25 ga needle. The probe permitted transudation of molecules with masses <36 kDa. A micro infusion pump connected to the inflow line was used to equilibrate the probe with 0.9% NaCl vehicle at 5 μl/min for 1 h.

2.1.3. Assessment of vagal bradycardia and vagal δ-OR modulation

Following 1 h post-surgical equilibration, the right vagus nerve was stimulated at a supramaximal voltage (e.g., 15 V) at 3 Hz for 15 s. Heart rate was recorded when it reached a steady state during the stimulus, and then was allowed 105 s for complete post-stimulus recovery. Increasing doses of MEAP ($5 \cdot 10^{-15}$ to $1.5 \cdot 10^{-9}$ mol/min) were then infused by sinoatrial nodal microdialysis and the heart rate response to vagal stimulation was recorded at each MEAP dose. Each new dose was infused for 5 min at 5 μl/min before testing vagal responses. The probe was then perfused with 0.9% NaCl until the baseline vagal response was reconfirmed.

2.1.4. Assessment of gangliolytic δ_2-OR responses on hind limb conductance

Thirty minutes after discontinuing the nodal MEAP, increasing doses of met-enkephalin ($5 \cdot 10^{-8}$ to $1 \cdot 10^{-5}$ mol/kg) were injected into the descending aorta to construct a δ_2-OR dose-response relationship using femoral arterial flow and conductance as the outcome measures. Bolus doses (1 ml) were injected just proximal to the final segmental arteries providing direct access to the L5 sympathetic ganglia controlling femoral conductance [Barlow et al. 2011], and flushed with 5 ml 0.9% saline. The femoral hemodynamic effect of each dose was recorded and allowed to return to baseline for 5–10 min before applying the next dose.

2.2. Biopsy preparation

The heart was stopped by applying 9 V current to the epicardium, and biopsies of sinoatrial node, atrium and left ventricle were taken. Biopsies were subdivided; one portion was snap-frozen in liquid N_2 and stored at −80 °C, and the other portion was immersion-fixed in 4% paraformaldehyde for 8 h. Fixed tissues were cryoprotected with serial sucrose solutions (10–30%) and then stored at −80 °C.

2.3. Protein extraction

Snap-frozen atrial and left ventricular biopsies were pulverized in a liquid N_2-cooled porcelain mortar [von Ziegler et al. 2013] and homogenized in 10 vol (v/w) extraction buffer (1% IGEPAL CA-630, 0.5% cholate, 0.5% SDS, 150 mM NaCl, 10 mM Tris-Cl, 1 mM EDTA, 1 mM PMSF, pH 7.6; Sigma-Aldrich, St. Louis, MO, USA) containing protease and phosphatase inhibitors (Cell Signaling Technology, Danvers, MA, USA) at 4 °C. Homogenates were then sequentially drawn through 18, 21 and 23 ga needles to decrease sample viscosity by shearing nucleic acids. Cellular debris was sedimented by 15 min centrifugation (Eppendorf, Hauppage, NY, USA) at 15,000 *g* and 4 °C. Total protein concentrations were measured by bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL, USA) in a microplate reader (Bio-Rad, Hercules, CA, USA).

2.4. Protein electrophoresis and immunoblotting

SDS-polyacrylamide (4% *bis*-acrylamide stacking, 10% *bis*-acrylamide resolving) gels (1.0 mm thick) were prepared from stock reagents (Bio-Rad) according to the manufacturer's instructions. Running and transfer buffers were prepared as described [Towbin 2009] with addition of 0.02% w/v SDS to the transfer buffer. Radio-immunoprecipitation assay buffer lysates were combined with concentrated sample loading buffer yielding a mixture containing 250 mM Tris-HCl, pH 8.5, 2% w/v lithium dodecyl sulphate, 100 mM dithiothreitol, 0.4 mM EDTA, 10% v/v glycerol, 0.2 mM phenol red, 0.2 mM Brilliant Blue G [Cubillos-Rojas et al., 2010] and 1 mg/ml protein. Samples were heated at 70 °C for 10 min, and then cooled on ice. Proteins (5–10 μg/well) were electrophoresed at 60 V through the stacking gel and at 100 V through the resolving gel, and then electroblotted onto vinyl membranes (Bio-Rad) at 70 V for 60–90 min, rinsed with deionized water to remove buffer salts, and dried 1 h at room temperature.