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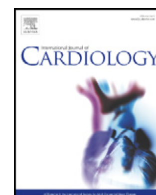
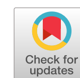


Chronic exercise induces pathological left ventricular hypertrophy in adrenaline-deficient mice



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ABSTRACT

Adrenaline-deficient phenylethanolamine-*N*-methyltransferase-knockout mice (Pnmt-KO) have concentric heart remodeling and though their resting blood pressure is normal, it becomes higher during acute exercise. The aim of this study was to evaluate cardiac morphological, functional and molecular alterations after chronic exercise in adrenaline-deficient mice.

Genotypes at the Pnmt locus were verified by polymerase chain reaction (PCR) of ear samples of Pnmt-KO and wild-type (WT) mice. These mice were submitted to chronic exercise training during 6 weeks. Blood pressure was determined by a photoelectric pulse detector. Mice were anesthetized and cardiac morphology and function were evaluated by echocardiography and hemodynamics. IGF-1, IGF-1R, ANP and BNP mRNA were quantified by real-time PCR in left ventricle (LV) samples.

Pnmt-KO mice showed increased systolic blood pressure compared with WT mice. A significant increase was found in LV mass, and LV posterior wall thickness in trained Pnmt-KO compared to trained WT mice, without significant differences in LV volumes. Acute β_1-adrenergic stimulation with dobutamine increased systolic function indexes in WT mice, but not in Pnmt-KO mice. LV expression of IGF-1 and ANP was increased in trained Pnmt-KO mice when compared to trained WT mice.

In conclusion, in response to chronic exercise adrenaline-deficient Pnmt-KO mice show concentric LV hypertrophy and impaired response to dobutamine, suggesting an initial stage of pathological cardiac hypertrophic remodeling. These results support the need for an efficient partial conversion of noradrenaline into adrenaline for prevention of blood pressure overshoot and thus pathological cardiac hypertrophic remodeling in chronic exercise.

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1. Introduction

Mass activation of sympathetic neurons, with adrenaline and noradrenaline release, is the physiological basis of the fight-or-flight response. This response enables adaption to a stressful event, culminating in increased heart rate and blood pressure, enhanced energy mobilization, and neural reflexes [1]. A hyperactive sympathetic system is associated with various pathological conditions, in particular cardiovascular diseases [2–4].

Noradrenaline from post-ganglionic nerve terminals, and noradrenaline and adrenaline from the adrenal medulla, are the main endogenous catecholamines acting on adrenoceptors in the blood vessels to adjust arteriolar resistance and venous capacitance, and in the heart to regulate cardiac function [5]. The final step in catecholamine biosynthesis is the conversion of noradrenaline to adrenaline by phenylethanolamine-*N*-methyltransferase (Pnmt), a cytoplasmic enzyme [6,7]. Adrenaline synthesis (and Pnmt) is also present in some tissues outside of the adrenal medulla, such as the heart [8,9].

It has been difficult to decipher the role of adrenaline. Adrenal medullectomy can damage the adrenal cortex, altering the release of corticosteroids, and it also impairs the release of other adrenal amines and peptides, such as noradrenaline, chromogranin A, catestatin and

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neuropeptide Y [10]. An alternative methodology is the use of Pnmt inhibitors to prevent adrenaline synthesis *in vivo* [11], but most of them also block monoamine oxidase [12] and α-adrenoceptors [13].

Nowadays, loss-of-function studies are widely used to clarify the role of a specific gene or protein. The Pnmt knockout (Pnmt-KO) mice ($Pnmt^{-/-}$) is an adrenaline-deficient mice model that allows to clarify the role of adrenaline [8,14]. These animals are viable and fertile, and they do not display gross abnormalities [8]. They show absent adrenaline in the adrenal medulla, heart, and plasma [14–16]. On simple echocardiographic evaluation, Pnmt-KO mice seem to present lower filling volumes and cardiac output, as well as increased wall thickness, suggesting a pattern of cardiac remodeling [14]. However, a precise pressure-volume hemodynamic evaluation with gold standard load-independent indexes of contractility and diastolic function is lacking.

In response to acute stress induced by restraint or treadmill exercise, as well as after denervation by ganglionic blockade, Pnmt-KO mice show impaired vasodilator response, which could be due both to lack of adrenaline [17] and abrogated β_2-adrenoceptor signaling [16]. Although blood pressure was normal at rest in Pnmt-KO mice, an exaggerated blood pressure response was demonstrated during acute treadmill exercise [14]. However, the role of adrenaline in response to chronic exercise training has also not been elucidated. The aim of this study is to evaluate cardiac morphological, functional and molecular alterations after chronic exercise training in adrenaline-deficient mice.

2. Methods

2.1. Animals

All animal care and experimental procedures were performed in accordance with the European Directive 63/2010/EU, transposed to the Portuguese legislation by the Directive Law 113/2013. Ten week-old wild type (WT, $Pnmt^{+/+}$, n = 20) and Pnmt-KO ($Pnmt^{-/-}$, n = 17) male mice (129 × 1/SvJ) were kept in cages under controlled environmental conditions (12 h light/dark cycle, room temperature 23 ± 1 °C, humidity 50%, autoclaved drinking water, and mice breeding diet 4RF25/I; Ultragene, Porto, Portugal), and housed with the respective litter. Pnmt-KO mice were produced by disruption of Pnmt locus by insertion of Cre-recombinase in exon 1 [8]. A couple of Pnmt-KO mice were kindly provided by Steven N. Ebert and animals were bred in our conventional vivarium. Genotyping was performed by PCR amplification analysis of the Pnmt gene in ear samples.

2.2. Chronic exercise training

WT and Pnmt-KO mice were randomly allocated to chronic exercise training or their regular physical activity. Before each training session, animals were allowed to adapt to the treadmill for 15 min. Exercise training was performed on a motor treadmill (Panlab Harvard Apparatus, Barcelona, Spain) at the same time of the day, 5 days/week for 6 weeks. Exercise started at 6 cm/s for 10 min, with an increase in speed of 3 cm/s every 2 min until 20 m/min, for 55 min [18]. Training was interrupted if exhaustion occurred, as defined by permanence in the shock grid (0.2 mA) for >5 consecutive seconds or the third time that the animal stays 2 or more seconds in the shock grid (without any attempt to return to the treadmill).

2.3. Blood pressure and heart rate measurements

Systolic and diastolic blood pressure, and heart rate were measured in conscious restrained animals, in a temperature control box at 37–38 °C, using a photoelectric tail-cuff pulse detector (Kent Scientific, CT, USA), as previously described [19]. Four determinations were made and averaged.

2.4. Echocardiographic and hemodynamic evaluation

Upon sedation and analgesia with fentanyl (50 μg/kg, i.p.) and midazolam (5 mg/kg, i.p.), mice were anesthetized by inhalation of 8% sevoflurane in vented containers, orotracheally intubated (20G) and mechanically ventilated using a MouseVent™ Automatic Ventilator (Physiosuite, Kent Scientific, CT, USA). Anesthesia was maintained with sevoflurane (2.5–3.5%) titrated according to the toe pinch reflex. Mice were placed in left-lateral decubitus on a heating pad, the electrocardiogram was monitored (Animal Bio Amp, FE136, ADInstruments, Dunedin, New Zealand) and their temperature was automatically kept at 38 °C (RightTemp™ Temperature Monitor & Homeothermic Controller, Physiosuite, Kent Scientific, CT, USA). The peripheral oximetry (MouseSTAT™ - Pulse Oximeter & Heart Rate Monitor, Physiosuite, Kent Scientific, CT, USA), capnography, respiratory rate and minute ventilation (CapnoScan™ - End-Tidal CO_2 Monitor, Physiosuite, Kent Scientific, CT, USA) were continuously motorized. Cardiac ultrasound was performed using a 15 MHz linear probe (15 MHz ACUSON™ Sequoia 15L8W, Siemens Medical Solutions, CA, USA) on an ultrasound system (ACUSON Sequoia™ C512, Siemens Medical Solutions, CA, USA). Before applying pre-warmed echocardiographic gel, the chest was shaved and depilated. LV end-systolic and end-diastolic cavity dimensions and wall thickness were measured using M-mode tracings and 2-D echocardiography, just above the papillary muscles in parasternal short-axis view. LV volumes, ejection fraction (EF) and fractional shortening (FS) were calculated according to Teichholz formula, and LV mass was determined in diastole through an uncorrected cube method [20]. The parasternal long axis view was used to measure LV long axis, from the mitral annulus to LV endocardial surface at the apex, and to record aortic root dimensions in M-mode. Pulsed-wave Doppler velocity tracings in the 4-chamber view allowed an assessment of LV filling with the peak early (E) and late (A) wave velocities of mitral inflow. Peak early diastolic (E′) and systolic (S′) mitral annular velocities were measured with tissue Doppler (TDI) at the lateral mitral annulus, and the E/E′ ratio was calculated. All the recordings were averaged from three consecutive heartbeats.

For hemodynamic evaluation, the right jugular vein was catheterized (24G) under surgical microscopy for fluid replacement with warm Ringer's lactate solution at 64 ml/Kg/h (NE-1000, New Era Pump Systems, NY, USA). After left thoracotomy, a pressure volume (PV) catheter (PV-1035, Millar Instruments, TX, USA), was inserted through the apex in left ventricle (LV) and signals were continuously acquired (MPVS 3000, Millar Instruments) and digitized at 1000 Hz (ML880 PowerLab 16/30, ADInstruments, Dunedin, New Zealand). Parallel conductance was assessed by injection of 10 μl of 10% hypertonic saline and slope factor α was derived by the measurement of cardiac output (CO) with echocardiography, immediately before. Baseline recordings were obtained after a stabilization period of 30 min and inferior vena cava occlusion with a 5–0 silk lace was also obtained to derive load-independent indexes of contractility and compliance by linear and exponential fitting of the end-systolic and end-diastolic PV relationships (ESPVR and EDPVR). After an intravenous infusion of dobutamine (β_1-adrenoceptor agonist, 5 μg/Kg/min), inferior vena cava occlusion recordings were repeated upon obtaining a stable effect of at least 10 min. All acquisitions were performed with ventilation suspended at end-expiration. Echocardiographic and hemodynamic stroke volume (SV) and CO were defined as SV = LVEDV − LVESV (LVEDV, LV end-diastolic volume; LVESV, LV end-systolic volume) and CO = HR × SV. EF was defined as EF = (LVEDV − LVESV)/LVEDV × 100 [14]. To account for large differences in body weight between groups, some parameters were indexed for body surface area (BSA) (LVM_i, $LVEDV_i$, SV_i and cardiac index), as estimated by 9.82 ∗ body weight$^{2/3}$ in grams [21]. Indexed systemic vascular resistances (SVRI) were calculated by dividing end-systolic pressure (used as surrogate of mean blood pressure [22]) and cardiac index, neglecting right atrial pressure. Upon completion of experiments, animals were euthanized by exsanguination under anesthesia and hearts were removed and dissected. LV samples were collected and snap frozen in liquid nitrogen and stored at −80 °C.

2.5. RNA isolation and relative quantification of mRNA expression

Real-time PCR was performed in LV samples, as previously detailed [23]. In brief, samples were homogenized using the MagNA Lyser Instrument homogenizer (Roche Diagnostics, Basel, Switzerland). Total RNA isolation was carried out with the SV Total RNA Isolation System kit (Promega, WI, USA). Concentration and purity of the isolated RNA were measured using the NanoDrop 2000 spectrophotometer (Thermo Scientific, MA, USA). Reverse transcription was performed in a T100™ Thermal Cycler (Bio-Rad, CA, USA). A StepOne™ real-time PCR System (Applied BioSystems, MA, USA) was used in real-time PCR experiments. For each studied mRNA molecule, standard curves were generated from the correlation between the amount of starting total mRNA and PCR threshold cycle of graded dilutions from a pool of all samples. Maxima SYBR Green qPCR Master Mix (2×) (Thermo Scientific, MA, USA), Nuclease-free H_2O (Thermo Scientific, MA, USA) and gene specific primers (5 μM) were mixed before adding cDNA (1:20). Instead of cDNA, Nuclease-free H_2O (Thermo Scientific, MA, USA) was added as negative control. Gene specific primers were as follows: Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) FWD, CCATCACCATCTTCCAGGAG; GAPDH REV, GCATGGACTGTGGTCATGAG; type-A natriuretic peptide (ANP) FWD, AGGCAGTCGATTCTGCTTGA; ANP REV, CGTGATAGATGAAGGCAGGAAG; type-B natriuretic peptide (BNP) FWD, TAGCCAGTCTCCAGAGCAATTC; BNP REV, TTGGTCCTTCAAGAGCTGTCTC; insulin-like growth factor-1 (IGF-1) FWD, GAAGTCCCCGTCCCTATCGA; IGF-1 REV, CCTTCTCCTTTGCAGCTTCG; IGF-1 receptor (IGF-1R) FWD, AGTGACTCGGATGGCTTCGTT; IGF-1R REV, TTTCACAGGAAGCTCGCTCTC. GAPDH mRNA levels were similar in all experimental groups, which enabled the use of this gene as an internal control. Results of mRNA quantification are expressed in an arbitrary unit (AU) after normalization for GAPDH.

2.6. Statistics

All results are expressed as mean ± standard error of the means (SEM) for the indicated number of determinations. Student's unpaired *t*-test was applied to data regarding distance and total time run during chronic exercise training. Other results were assessed by two-way ANOVA and two-way repeated measures ANOVA. Residuals normality was checked by Shapiro-Wilk's test, homogeneity of variances by Levene's or Box's M test. *Post hoc* analysis was performed using Newman-Keuls' test. For the ESPVR and EDPVR analysis, volume intercept and scaling constant were included as covariates, respectively. Log transformation of variables was applied whenever necessary to overcome violation of assumptions. Comparisons were made using *STATISTICA* (StatSoft, Inc., OK, USA) and

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