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Gender-specific impairment of *in vitro* sinoatrial node chronotropic responses and of myocardial ischemia tolerance in rats exposed prenatally to betamethasone

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ABSTRACT

Excessive fetal glucocorticoid exposure has been linked to increased susceptibility to hypertension and cardiac diseases in the adult life, a process called fetal programming. The cardiac contribution to the hypertensive phenotype of glucocorticoid-programmed progeny is less known, therefore, we investigated *in vitro* cardiac functional parameters from rats exposed *in utero* to betamethasone.

Pregnant Wistar rats received vehicle (VEH) or betamethasone (BET, 0.1 mg/kg, i.m.) at gestational days 12, 13, 18 and 19. Male and female offspring were killed at post-natal day 30 and the right atrium (RA) was isolated to *in vitro* evaluation of drug-induced chronotropic responses. Additionally, whole hearts were retrograde-perfused in a Langendorff apparatus and infarct size in response to *in vitro* ischemia/reperfusion (I/R) protocol was evaluated.

Male and female progeny from BET-exposed pregnant rats had reduced birth weight, a hallmark of fetal programming. Male BET-progeny had increased basal RA rate, impaired chronotropic responses to noradrenaline and adenosine, and increased myocardial damage to I/R. Though a 12-fold reduction in the negative chronotropic responses to adenosine, the effects of non-metabolisable adenosine receptor agonists 5′-(*N*-ethylcarboxamido)adenosine or 2-Chloro-adenosine were not different between VEH- and BET-exposed male rats. BET-exposed female offspring presented no cardiac dysfunction.

Prenatal BET exposure engenders male-specific impairment of sinoatrial node function and on myocardial ischemia tolerance resulting, at least in part, from an increased adenosine metabolism in the heart. In light of the importance of adenosine in the cardiac physiology our results suggest a link between reduced adenosinergic signaling and the cardiac dysfunctions observed in glucocorticoid-induced fetal programming.

1. Introduction

Adverse intrauterine environment can lead to developmental changes that cause long-term alterations in postnatal life physiology and risk to development of diseases, a process known as fetal programming (Godfrey and Barker, 2001). Different stressors were shown to cause intrauterine programming of disease in experimental models including maternal undernutrition, intrauterine hypoxia and exposure to abuse drugs (Harris and Seckl, 2011; Dasinger and Alexander, 2016). Antenatal glucocorticoid administration, whose increasing evidence suggests to be triggers and mediators of intrauterine programming, is an established obstetric practice employed to enhance fetal lung maturation in pregnancies at risk of preterm delivery (Roberts and Dalziel, 2006). Apart the benefits of antenatal glucocorticoid administration to

Abbreviations: BET, betamethasone; RA, right atrium; I/R, ischemia/reperfusion; NECA, 5′-(*N*-ethylcarboxamido)adenosine; 2-Cl-adenosine, 2-chloro-adenosine; SAN, sinoatrial node; TTC, 2,3,5-Triphenyl-tetrazolium chloride; A1R, adenosine A1 receptor

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neonate survival, excessive glucocorticoid exposure has been associated with decreased birth weight and increased risk of cardiometabolic and brain disorders (Reynolds, 2013).

Changes in offspring postnatal cardiovascular homeostasis are a well defined facet of fetal programming (Barker, 1993; Barker and Fall, 1993). In particular, substantial data from rodent and human studies show a positive relationship between low birth weight and increased risk of hypertension and ischemic heart disease in adult life (Barker et al., 1989; Mercuro et al., 2013; do Carmo Pinho Franco et al., 2003). Mechanistically, altered vasomotor responses of arteries taken from intrauterine programmed animals were linked to the hypertensive phenotype (Brawley et al., 2003; Franco Mdo et al., 2002; Lamireau et al., 2002; Xiao et al., 2009). Less knowledge is available about any cardiac contribution to the hypertensive phenotype of these programmed animals.

Endogenous glucocorticoid surge in the late gestation is important to heart maturation but excessive glucocorticoid exposure may alter the normal trajectory of this process (Rog-Zielinska et al., 2014). To gain further insight on the cardiac effects of excessive prenatal glucocorticoid exposure we investigated the *in vitro* function of sinoatrial node (SAN) and the function and ischemia tolerance of Langendorff-perfused hearts in rats programmed by intrauterine BET administration. Additionally, in light of the reported gender-specific impairment of cardiovascular responses, the cardiac functional parameters from both, male and female rats, were assessed.

2. Materials and methods

2.1. Animals

The experimental procedures were approved by the local Ethics Committee for the Use of Experimental Animals of the University of São Paulo State (protocol number 451-CEEA) in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health). The euthanasia was performed by decapitation and all efforts were made to minimize the number and suffering of animals.

Male (90 days old/300–350 g) and female (90 days old/225–230 g) Wistar rats were obtained from the Multidisciplinary Center for Biological Investigation, State University of Campinas and maintained under controlled conditions (25 °C, 30% air humidity, 12/12-h light/dark cycle) with food and water available *ad libitum*.

The experimental design was performed as published by C.S. Borges et al. (2016) and C.D. Borges et al. (2017). Briefly, each sexually experienced male rat ($n = 10$) was paired with two nulliparous female rats ($n = 20$) during the dark cycle of the photoperiod and allowed to stay together until sperm were detected in the daily vaginal smears as evidence of copulation. The detection of sperm in the vaginal smear of rats in estrus was considered as gestational day 1 (GD1). Pregnant and lactating rats ($n = 15$) were maintained in individual cages and were randomly allocated into two experimental groups: VEH (vehicle; saline solution; $n = 6$) and BET (0.1 mg/kg of betamethasone, Sigma; $n = 9$) treated by intramuscular injection on days 12, 13, 18 and 19 of pregnancy. This injection protocol was based on the maternal corticosteroid therapy previously adopted by Piffer et al. (2009a).

After birth, during 21 days, the pups were maintained with dams and on post-natal day 21 (PND21, weaning) they were housed in separate cages ($n = 3$ pups/litter). For each set of experiments, one male and female rat from each litter was sampled in order to prevent potential variation due to litter effects.

2.2. In vivo mean arterial blood pressure (MAP) and heart rate (HR) recording in conscious unrestrained rats

Due to the limited number of animals available to the realization of all experiments of this study the *in vivo* recording of MAP and HR was done with the male offspring, only. Thirty-day old VEH- and BET-exposed male rats ($n = 5$ for both groups) were anesthetized with 2% isoflurane in 100% O_2 and the left carotid artery was catheterized with a heparin-filled (500 IU/ml) polyethylene catheter (PE50 connected to PE10). The catheter was driven subcutaneously and exteriorized in the dorsum of the rat. Twenty-four hours after the surgery the carotid catheter was connected to a pressure transducer in a MP150 system (Biopac, Santa Barbara, CA, USA) and the MAP and HR were recorded continuously during 15 min in conscious unrestrained rats. All the recordings of hemodynamic parameters were done between 8 am and 12 pm. Due to the known ischemic preconditioning effect of isoflurane (Liu et al., 2015; Lotz et al., 2015) animals included in this experiment were not employed in other assays of this study.

2.3. In vitro RA contraction

Thirty day-old VEH- and BET-exposed male and female rats were killed by decapitation and the heart was excised and immersed in ice-cold Krebs-Henseleit solution of following composition (mM): 119 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 25 $NaHCO_3$, 11 dextrose, pH 7.4. The RA was isolated, washed from remaining blood and attached to isometric force transducers under 1 gram resting tension in 10 ml organ baths filled with Krebs-Henseleit solution (pH 7.4) at 30 °C constantly bubbled with 95%O_2/5%CO_2. Tissues were allowed a 1 hour equilibration period with solution changes every 15 min. After the equilibration period the spontaneous basal RA rate was recorded and cumulative concentration-response curves to noradrenaline, adenosine and acetylcholine were obtained in each tissue at 30 minute interval. In another series of experiments the RA chronotropic responses to 5′-(*N*-ethylcarboxamido)adenosine (NECA) and 2-chloro-adenosine (2-Cl-adenosine), two adenosine receptor agonists resistant to adenosine uptake/metabolism, were investigated in RA from VEH- and BET-exposed male rats.

Data from all rats of the same experimental group were plotted together in GraphPad Prism 5 software (GraphPad, CA, USA) and subjected to non-linear regression analysis using a sigmoidal dose-response curve fitting and the averaged maximal chronotropic responses (E_{max}) expressed as changes of beating rate over the basal atrial rate (Δ atrial rate, in beats per minute (bpm)) and the agonist potencies expressed as pD_2, *i.e.* the negative of logarithm of agonist concentration inducing 50% of maximal response, were evaluated.

2.4. Langendorff-perfused hearts

Male and female VEH- and BET-exposed rats were anesthetized with ketamin + xylazine (70 + 15 mg/kg, respectively). Once anesthesia was achieved heparin (100 IU) were injected intraperitonialy. Then, the heart was excised *via* thoracotomy and immediately immersed in ice-cold Krebs-Henseleit solution of above cited composition except the $CaCl_2$ concentration that was reduced to 1.8 mM, a modification previously used in myocardial ischemia studies to better reflect the physiologically ionized blood Ca^{2+} concentration (Sutherland and Hearse, 2000). The adherent tissues were removed, the aorta was cannulated with a 17 Gauge needle and the heart retrograde-perfused with oxygenated Krebs-Henseleit solution (with 1.8 mM $CaCl_2$) at 37 °C. As altered coronary contraction/relaxation from antenatal BET-exposed lambs was described previously (Segar et al., 2006), the constant flow perfusion mode was employed to allow the measurement of different functional endpoints under the same experimental conditions. As preliminary experiments showed hearts from vehicle or betamethasone-exposed 30 day-old rats averaged approximately 0.8 g, the perfusion was set at constant flow of 8 ml/min in accordance to previously published value of 10 ml/min/g of heart wet weight (Kolar et al., 1990).

To avoid unintended ischemic preconditioning all the procedures from the heart excision to commencing of perfusion were done in < 3 min and hearts out of this time limit were not used. After the perfusion was established, the left auricle was removed and a saline-filled