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Dietary calcium supplementation in adult rats reverts brown adipose tissue dysfunction programmed by postnatal early overfeeding

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Abstract

Brown adipose tissue (BAT) dysfunction is associated with obesity and its comorbidities, such as hypertension, and the improvement of BAT function seems important for obesity management. Here we investigated the effects of dietary calcium supplementation on BAT autonomic nerve activity, sympathoadrenal function and cardiovascular parameters in adult obese rats that were raised in small litters (SL group). Three days after birth, SL litters were adjusted to three pups to induce early overfeeding. The control group remained with 10 pups/litter until weaning (NL group). At PN120, the SL group was randomly divided into the following: rats fed with standard chow (SL) and rats fed with dietary calcium carbonate supplementation (SL-Ca, 10 g/kg chow). Animals were killed either at PN120 or PN180. At both ages, SL rats had higher BAT autonomic nervous system activity, mass and adipocyte area, as well as increased heart rate and blood pressure (systolic and diastolic); 2 months of calcium supplementation normalized these parameters. At PN180 only, UCP1 and TRβ1 in BAT were decreased in SL rats. These changes were also prevented by calcium treatment. Also at PN180, the SL group presented higher tyrosine hydroxylase and adrenal catecholamine contents, as well as lower hypothalamic POMC and MC4R contents. Calcium supplementation did not revert these alterations. Thus, we demonstrated that dietary calcium supplementation was able to improve cardiovascular parameters and BAT thermogenesis capacity in adult animals that were early overfed during lactation.

Keywords: Small litter; Overnutrition; Obesity; Autonomic function; Calcium supplementation; Rat

1. Introduction

The brown adipose tissue (BAT) is the major contributor to body thermoregulation, producing heat through the oxidation of fatty acids and glucose unassociated with ATP production [1]. BAT adipocytes are composed by multilocular small lipid droplets and a considerable number of mitochondria, which are rich in uncoupling protein 1 (UCP1) [1]. UCP1 is present in the mitochondrial inner membrane; it dissipates the mitochondrial proton gradient, leading to heat production instead of ATP synthesis [2]. Sympathetic activation and thyroid hormones enhance UCP1 expression, thus increasing the thermogenesis and systemic energy expenditure [3].

Sympathetic nerve activity regulation of BAT function is also dependent on melanocortin signaling [4]. The alpha-melanocyte-stimulating hormone (α-MSH) produced in the arcuate nucleus (ARC) is derived from the prepropeptide hormone proopiomelanocortin (POMC) and is an endogenous agonist for melanocortin receptors (MCRs), which are mainly found in the hypothalamic paraventricular nucleus (PVN) [5]. The MCR has the agouti-related protein as an antagonist, which is also produced in the ARC. The activation of the MC4R isoform is the main regulator of energy balance and blood pressure, which is more important than the activation of the MC3R isoform [6], responsible for the increase in BAT sympathetic outflow, thereby promoting adipocyte proliferation and differentiation and reducing apoptosis. Also, it stimulates mitochondrial biogenesis and UCP1 mRNA production, thus improving BAT thermogenesis capacity [7].

Considering these aforementioned features, BAT hypofunction has been associated with obesity and type 2 diabetes Therefore, strategies to improve BAT function can be important in the treatment of obesity

Abbreviations: ADRβ3, beta 3 isoform adrenergic receptor; AgRP, agouti-related protein; ANS, autonomic nervous system; ARC, hypothalamic arcuate nucleus; BAT, brown adipose tissue; MCR, melanocortin receptors; NL, normal litter; POMC, proopiomelanocortin; PVN, hypothalamic paraventricular nucleus; SL, small litter; SL-Ca, small litter supplemented with calcium carbonate; TRβ1, beta 1 isoform thyroid hormone receptor; UCP1, uncoupling protein 1; α-MSH, alpha-melanocyte-stimulating hormone

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and its comorbidities [8]. To better understand the contribution of BAT function in obesity models, we used the well-known model of postnatal early overfeeding induced by litter size reduction, in which other groups and our own have previously demonstrated a programming effect for obesity, insulin and leptin resistance and higher oxidative stress [9–11]. This is an interesting model for child obesity during the first years of life, especially during lactation, a health problem that has reached high levels of prevalence in western societies [12] and that has been associated with long-standing obesity [13] and worsened health prognoses [14].

Animals raised in small litters (SL) are exposed to overfeeding due to the higher milk availability, with elevated triglyceride content, during lactation [15]. This intervention results in increased body mass from the first postnatal week onward, associated with the development of the metabolic syndrome [16]. Concerning BAT function, SL rats have no change in BAT sympathetic nerve activity at weaning, that is, at postnatal day (PN) 21 [17], despite the increased UCP1 mRNA expression [18]. At PN60, SL rats presented reduced UCP1 and ADRβ3 mRNA levels, as well as BAT thermogenesis [18], and at PN90, these rats also displayed higher lipid droplet distribution in BAT [19], meaning lower BAT thermogenic capacity. Regarding the sympathoadrenal system, which also plays an important function in body mass regulation, by stimulating energetic expenditure [20,21], we have previously reported an increase of total catecholamine production, content and secretion in the adrenal medulla of SL rats at PN180 [22]. In addition, these animals developed hypothyroidism when adults [10]. Early overfeeding may influence this profile through changes in glycemia, leptinemia and insulinemia during the critical period of neuronal plasticity, affecting melanocortin system function [23–25].

We have already demonstrated, in the SL model, that dietary calcium supplementation from PN120 to PN180 is able to prevent the development of overweight and associated metabolic dysfunctions (higher oxidative stress and liver microsteatosis), as well as preference for high-fat diets [26,27]. The present study continues the characterization of the SL animal phenotype, testing the hypothesis that lower BAT thermogenesis contributes to obesity in rats. We are using dietary calcium treatment as a tool to verify if the reversion of obesity is associated with normalization of BAT thermogenesis, through normalization of BAT sympathetic nerve activity, BAT catecholamine and thyroid hormone sensitivity, and POMC content in the ARC and MC4R in the PVN. Since important changes in sympathoadrenal function are known to present in this model, we use two parameters of cardiovascular function to evaluate if dietary calcium supplementation also normalizes sympathetic nervous function.

2. Materials and methods

The Animal Care and Use Committee of the Biology Institute of the State University of Rio de Janeiro approved our experimental protocol (CEUA/012/2014). Experiments were conducted following the ethical doctrine of the three "R's" reduction, refinement and replacement, to minimize the number of animals and the suffering caused by the experimental procedures, based on the principles established in the Brazilian Law No. 11.794/2008.

The Wistar rats employed in the experiment were housed under controlled temperature (23°C±1°C), light (12-h light/dark cycle), and had free access to water and food. Twenty nulliparous female rats were placed with 10 male rats (all the animals were approximately 120 days old) in a 2:1 ratio during 5 days. After mating, pregnant females were housed in individual cages until delivery. After birth, all litters were adjusted to 10 male pups for each dam. Male pups from other litters substituted female pups.

2.1. Experimental model of litter size reduction and calcium supplementation

To induce early overfeeding, at PN3, litters selected to comprise the Small Litter group (SL, $n=10$) were culled to three male pups per dam. The normal litter group (NL, $n=10$) was kept with 10 pups per dam until weaning (PN21). After weaning, food intake and body mass were monitored until PN180.

Calcium supplementation was administered from PN120 to PN180. NL rats received standard chow for rodents. SL rats were subdivided into two groups:

1) SL ($n=10$) — received standard chow;
2) SL-Ca ($n=10$) — received standard chow supplemented with calcium carbonate (to attain a final concentration of 10 g of $CaCO_3$/kg chow), which was prepared in our laboratory. Twice the amount of calcium recommended for rodents (5 g calcium/kg chow) [27]. This amount is based on the recommendation for calcium supplementation in humans. Previously, we have shown that this diet does not alter food consumption and body mass of control rats, indicating that calcium in this concentration does not change diet palatability [28,29].

2.2. Analysis of cardiovascular parameters

Animals were acclimated during 2 weeks in order to record the cardiovascular parameters with minimal restraint and stress. At the end of the acclimation period, the means of three measurements per parameter were recorded. Heart rate and systolic and diastolic blood pressure were evaluated at PN119 and PN179 by using a noninvasive method (tail-cuff plethysmograph — LE5001 Panlab, Barcelona, Spain).

2.3. Sympathetic autonomic nerve electrical activity

At PN120 and PN180 (different animals at each age), the groups were fasted for 12 h and then anesthetized (pentobarbital sodium, 90 mg/kg bw) for *in vivo* autonomic nerve activity assessment. BAT sympathetic autonomic nerve activity from the left intrascapsular nerve was exposed under a dissection microscope. The branches were placed on a pair of hook platinum electrodes connected to an electronic device (Bio-Amplificator, Insight, Ribeirão Preto, SP, Brazil) to record the electrical signals. To avoid dehydration, the nerve was covered with mineral oil. Nerve activity was amplified (10,000) and filtered (cutoff: 60 kHz). Data were analyzed using the PowerLab data acquisition system (8SP; ADInstruments, New South Wales, Australia). All nerve activity recordings were carried out inside a Faraday cage to avoid electromagnetic interference. Rats were kept under warming light. After 10 min of stabilization, the average of the number of spikes per 10-s intervals during a 10-min period was calculated [30]. The background noise level was determined in a nerve segment.

2.4. Euthanasia and tissue collection

After the autonomic nerve activity measurement, rats were euthanized by exsanguination. BAT was dissected, weighed and prepared for morphological and molecular studies. The adrenal glands were frozen for the sympathoadrenal assessment. The whole brain was removed and stored at −80°C until the dissection of the areas of interest. Blood samples were centrifuged (1000×*g*, 4°C, 20 min) to collect the plasma, which was then stored (−20°C) until analysis.

2.5. Morphological evaluation of BAT

The BAT was fixed in formaldehyde 0.1 M phosphate-buffered saline (pH 7.2). Then, tissues were dehydrated, cleared and paraffin-embedded. Nonconsecutive slices of 10-μm-thick sections were obtained and stained with hematoxylin/eosin to assess morphology. Digital images were with a Olympus BX40 microscope (Olympus, Tokyo, Japan) using a 40× objective. From each rat, three slides were obtained; each one resulted in five pictures of different fields. From each picture, the sectional multilocular lipid droplet percentage area was measured with the software Image-Pro Plus 5.0 (Media Cybernetics, Silver Spring, MD, USA) [17].

2.6. Isolation of the PVN and ARC

To perform the coronal sections of the brain, we used a cryostat (Hyrax C25; Zeiss, Oberkochen, Germany). The PVN (Bregma −1.8 to −2.1 mm) and ARC (Bregma −1.6 to −2.6 mm) were isolated according to the coordinates from the Paxinos and Watson [31] stereotaxic atlas. The samples were frozen (at −80°C) until Western blot analysis was performed.

2.7. Adrenal catecholamine measurement

Total catecholamines were quantified by the trihydroxyindole method [17]. Right adrenal glands were homogenized in 10% of acetic acid and centrifuged (1120×*g*, 5 min). Briefly, 50 μl of epinephrine standard and the adrenal supernatant were mixed with 250 μl of buffer phosphate (0.5 M, pH 7.0) and 25 μl of potassium ferricyanate (0.5%), and incubated for 20 min. The reaction was stopped with 500 μl of ascorbic acid (60 mg/ml)/NaOH (5 N) solution, and diluted with 2 ml of distilled water. The fluorescence was determined at 420 nm for excitation and 510 nm for emission (Hidex, Turku, Finland).