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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).

2.8. Western blotting analysis

Proteins contents in BAT, adrenal, PVN and ARC were evaluated by Western blotting. Briefly, BAT, PVN and ARC were homogenized in RIPA buffer [50 mM Tris–HCl (pH 7.4), 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF] and protease inhibitor cocktail (F. Hoffmann-La Roche Ltd., Basel, Switzerland). This was followed by ultrasonic processing (three times, 10 s on and 15 s off; range of 40%). Homogenates were centrifuged (1120×*g*, 4°C, 5 min). The adrenal glands were homogenized in 1 ml phosphate buffer (pH 7.4), containing a protease inhibitor cocktail (F. Hoffmann-La Roche Ltd.), and centrifuged (7500×*g*, 4°C, 5 min). Protein concentration in supernatants was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, San Diego, CA, USA). Then, homogenates were analyzed by SDS-PAGE using 30 mg total protein. Samples were electroblotted onto nitrocellulose membranes (Hybond ECL; Amersham Pharmacia Biotech, London, UK). Membranes were incubated with Tris-buffered saline (TBS) containing 2% albumin for 90 min. Subsequently, membranes were washed with TBS and incubated with a specific primary antibody diluted to 1:500 (anti-UCP1 — SAB1404511 from Sigma-Aldrich Inc., St. Louis, MO, USA; anti-ADRβ3, sc-50,436 from Santa Cruz Biotechnology Inc., Wembley, UK; anti-TRβ1, from Santa Cruz; anti-TH, T2928 from Sigma-Aldrich; anti-POMC, sc-20,148 from Santa Cruz; anti-MC4R, ab24233 from Abcam, Cambridge, MA, USA; anti-β actin, A2228 from Sigma-Aldrich) overnight at 4°C. Membranes were washed and incubated with the secondary antibody diluted to 1:5000 (anti-mouse, B8520 from Sigma-Aldrich; anti-goat, 62-6540; anti-rabbit, 65-6140 from Invitrogen Corporation, Carlsbad, CA, USA) conjugated with horseradish peroxidase in an adequate dilution for 1 h at room temperature. The protein bands were visualized by chemiluminescence (Kit ECL plus; Amersham Biosciences, London, UK) followed by exposure to ImageQuant LAS (GE Healthcare, Buckinghamshire, UK). Area and density of the bands were quantified by the Image J software (Wayne Rasband; National Institute of Health, Cambridge, MA, USA). Results were expressed as relative (%) to the control group.

2.9. Statistical analysis

The statistical analyses were carried out using the Graph Pad Prism 5.0 for Windows statistical software (GraphPad Software, La Jolla, CA, USA). At PN120, comparisons between the groups were performed using Student's unpaired *t* test, whereas at PN 180, the comparisons among the groups were analyzed by one-way analysis of variance (ANOVA; group as the between-subjects factor: NL, SL, SL-Ca. Results presented in Table 1), followed by the Newman–Keuls multiple comparison tests. For all analyses, the data were given as mean and standard error of the mean (S.E.M.). Differences were considered significant when $P<.05$.

3. Results

3.1. Body mass and food intake

At PN120, the body mass of the SL group was higher when compared to that of the NL group (NL: 362.2±9.1 g, SL: 421.7±7.5 g; $P<.001$). At PN180, both SL and SL-Ca groups were heavier than the NL one (NL: 403.0±8.3 g, SL: 486.7±16.6 g, SL-Ca: 459.8±7.7 g; $P<.001$).

The SL group presented higher food intake compared with the NL group from weaning until PN120 (1.6 fold-increase; $P<.001$), as well as at PN180 (+77%; $P<.001$). The dietary calcium supplementation, which was offered from PN121 until PN180, reduced the food consumption of the SL-Ca group when compared with the SL group (−34%; $P<.001$), although it was still greater than that of the NL group (+16%; $P<.001$).

Table 1
Results from the ANOVAs

Parameter	*F* value	d.f.	*P*
Heart rate	7.7	2	**
Systolic blood pressure	12.9	2	***
Diastolic blood pressure	10.1	2	***
BAT ANS	4.0	2	*
BAT lipid droplets sectional area	4.5	2	*
BAT mass	3.2	2	
BAT UCP1	4.5	2	*
BAT β3ADR	0.4	2	
BAT TRβ1	4.6	2	*
Adrenal TH	3.9	2	*
Adrenal catecholamine	4.8	2	*
ARC POMC	6.4	2	**
PVN MC4R	5.0	2	*

* $P<.05$.
** $P<.01$.
*** $P<.001$.

3.2. Cardiovascular parameters

The heart rate was higher in the SL group when compared to that of the NL group in both ages (PN119: +17%; $P<.001$. PN179: +10%; $P<.002$. Fig. 1A). Also, both systolic (PN120: +28%; $P<.0027$. PN180: +35%; $P<.0001$; Fig. 1B) and diastolic blood pressures (PN119: +17%; $P=.04$. PN179: +37%; $P<.001$; Fig. 1C) were higher in SL rats than in NL ones. Two months of calcium supplementation was able to reverse these changes (Fig. 1A–C).

3.3. BAT autonomic nervous system activity at basal condition

At both ages (Fig. 2), SL rats presented greater autonomic nerve activity in the BAT when compared to that of the NL group, as indicated by the higher number of spikes (PN120: +122%; $P=.006$. PN180: +63%; $P=.03$). Two months of calcium supplementation was able to normalize autonomic nervous system (ANS) nerve activity in the SL-Ca group (Fig. 2).

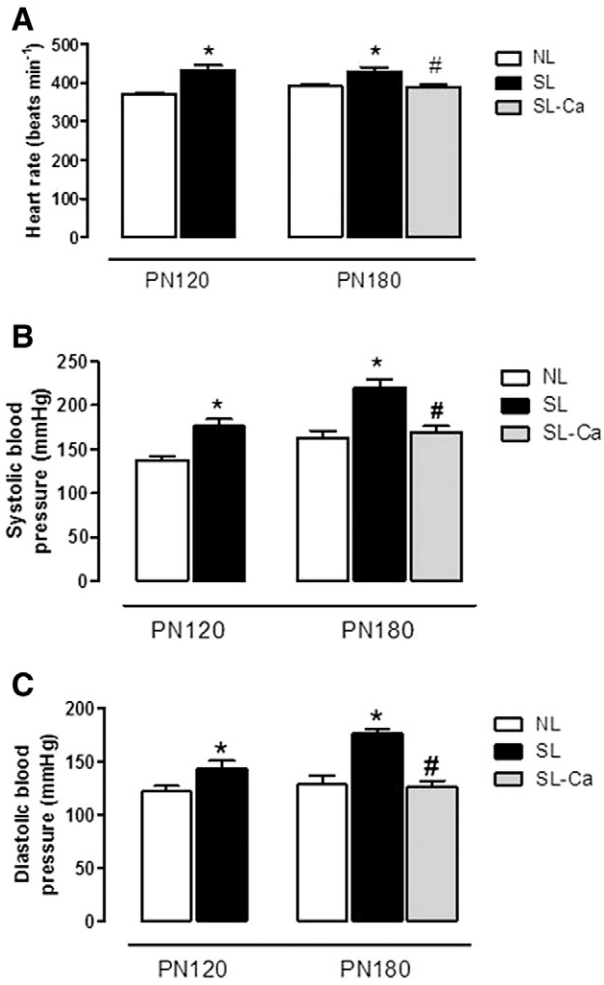

Fig. 1. Long-term effects of reducing litter size on cardiovascular parameters. The heart rate (A), systolic blood pressure (B) and diastolic blood pressure (C) at PN120 and PN180. Rats raised in normal (NL) and small (SL) litters during lactation. SL rats supplemented with calcium from PN120 until PN180 (SL-Ca). Results are expressed as mean±S.E.M.; $n=10$ per group; * $P<.05$.

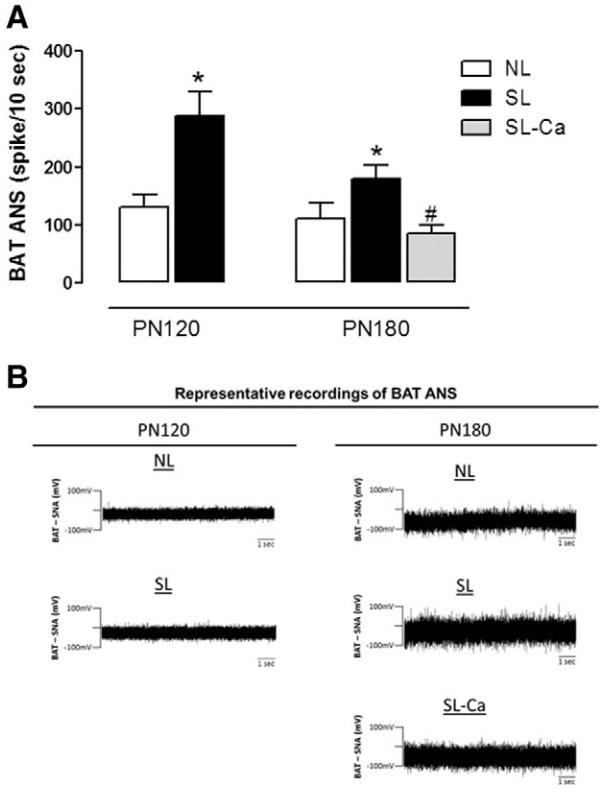

Fig. 2. Long-term effects of reducing litter size on BAT ANS. Number of spikes in 10 s at PN120 and PN180 (A) and representative recordings (B). Rats raised in normal (NL) and small (SL) litters during lactation. SL rats supplemented with calcium from PN120 until PN180 (SL-Ca). Results are expressed as mean±S.E.M.; $n=10$ per group; * $P<.05$.

3.4. BAT morphology

At both ages (Figs. 3 and 4), SL rats showed a larger lipid droplet sectional area in the BAT than the NL group (PN120: +45%; $P=.04$. PN180: +33%; $P=.03$). Two months of dietary calcium treatment was able to restore the size of the lipid droplet (Fig. 4).

3.5. BAT mass and protein content

The BAT mass (Fig. 5A) was increased in SL rats when compared to NL rats (PN120: 1.2 fold-increase; $P=.002$. PN180: +24%; $P=.05$). At PN120, UCP1 protein content (Fig. 5B) was unaffected in the SL group, whereas, at PN180, its content was lower in SL rats when compared to NL rats (−40%; $P=.02$). At PN120, β3ADR protein content (Fig. 5C) was higher in SL animals when compared to NL ones (+92%; $P=.05$). However, at PN180, no difference was observed between these groups. At PN120, TRβ1 protein content (Fig. 5D) was unaffected in the SL group, while at PN180, its content was reduced in SL rats when compared to NL rats (−48%; $P=.02$). Calcium supplementation for 2 months was able to normalize BAT mass (Fig. 5A), UCP1 content (Fig. 5B) and TRβ1 content (Fig. 5D) in the SL-Ca group.

3.6. Adrenal medulla function

At PN120, TH protein content (Fig. 6A) was unaffected in the SL group, but at PN180, its content was higher in SL rats than in NL ones (+98%; $P=.005$). At both ages, total catecholamine content (Fig. 6B) in the adrenal gland was higher in SL animals when compared to NL ones (PN120: +47%; $P<.01$. PN180: +49%; $P<.01$). Dietary calcium therapy was not able to restore TH and catecholamine contents in the SL-Ca group.

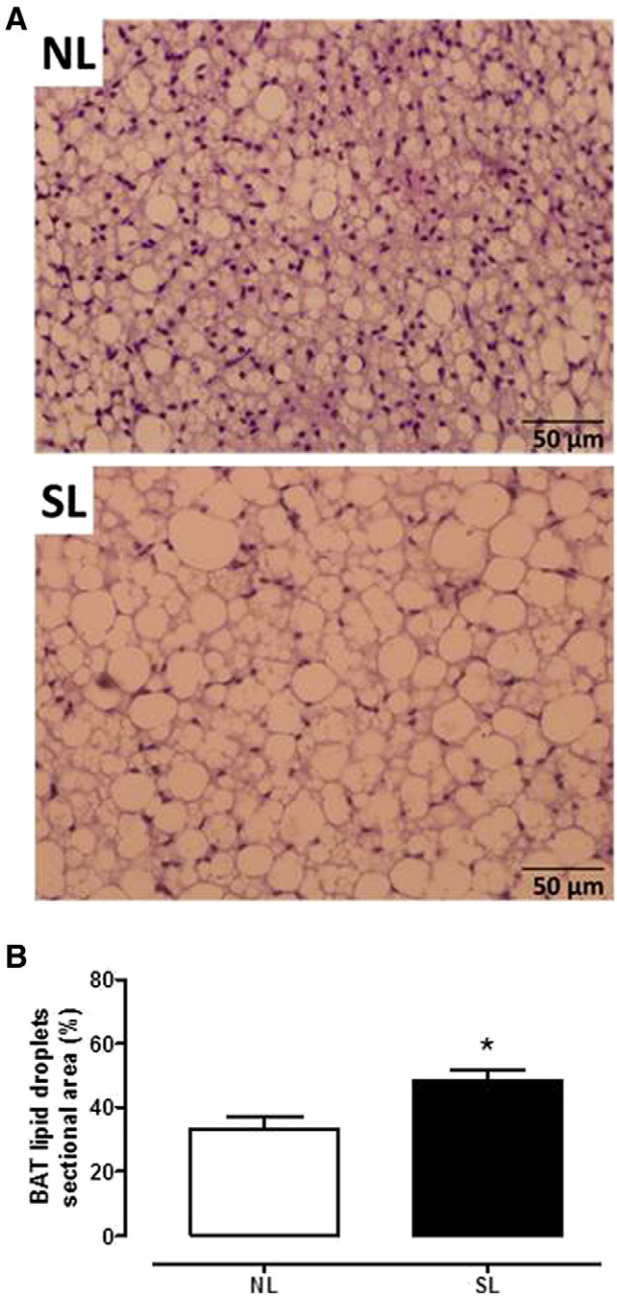

Fig. 3. Long-term effects of reducing litter size on BAT morphology at PN120. Representative hematoxylin and eosin staining (A) of BAT at PN120 in rats raised in normal litters (NL) and small litters (SL) during lactation. The quantitative analysis of the sectional areas (B) of BAT lipid vacuoles is shown. Results are expressed as mean± S.E.M.; $n=10$ per group; * $P<.05$.

3.7. Hypothalamic POMC and MC4R protein content

At PN120, POMC protein content (Fig. 7A) in the ARC was unaffected in the SL group, although at PN180, its content was lower in SL rats than in NL ones (−44%; $P=.006$). At both ages, MC4R protein content (Fig. 7B) in the PVN was reduced in SL animals when compared to NL ones (PN120: −32%; $P<.01$. PN180: −48%; $P<.01$). Two months of calcium supplementation did not reverse POMC and MC4R contents in the SL-Ca group.

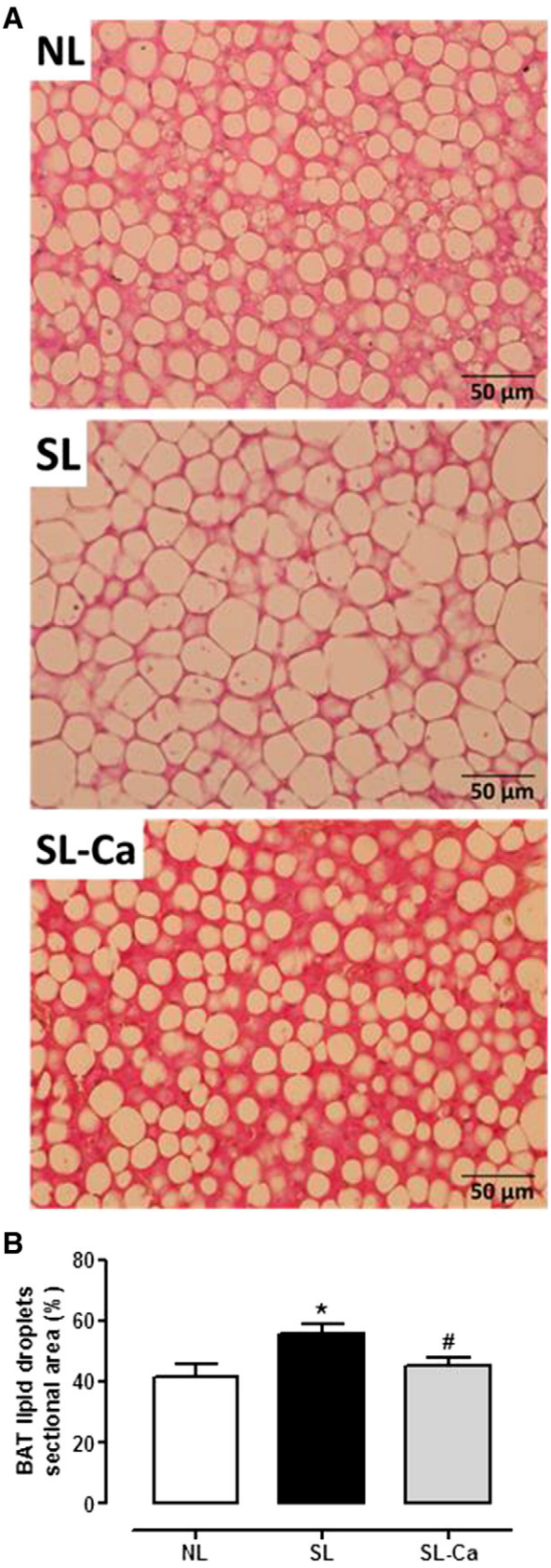

Fig. 4. Long-term effects of reducing litter size on BAT morphology at PN180. Representative hematoxylin and eosin staining (A) of BAT at PN180 in rats raised in normal litters (NL), small litters (SL) during lactation, and in SL rats supplemented with calcium from PN120 until PN180 (SL-Ca). The quantitative analysis of the sectional areas (B) of BAT lipid vacuoles is shown. Results are expressed as mean±S.E.M.; $n=10$ per group; * $P<.05$.

4. Discussion

It is well known that an imbalance of the autonomic nerve system plays an important contribution in the development of obesity and cardiovascular diseases. The reduced litter size, which is a model of childhood obesity, results in a significant increase in cardiovascular risk during development. In fact, Habbout and colleagues [32] have shown that SL rats have cardiac hypertrophy and high sensitivity to ischemia at PN180. Furthermore, these rats have hypertension [33] and microvascular dysfunction at PN147 [34], probably due to a disruption in autonomic function. Recently, we have shown, in rats, that obesity at weaning (PN21), caused by reducing litter size, does not alter autonomic nerve activity in the BAT and catecholamine production in the adrenal medulla [17]. However, at adulthood (PN180), these rats develop increased adrenal catecholamine production and secretion, evidencing a sympathoadrenal disruption [22]. The present work reproduced these findings (higher TH and catecholamine content), suggesting that SL rats have a higher serum catecholamine, possibly a contributing factor for the higher heart frequency and blood pressure observed in the present study. The blood pressure seems to become worse with age in the SL group.

Autonomic dysfunction has an important role in obesity development [35,36] since BAT expends energy as heat when it is stimulated by the ANS [37], contributing to the control of body mass and adiposity. Norepinephrine stimulates BAT function, increasing UCP1 content, mitochondriogenesis, blood flow, glucose uptake and thermogenesis [38]. It has been previously reported [10] that PN180 SL rats have hypothyroidism. Here, we showed that they also present reduced TRβ1 content. It is known that thyroid hormones upregulate BAT UCP1 [39]. Thus, the lack of thyroid hormone action in this model may be responsible for the lower BAT thermogenesis. This finding is in accordance with the observed increase in BAT mass associated with larger lipid droplet vacuoles in the SL group, a finding that has been previously reported in some models of hypothyroidism [40,41].

The main BAT function is body thermoregulation preventing against hypothermia. Therefore, BAT ANS activity also depends on the perception of environmental temperature by peripheral nerves and its integration with central pathways of thermoregulation [42]. SL rats presented an increase in nerve firing rate both at PN120 and PN180. However, it seems that this higher activity in the overweight animals undergoes attenuation with increasing age, which was has not been observed in the NL group. This pattern is also observed for BAT β3ADR, since PN120 SL rats presented higher β3ADR, while at PN180, no increase was observed. It is possible that the development of hypothyroidism in the older animals explains the impairment in BAT ANS activation and action, since it is known that thyroid hormones increase the amount of BAT adrenergic receptors. These data help to explain why those animals during aging did not maintain adequately BAT UCP1.

The levels of endogenous MC4R agonist, α-MSH derived of the proteolytic cleavage from POMC, is a good indicator of central sympathetic activity [43]. A reduced POMC level has been associated with the obese phenotype in humans [44] and animals [45,46]. SL rats presented lower POMC content at PN180, which is in agreement with their overweight and hyperphagia. In fact, Plagemann and colleagues [47] reported that SL rats present POMC promotor hypermethylation at PN21, which represents loss of function during development. In addition, these rats do not have the anorexigenic neuronal ARC response to leptin and insulin at weaning [47]. Our present results suggest that these characteristics persist until PN180.

As POMC neurons in the ARC projects to the PVN, the integrity of the melanocortin system also depends on the PVN sensitivity mediated by the MCRs content [43]. It has been reported that MC4R deficiency causes hyperphagia and reduced energy expenditure in mice [48] and humans [49]. Our data showed that MC4R content in the PVN was reduced in SL rats at both PN120 and PN180. Our

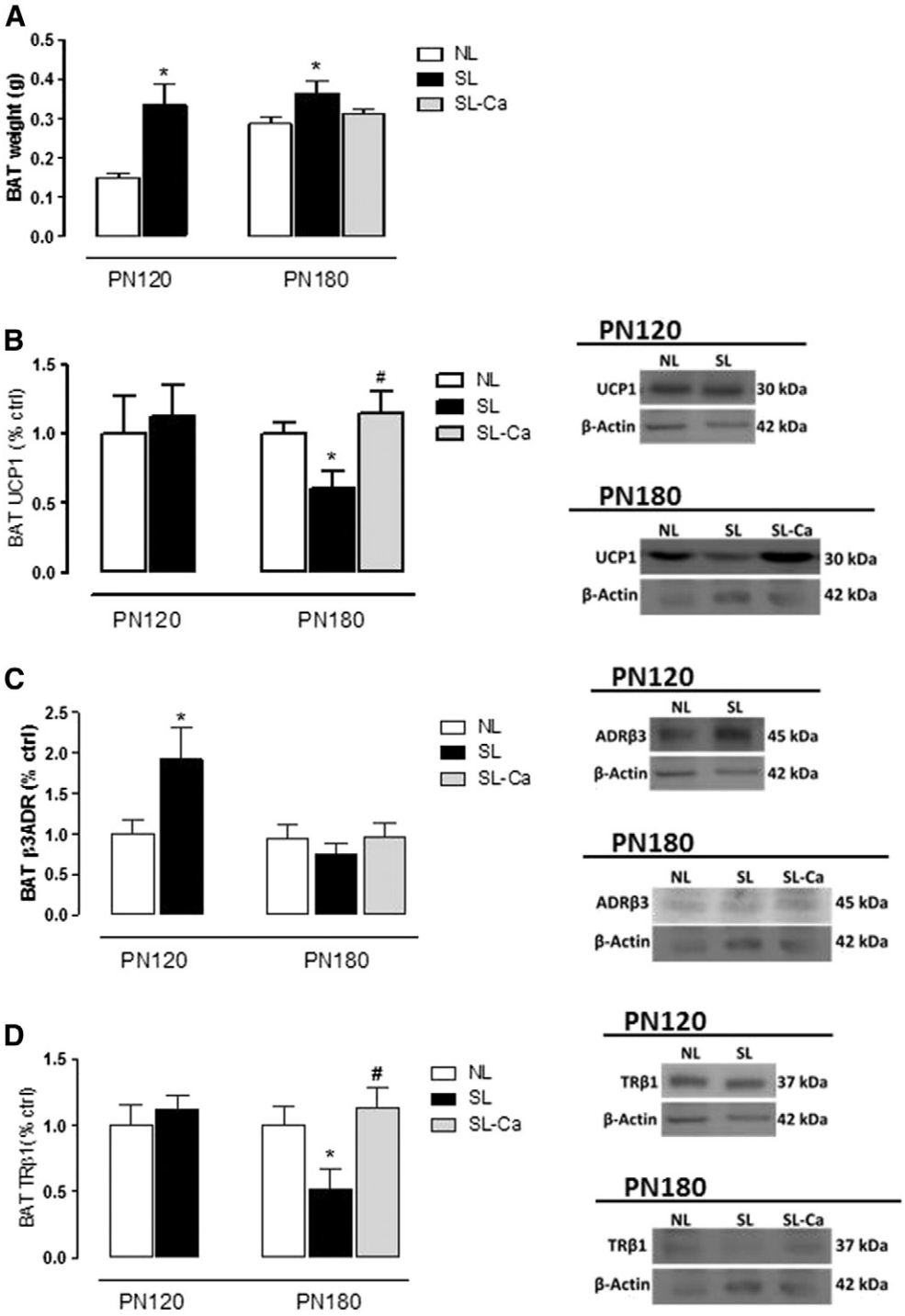

Fig. 5. Long-term effects of reducing litter size on BAT functional parameters. BAT weight (A), UCP1 (B), β3ADR (C) and TRβ1 (D) protein content at PN120 and PN180. Rats raised in normal (NL) and small (SL) litters during lactation. SL rats supplemented with calcium from PN120 until PN180 (SL-Ca). Representative blots of the proteins are shown beside the graphs. β-Actin content was used as control loading. Results are expressed as relative (%) to the control group and as mean±S.E.M.; n=7–10 per group; * P<.05.

findings corroborate a previous study in MC4R-null mice [50], which presented higher body mass, visceral fat and hyperphagia. In addition, Davidowa and colleagues [51] demonstrated that SL rats have reduced neuronal response to melanocortin at PN21 (weaning). These data suggest that precocious obesity causes a long-term "malprogramming" of the PVN response to melacortins.

The central control of BAT ANS occurs through the activation of MC4R in the PVN [41]. Microinjection of melanotan II (a MC3R and MC4R agonist) into the third ventricle and PVN increases BAT norepinephrine turnover [52] and BAT temperature [53]. Considering the reduced POMC and MC4R contents, a decrease in BAT ANS activity would be expected. However, as we have shown, both an increase in ANS firing rate and that in β3ADR content in the BAT were present at PN120. Indeed, a similar finding was observed in MC4R KO mice, which are obese and present increased BAT temperature [54]. It must be pointed out that this group did not evaluate ANS firing rate. Thus,

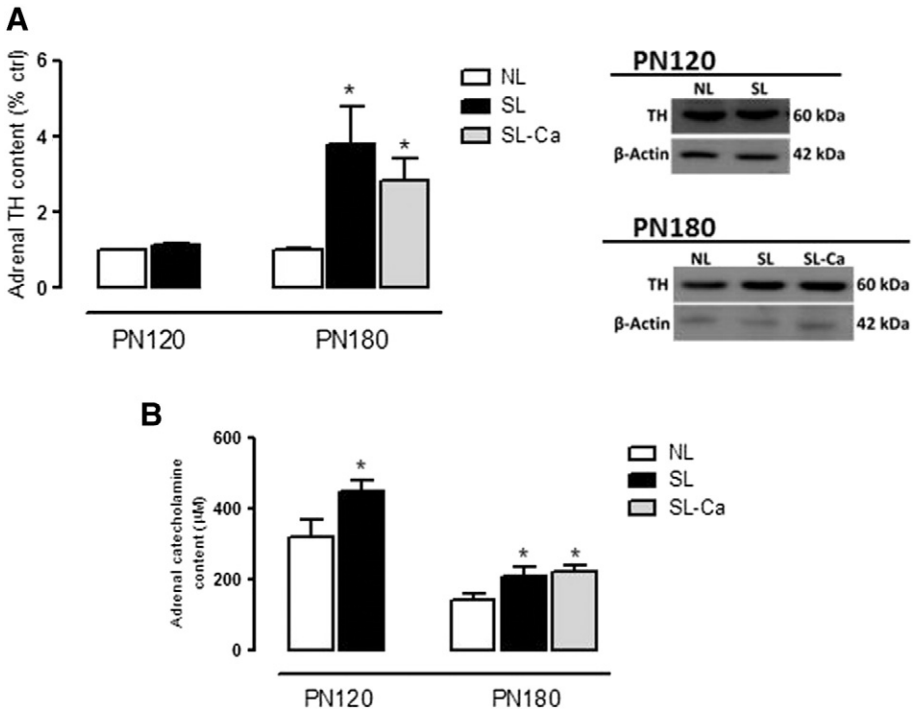

Fig. 6. Long-term effects of reducing litter size on adrenal function. Adrenal tyrosine hydroxylase (TH) protein content (A) and total adrenal catecholamine content (B) at PN120 and PN180. Rats raised in normal (NL) and small (SL) litters during lactation. SL rats supplemented with calcium from PN120 until PN180 (SL-Ca). Representative blots of proteins are shown beside the graphs. β-Actin content was used as control loading. Results are expressed as relative (%) to the control group and as mean±S.E.M.; n=7–10 per group; * P<.05.

these data indicate that the melanocortin system in the ARC and PVN is not the unique regulator of BAT ANS activity. Notwithstanding, the impairment in POMC at PN180 may help explain why the increased ANS activity in overweight animals is attenuated with age.

4.1. Effects of dietary calcium supplementation

The calcium supplementation for 6 weeks reduced body mass gain in mice [55]. Torres and colleagues [56] showed that a calcium-rich

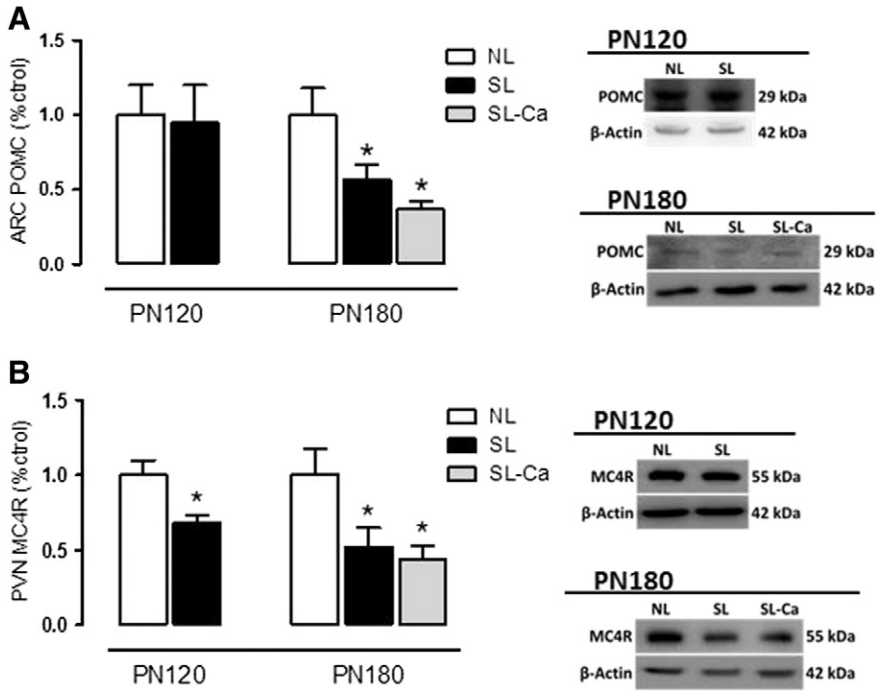

Fig. 7. Long-term effects of reducing litter size on POMC and MC4R in hypothalamus. Protein content of POMC (A) in the ARC and MC4R (B) in the PVN at PN120 and PN180. Rats raised in normal (NL) and small (SL) litters during lactation. SL rats supplemented with calcium from PN120 until PN180 (SL-Ca). Representative blots of proteins are shown beside the graphs. β-Actin content was used as control loading. Results are expressed as relative (%) to the control group and as mean±S.E.M.; n=6–9 per group; * P<.05.

diet in hypertensive patients leads to body mass loss and lower adiposity. In a similar study, the same group, evaluating postmenopausal women, showed improvement in body mass, abdominal circumference, body fat, insulin resistance and mean and diastolic blood pressures [57]. Here, we evidence that 2 months of dietary calcium supplementation also has an antiobesogenic effect in adult hyperphagic obese animals that were overfed during lactation. It has already been described that calcium supplementation has relevant results in the treatment of cardiovascular diseases [58,59]. On the other hand, a meta-analysis study suggested adverse effects of calcium supplementation, increasing cardiovascular risk [60]. In our study, calcium supplementation had no undesirable cardiovascular effects. On the contrary, it improved, in SL rats, both systolic and diastolic blood pressures, as well as heart rate. In the present experiment, since BAT ANS activity was normalized by calcium, the same phenomenon may be occurring in the sympathetic innervation of the heart and vessels, explaining the effects upon cardiovascular parameters. In addition, our group has previously reported that adrenal medulla hyperfunction in adult obese rats from dams exposed to nicotine during lactation is reverted by calcium supplementation [29]. Here, however, dietary calcium treatment was unable to revert the higher adrenal catecholamine production and BAT β3ADR, as well as the lower expression of hypothalamic POMC and MC4R in the SL groups. Nevertheless, calcium restored BAT morphology, UCP-1 and TRβ1 contents, which could be attributed to the normalization of the BAT ANS activity and, possibly, the thyroid function. One limitation of our study was not to measure thyroid hormones after dietary calcium. In humans, dietary calcium supplementation did not change serum calcium or PTH, but caused an increase in thermogenesis, measured by indirect calorimetry, and lower caloric intake, by reducing the carbohydrate intake, without adverse effect [61]. The current study is the first to characterize the repercussion of dietary calcium supplementation on heart frequency, blood pressure, BAT sympathetic nerve activity, BAT morphology and function in obese rats that were raised in small litters.

Our results show that BAT ANS stimulation is important as a defense mechanism against the development of obesity that is gradually lost during aging. Impairment in POMC production and action in old overweighed animals may be partially responsible for BAT function and morphology, which is suggestive of a "whitening" effect on the brown fat [62]. Concerning dietary calcium supplementation, dietary calcium supplementation was able to normalize the BAT ANS activity and morphology, suggesting the restoration of this tissue's normal morphology and function. Therefore, the present study suggests another mechanism, induced by calcium supplementation, which results in a better metabolic profile through the correction of BAT dysfunction, independently of BAT melanocortin system regulation.

Competing interests

The authors declare that there is no competing interest that could be perceived as prejudicing the impartiality of the research reported.

Author contributions

Conception and design: EPSC, EGM, PCL. Animal treatment, collection and measurements: EPSC, DSG, MSF, FTQ, CC. Analysis and interpretation of data: EPSC, EGM, EO, PCFM, ACM, PCL. Drafting and/or revising the article critically for important intellectual content: EPSC, EGM, EO, ACM, PCL.

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References

[1] Medina-Gómez G. Mitochondria and endocrine function of adipose tissue. Best Pract Res Clin Endocrinol Metab 2012;26:791–804.
[2] Oelkrug R, Polymeropoulos ET, Jastroch M. Brown adipose tissue: physiological function and evolutionary significance. J Comp Physiol B 2015;185:587–606.
[3] Schulz TJ, Tseng YH. Systemic control of brown fat thermogenesis: integration of peripheral and central signals. Ann N Y Acad Sci 2013;1302:35–41.
[4] Vaughan CH, Shrestha YB, Bartness TJ. Characterization of a novel melanocortin receptor-containing node in the SNS outflow circuitry to brown adipose tissue involved in thermogenesis. Brain Res 2011;1411:17–27.
[5] Ward KR, Bardgett JF, Wolfgang L, Stocker SD. Sympathetic response to insulin is mediated by melanocortin 3/4 receptors in the hypothalamic paraventricular nucleus. Hypertension 2011;57:435–41.
[6] Li P, Cui BP, Zhang LL, Sun HJ, Liu TY, Zhu GQ. Melanocortin 3/4 receptors in paraventricular nucleus modulate sympathetic outflow and blood pressure. Exp Physiol 2013;98:435–43.
[7] Kooijman S, Boon MR, Parlevliet ET, Geerling JJ, van de Pol V, Romijn JA, et al. Inhibition of the central melanocortin system decreases brown adipose tissue activity. J Lipid Res 2014;55:2022–32.
[8] Wu C, Cheng W, Sun Y, Dang Y, Gong F, Zhu H, et al. Activating brown adipose tissue for weight loss and lowering of blood glucose levels: a microPET study using obese and diabetic model mice. PLoS One 2014;9, e113742.
[9] Plagemann A, Heidrich I, Götz F, Rohde W, Dörner G. Obesity and enhanced diabetes and cardiovascular risk in adult rats due to early postnatal overfeeding. Exp Clin Endocrinol 1992;99:154–8.
[10] Rodrigues AL, de Moura EG, Passos MC, Dutra SC, Lisboa PC. Postnatal early overnutrition changes the leptin signalling pathway in the hypothalamic–pituitary-thyroid axis of young and adult rats. J Physiol 2009;587:2647–61.
[11] Conceição EP, Franco JG, Oliveira E, Resende AC, Amaral TA, Peixoto-Silva N, et al. Oxidative stress programming in a rat model of postnatal early overnutrition — role of insulin resistance. J Nutr Biochem 2013;24:81–7.
[12] Ozturk Y, Soylu OB. Fatty liver in childhood. World J Hepatol 2014;27:33–40.
[13] Tounian P. Programming towards childhood obesity. Ann Nutr Metab 2011;58:30–41.
[14] Sabin MA, Kiess W. Childhood obesity: current and novel approaches. Best Pract Res Clin Endocrinol Metab 2015;29:327–38.
[15] Cunha AC, Pereira RO, Pereira MJ, Soares VdM, Martins MR, Teixeira MT, et al. Long-term effects of overfeeding during lactation on insulin secretion—the role of GLUT-2. J Nutr Biochem 2009;20:435–42.
[16] Habbout A, Guenancia C, Lorin J, Rigal E, Fassot C, Rochette L, et al. Postnatal overfeeding causes early shifts in gene expression in the heart and long-term alterations in cardiometabolic and oxidative parameters. PLoS One 2013;8, e56981.
[17] Conceição EP, Moura EG, Carvalho JC, Oliveira E, Lisboa PC. Early redox imbalance is associated with liver dysfunction at weaning in overfed rats. J Physiol 2015;593:4799–811.
[18] Xiao XQ, Williams SM, Grayson BE, Glavas MM, Cowley MA, Smith MS, et al. Excess weight gain during the early postnatal period is associated with permanent reprogramming of brown adipose tissue adaptive thermogenesis. Endocrinology 2007;148:4150–9.
[19] Almeida DL, Fabricio GS, Trombini AB, Pavanello A, Ribeiro TAS, Mathias PCF, et al. Early overfeed-induced obesity leads to brown adipose tissue hypoactivity in rats. Cell Physiol Biochem 2013;32:1621–30.
[20] Troisi RJ, Weiss ST, Parker DR, Sparrow D, Young JB, Landsberg L. Relation of obesity and diet to sympathetic nervous system activity. Hypertension 1991;17:669–77.
[21] Del Rio G. Adrenomedullary function and its regulation in obesity. Int J Obes Relat Metab Disord 2000;24:S89–91.
[22] Conceição EP, Moura EG, Trevenzoli IH, Peixoto-Silva N, Pinheiro CR, Younes-Rapozo V, et al. Neonatal overfeeding causes higher adrenal catecholamine content and basal secretion and liver dysfunction in adult rats. Eur J Nutr 2013;52:1393–404.
[23] Zheng J, Xiao X, Zhang Q, Yu M, Xu J, Wang Z, et al. Maternal and post-weaning high-fat, high-sucrose diet modulates glucose homeostasis and hypothalamic POMC promoter methylation in mouse offspring. Metab Brain Dis 2015;30:1129–37.

[24] Kim JD, Leyva S, Diano S. Hormonal regulation of the hypothalamic melanocortin system. Front Physiol 2014;9:480.
[25] Hu J, Jiang L, Low MJ, Rui L. Glucose rapidly induces different forms of excitatory synaptic plasticity in hypothalamic POMC neurons. PLoS One 2014;9, e105080.
[26] Conceição EP, Moura EG, Soares PN, Ai XX, Figueiredo MS, Oliveira E, et al. High calcium diet improves the liver oxidative stress and microsteatosis in adult obese rats that were overfed during lactation. Food Chem Toxicol 2016;92:245–55. http://dx.doi.org/10.1016/j.fct.2016.04.015.
[27] Conceição EP, Carvalho JC, Manhães AC, Guarda DS, Figueiredo MS, Quitete FT, et al. Effect of early overfeeding on palatable food preference and brain dopaminergic reward system at adulthood: role of calcium supplementation. J Neuroendocrinol 2016;28(5). http://dx.doi.org/10.1111/jne.12380.
[28] Nobre JL, Lisboa PC, Lima Nda S, Franco JG, Nogueira Neto JF, de Moura EG, et al. Calcium supplementation prevents obesity, hyperleptinaemia and hyperglycaemia in adult rats programmed by early weaning. Br J Nutr 2012;107:979–88.
[29] Nobre JL, Lisboa PC, Santos-Silva AP, Lima NS, Manhães AC, Nogueira-Neto JF, et al. Calcium supplementation reverts central adiposity, leptin, and insulin resistance in adult offspring programed by neonatal nicotine exposure. J Endocrinol 2011;210:349–59.
[30] Scomparin DX, Gomes RM, Grassiolli S, Rinaldi W, Martins AG, de Oliveira JC, et al. Autonomic activity and glycemic homeostasis are maintained by precocious and low intensity training exercises in MSG-programmed obese mice. Endocrine 2009;36:510–7.
[31] Paxinos G, Watson C. The rat brain in stereotaxic coordinates. 4th ed. Academic Press; 1998.
[32] Habbout A, Delemasure S, Goirand F, Guilland JC, Chabod F, Sediki M, et al. Postnatal overfeeding in rats leads to moderate overweight and to cardiometabolic and oxidative alterations in adulthood. Biochimie 2012;94:117–24.
[33] Boubred F, Daniel L, Buffat C, Feuerstein JM, Tsimaratos M, Oliver C, et al. Early postnatal overfeeding induces early chronic renal dysfunction in adult male rats. Am J Physiol Renal Physiol 2009;297:F943–51.
[34] Leite RD, Kraemer-Aguiar LG, Boa BC, Cyrino FZ, Nivoit P, Bouskela E. Muscle endothelial-dependent microvascular dysfunction in adult-hood due to early postnatal overnutrition. Microvasc Res 2012;84:94–8.
[35] Lima JJ, Feng H, Duckworth L, Wang J, Sylvester JE, Kissoon N, et al. Association analyses of adrenergic receptor polymorphisms with obesity and metabolic alterations. Metabolism 2007;56:757–65.
[36] Messina G, De Luca V, Viggiano A, Ascione A, Iannaccone T, Chieffi S, et al. Autonomic nervous system in the control of energy balance and body weight: personal contributions. Neurol Res Int 2013;639280.
[37] Tan CY, Ishikawa K, Virtue S, Vidal-Puig A. Brown adipose tissue in the treatment of obesity and diabetes: are we hot enough? J Diabetes Investig 2011;2:341–50.
[38] Bartness TJ, Vaughan CH, Song CK. Sympathetic and sensory innervation of brown adipose tissue. Int J Obes (Lond) 2010;34:S36–42.
[39] Obregon MJ. Adipose tissues and thyroid hormones. Front Physiol 2014;5:479.
[40] Pazos-Moura CC, Moura EG, Dorris ML, Rehnmark S, Melendez L, Silva JE, et al. Effect of iodine deficiency and cold exposure on thyroxine 5′-deiodinase activity in various rat tissues. Am J Physiol 1991;260:E175–82.
[41] Lombardi A, Senese R, De Matteis R, Busiello RA, Cioffi F, Goglia F, et al. 3,5-Diiodo-L-thyronine activates brown adipose tissue thermogenesis in hypothyroid rats. PLoS One 2015;10, e0116498.
[42] Morrison SF, Madden CJ, Tupone D. Central neural regulation of brown adipose tissue thermogenesis and energy expenditure. Cell Metab 2014;19:741–56.
[43] Millington GW. The role of proopiomelanocortin (POMC) neurones in feeding behaviour. Nutr Metab (Lond) 2007;1:4–18.
[44] Krude H, Biebermann H, Luck W, Horn R, Brabant G, Gruters A. Severe early-onset obesity, adrenalin sufficiency and red hair pigmentation caused by POMC mutations in humans. Nat Genet 1998;19:155–7.
[45] Greenman Y, Kuperman Y, Drori Y, Asa SL, Navon I, Forkosh O, et al. Postnatal ablation of POMC neurons induces an obese phenotype characterized by decreased food intake and enhanced anxiety-like behavior. Mol Endocrinol 2013;27:1091–102.
[46] Gropp E, Shanabrough M, Borok E, Xu AW, Janoschek R, Buch T, et al. Agouti-related peptide-expressing neurons are mandatory for feeding. Nat Neurosci 2005;8:1289–91.
[47] Plagemann A, Harder T, Brunn M, Harder A, Roepke K, Wittrock-Staar M, et al. Hypothalamic proopiomelanocortin promoter methylation becomes altered by early overfeeding: an epigenetic model of obesity and the metabolic syndrome. J Physiol 2009;587:4963–76.
[48] Huszar D, Lynch CA, Fairchild-Huntress V, Dunmore JH, Fang Q, Berkemeier LR, et al. Targeted disruption of the melanocortin-4 receptor results in obesity in mice. Cell 1997;88:131–41.
[49] Ho G, MacKenzie RG. Functional characterization of mutations in melanocortin-4 receptor associated with human obesity. J Biol Chem 1999;274:35816–22.
[50] Ste Marie L, Miura GI, Marsh DJ, Yagaloff K, Palmiter RD. A metabolic defect promotes obesity in mice lacking melanocortin-4 receptors. Proc Natl Acad Sci U S A 2000;97:12339–44.
[51] Davidowa H, Li Y, Plagemann A. Altered responses to orexigenic (AGRP, MCH) and anorexigenic (alpha-MSH, CART) neuropeptides of paraventricular hypothalamic neurons in early postnatally overfed rats. Eur J Neurosci 2003;18:613–21.
[52] Brito MN, Brito NA, Baro DJ, Song CK, Bartness TJ. Differential activation of the sympathetic innervation of adipose tissues by melanocortin receptor stimulation. Endocrinology 2007;148:5339–47.
[53] Song CK, Vaughan CH, Keen-Rhinehart E, Harris RB, Richard D, Bartness TJ. Melanocortin-4 receptor mRNA expressed in sympathetic outflow neurons to brown adipose tissue: neuroanatomical and functional evidence. Am J Physiol 2008;295:R417–28.
[54] Enriori PJ, Sinnayah P, Simonds SE, Garcia Rudaz C, Cowley MA. Leptin action in the dorsomedial hypothalamus increases sympathetic tone to brown adipose tissue in spite of systemic leptin resistance. J Neurosci 2011;31:12189–97.
[55] Zemel MB, Shi H, Greer B, Dirienzo D, Zemel PC. Regulation of adiposity by dietary calcium. FASEB J 2000;14(9):1132–8.
[56] Torres MR, Ferreira T, da S, Carvalho DC, Sanjuliani AF. Dietary calcium intake and its relationship with adiposity and metabolic profile in hypertensive patients. Nutrition 2011;27(6):666–71. http://dx.doi.org/10.1016/j.nut.2010.07.012.
[57] da Silva Ferreira T, Torres MR, Sanjuliani AF. Dietary calcium intake is associated with adiposity, metabolic profile, inflammatory state and blood pressure, but not with erythrocyte intracellular calcium and endothelial function in healthy premenopausal women. Br J Nutr 2013;110(6):1079–88. http://dx.doi.org/10.1017/S0007114513000111.
[58] Waldman T, Sarbaziha R, Merz CN, Shufelt C. Calcium supplements and cardiovascular disease: a review. Am J Lifestyle Med 2015;9:298–307.
[59] Cormick G, Ciapponi A, Cafferata ML, Belizán JM. Calcium supplementation for prevention of primary hypertension. Cochrane Database Syst Rev 2015;30, CD010037.
[60] Bolland MJ, Avenell A, Baron JA, Grey A, MacLennan GS, Gamble GD, et al. Effect of calcium supplements on risk of myocardial infarction and cardiovascular events: meta-analysis. BMJ 2010;341:c3691.
[61] Ping-Delfos WC, Soares M. Diet induced thermogenesis, fat oxidation and food intake following sequential meals: influence of calcium and vitamin D. Clin Nutr 2011;30(3):376–83. http://dx.doi.org/10.1016/j.clnu.2010.11.006.
[62] Shimizu I, Walsh K. The whitening of Brown fat and its implications for weight management in obesity. Curr Obes Rep 2015;4(2):224–9. http://dx.doi.org/10.1007/s13679-015-0157-8.