Calcium supplementation reverts central adiposity, leptin, and insulin resistance in adult offspring programmed by neonatal nicotine exposure

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Abstract

Obesity is a worldwide epidemic. Calcium influences energy metabolism regulation, causing body weight loss. Because maternal nicotine exposure during lactation programs for obesity, hyperleptinemia, insulin resistance (IR), and hypothyroidism, we decided to evaluate the possible effect of dietary calcium supplementation on these endocrine dysfunctions in this experimental model. Osmotic minipumps containing nicotine solution (N: 6 mg/kg per day for 14 days) or saline (C) were s.c. implanted in lactating rats 2 days after giving birth (P2). At P120, N and C offspring were subdivided into four groups: 1) C – standard diet; 2) C with calcium supplementation (CCa, 10 g calcium carbonate/kg rat chow); 3) N – standard diet; and 4) N with calcium supplementation (NCa). Rats were killed at P180. As expected, N offspring showed higher visceral and total body fat, hyperleptinemia, lower hypothalamus leptin receptor

(OB-R) content, hyperinsulinemia, and higher IR index. Also, higher tyrosine hydroxylase (TH) expression (+51%), catecholamine content (+37%), and serum 25-hydroxy-vitamin D₃ (+76%) were observed in N offspring. Dietary calcium supplementation reversed adiposity, hyperleptinemia, OB-R underexpression, IR, TH overexpression, and vitamin D. However, this supplementation did not reverse hypothyroidism. In NCa offspring, *Sirt1* mRNA was lower in visceral fat (–37%) and higher in liver (+42%). In conclusion, dietary calcium supplementation seems to revert most of the metabolic syndrome parameters observed in adult offspring programmed by maternal nicotine exposure during lactation. It is conceivable that the reduction in fat mass *per se*, induced by calcium therapy, is the main mechanism that leads to the increment of insulin action.

Journal of Endocrinology (2011) **210**, 349–359

Introduction

According to the World Health Organization (WHO), obesity is considered a global epidemic surpassing malnutrition (Kosti & Panagiotakos 2006, WHO. Obesity e overweight. <http://www.who.int/dietphysicalactivity/childhood/en> 2007). Obesity is associated to dyslipidemia, insulin resistance (IR), and hypertension, which play key roles in raising the morbidity and mortality rates in cardiovascular disease in adulthood (Schulze *et al.* 2004). In several cases, some of these alterations can simultaneously appear, a condition called metabolic syndrome, which presents central obesity and peripheral IR as the major components (Weiss *et al.* 2004).

Experimental and epidemiological studies show an association between exposure to nutritional or hormonal factors during critical periods of life (such as pregnancy and/or lactation) with the development of chronic diseases in adulthood, such as obesity and type 2 diabetes. This biological phenomenon is known as metabolic programming

(Barker 2003, Moura & Passos 2005, de Moura *et al.* 2008). Exposure to environmental pollutants during perinatal life can lead to several changes in both the short and the long term, acting as endocrine disruptors (Newbold 2010). Smoking during pregnancy is a known risk factor for obesity in adult life (Blake *et al.* 2000, Bergmann *et al.* 2003, Goldani *et al.* 2007). Recently, we have found that maternal exposure to nicotine during lactation programs for obesity in adulthood, along with additional dysfunctions such as higher central adiposity, hyperleptinemia, IR and leptin resistance, and secondary hypothyroidism (Oliveira *et al.* 2009, de Oliveira *et al.* 2010). Maternal smoking increases catecholamine levels in the amniotic fluid (Divers *et al.* 1981). Recently, we have shown that maternal nicotine during lactation increased both leptin serum levels and catecholamine content in the adrenal gland of suckling pups (Oliveira *et al.* 2010); however, it is unknown whether catecholamine function is programmed in the adult animal. It is suggested that this could happen, because in another model of programming by neonatal hyperleptinemia, the adrenal catecholamine content was

higher after leptin administration and this primed those animals to develop hyperleptinemia and higher adrenal catecholamine content in adulthood (Trevenzoli *et al.* 2007).

It was shown that individuals who have poor calcium intake present higher body weight (BW; Zemel 2002). Furthermore, a calcium-rich diet is known to improve insulin sensitivity (Choi *et al.* 2005, Ma *et al.* 2006, Pittas *et al.* 2007) and lipid profile (Jacqmain *et al.* 2003, Reid *et al.* 2010). One of the hypotheses that may explain the beneficial effects of dietary calcium supplementation relies on the fact that calcium has the ability to modulate energy metabolism through calcitropic hormone concentrations: calcitriol (1,25-dihydroxyvitamin D (1,25(OH)₂D)) and parathyroid hormone (PTH; Zemel 2002). Vitamin D increases calcium uptake by the adipocyte, decreasing UCP activity, lipolysis, and apoptosis. With high-calcium ingestion, PTH and vitamin D levels are decreased and the opposite effects are reported for the adipocyte. A diet that is poor in calcium could inhibit lipolysis, stimulate lipogenesis, and decrease lipid oxidation. Thus, a calcium-rich diet could decrease these hormone concentrations, thus decreasing lipid storage (Zemel 2002, Xiaoyu *et al.* 2007). Another hypothesis is that calcium has the ability to form insoluble complexes with lipids in the intestine, increasing fecal excretion and decreasing their absorption, which reduces the available energy to the organism, contributing to its anti-obesity effect (Zemel 2005, Teegarden *et al.* 2008).

Considering that dietary calcium therapy could have a role in weight loss, this study was designed to test the possible beneficial effects of calcium supplementation in reversing some endocrine-metabolic changes, such as central adiposity, leptin and IR, and thyroid hypofunction, which have been previously detected in adult rat offspring whose mothers were exposed to nicotine during lactation. As catecholamine was affected in the suckling pups by maternal nicotine exposure, we also intend to evaluate catecholamine function in the adult animal, because both catecholamine and thyroid hormones affect body adiposity. In this study, we specifically studied the effects of dietary calcium supplementation in animals exposed to nicotine during the lactation period instead of during pregnancy, because there is a higher rate of smoking relapse among lactating women (McBride & Pirie 1990). In addition, in order to have some insight into possible factors involved in priming mechanisms, we studied sirtuin 1 (SIRT1) expression, a class III histone/protein NAD⁺-dependent deacetylase that has been implicated in the regulation of energy homeostasis (Elliott & Jirousek 2008, Liang *et al.* 2009) and that can also be suppressed by smoking (Rajendrasozhan *et al.* 2008). Furthermore, increased SIRT1 has been associated with lower adiposity and protection against diet-induced metabolic disorders (Banks *et al.* 2008, Pfluger *et al.* 2008). Therefore, we reasoned that SIRT1 expression may be altered by programming and/or calcium treatment, as we have previously reported for other programming models (Franco *et al.* 2010, Trotta *et al.* 2011).

Materials and Methods

Animal use and experimental procedures were approved by the Animal Care and Use Committee of the Biology Institute of the State University of Rio de Janeiro (CEA/189/2007 and CEA/015/2009), which based its analysis on the principles promulgated by the Brazilian Law no. 11.794/2008 (Marques *et al.* 2009). Wistar rats were kept in a temperature-controlled room (25 ± 1 °C) with artificial light-darkness cycles (lights on 0700 h, lights off 1900 h). Virgin female rats (3-month-old) were caged with male rats at the ratio of 3:1. After mating, each female rat was placed in an individual cage with free access to food and water until delivery.

Experimental model of programming by maternal nicotine exposure

In total, 2 days after giving birth, 20 lactating rats were randomly assigned to one of the following groups: nicotine (N) – 2 days after giving birth (P2), ten dams were lightly anesthetized with thiopental to allow s.c. insertion of osmotic minipumps (Alzet, 2ML2, Los Angeles, CA, USA). Minipumps were filled with nicotine (NIC) free base diluted in 0.9% NaCl so as to deliver a dose rate of 6 mg/kg per day for 14 days of lactation, as described previously (Oliveira *et al.* 2009). This dose produces plasma nicotine levels similar to those observed in moderate to heavy smokers, ~25 ng/ml (Lichtensteiger *et al.* 1988). Control (C) – ten dams were implanted with osmotic minipumps containing saline solution only, which were used for the same period mentioned above. According to the manufacturer's recommendation, minipumps must be filled with the solution of interest (in our case, nicotine or saline) and immersed in saline for 24 h prior to implantation to release substances continuously and homogeneously thereafter, a procedure that resulted in the P2 implantation.

In general, pregnant rats produce 10–12 pups. To avoid the influence of the litter size in the programming effect, we only used dams whose litter size was ten pups. At birth, to maximize lactation performance, litters were adjusted to six male pups per N or C dam.

Dietary calcium supplementation

We decided to start calcium supplementation at P120 to evaluate whether this substance has a role in reverting central adiposity, because we have previously shown that by P90 N offspring already present higher central obesity and BW (Oliveira *et al.* 2009). N and C offspring were subdivided into four groups (*n* = 10 offspring per group): 1) control (C) – received standard rat chow; 2) control calcium (CCa) – received standard rat chow supplemented with calcium carbonate (10 g/kg rat chow); 3) nicotine (N) – received standard rat chow; and 4) nicotine calcium (NCa) – received standard rat chow supplemented with calcium carbonate. BW and food intake of the offspring were evaluated every 4 days.

Calcium carbonate was added to the standard chow. The calcium-enriched diet provided twice the amount of calcium (in the form of calcium carbonate) that is recommended for rodents, which is 5 g calcium/kg of chow (Reeves 1997). This amount is based on the recommendation of supplementation for humans, where values up to two times the recommended amount have no toxic effect. Calcium was supplemented from P120 to P180, at which time all rats were killed by quick decapitation, with no prior anesthesia because it affects hormone and lipid metabolism (Chen *et al.* 2002). Blood, hypothalamus, liver, adrenal gland, carcass, and visceral fat were excised and kept frozen (-80°C). Calcemia was analyzed using colorimetric Biosystem commercial test kits. The metabolite 25-hydroxyvitamin D_3 was measured using a monoclonal antibody immunoassay (Elecsys and Cobas immunoassay analyzers, Roche Diagnostics GmbH), with a range of detection from 4 to 100 ng/ml. This hormone is generally measured to determine the overall vitamin D status. All measurements were performed in one assay.

Body composition

Visceral fat mass (VFM) was quickly collected and weighed for evaluation of central adiposity – mesenteric, epididymal, and retroperitoneal (Toste *et al.* 2006a,b, Fagundes *et al.* 2007) – and data were expressed as g/100 g BW. Body fat content was determined by carcass analysis (Toste *et al.* 2006a,b, Fagundes *et al.* 2007). All rats were eviscerated; carcasses were weighed, autoclaved for 1 h, and homogenized in distilled water (1:1). Homogenates were stored at 4°C for analysis. Homogenates (3 g) were used to determine fat content gravimetrically. Samples were hydrolyzed in a shaking water bath at 70°C for 2 h with 30% KOH and ethanol. The total fatty acids and nonesterified cholesterol were removed with three successive washings with petroleum ether. After drying overnight in vacuum, all tubes were weighed and data were expressed as g fat/100 g carcass. The estimate of the subcutaneous fat was calculated by subtracting the visceral fat from the total fat.

Hormonal determination by RIA

Blood samples were centrifuged (1500 g/20 min per 4°C) to obtain serum, which was kept at -20°C until assay. All measurements were performed in one assay and samples were analyzed in duplicate. Leptin was measured by specific RIA kit (Linco Research, St Charles, MO, USA) with a range of detection from 0.5 to 50 ng/ml; the intra-assay variation was 2.9%. Insulin was determined using a RIA kit (ICN Pharmaceuticals, Inc., Orangeburg, NY, USA) with an assay sensitivity of 0.1 ng/ml and an intra-assay variation of 4.1%. Adiponectin was measured by specific RIA kit (Linco Research) with an assay sensitivity of 0.5 ng/ml and an intra-assay variation of 7.1%. Thyroid hormones were

determined with a commercial RIA kit (ICN Pharmaceuticals, Inc.) with assay sensitivities of 0.045 ng/dl (free thyroxine (fT_4)) and 0.06 pg/ml (total triiodothyronine (tT_3)). Intra-assay variations were 2.8% (fT_4) and 3.6% (tT_3).

Lipid profile evaluation

Serum total cholesterol (TC), triglycerides (TG), and high-density lipoprotein (HDL) were analyzed using Biosystem commercial test kits. Low-density lipoprotein cholesterol (LDL-C) and very low-density lipoprotein cholesterol (VLDL-C) were obtained using Friedewald calculations:

$$\text{LDL-C (mg/dl)} = (\text{TC} - \text{HDL-C} - \text{TG})/5.$$

$$\text{VLDL-C (mg/dl)} = \text{TG}/5.$$

Castelli indexes I and II that correlate with atherogenicity were obtained using the following formulae:

$$\text{Castelli index I} = \text{TC}/\text{HDL}.$$

$$\text{Castelli index II} = \text{LDL}/\text{HDL}.$$

Glycemia and insulin sensitivity measurement

Blood glucose was determined from the tail vein of fasting rats using a glucometer, after fasting for 12 h (ACCU-CHEK Advantage; Roche Diagnostics).

To measure the insulin sensitivity, the IR index (IRI) was calculated as follows: fasting insulin (mIU/ml) $\times$ fasting glucose (mmol/l).

Catecholamine assays

Total catecholamine (adrenaline and noradrenaline) content in the adrenal medulla was measured by the trihydroxyindole fluorescence method (Trevanzoli *et al.* 2007). Left adrenal glands were homogenized in 500 μl 10% acetic acid using an ultrasonic processor and centrifuged (10 000 g for 1 min). To assay, 50 μl supernatant fraction was mixed with 250 μl 0.5 M PBS (pH 7.0) and 25 μl potassium ferricyanide (0.5%), followed by incubation (20 min). Reaction was stopped with 500 μl ascorbic acid–10 M NaOH (1:19 proportion). Parameters of the fluorometer were 420 nm to excitation and 510 nm to emission. Results were obtained by plotting the values into a linear regression of the standard adrenaline curve. Data were expressed as mmol catecholamines and mmol catecholamines/mg gland. Protein concentration was determined by the Bradford method.

Western blotting analysis for hypothalamic leptin receptor and adrenal tyrosine hydroxylase

Hypothalamus was isolated using the coordinates established by the Atlas of Neuroanatomy: with Systems Organization and Case Correlations (Warner 2001). To obtain cell extracts,

Figure 1 Calcaemia (A) and 25-hydroxyvitamin D₃ (B) of adult offspring whose mothers were exposed to nicotine or saline during lactation that were treated with dietary calcium supplement for 2 months. Values represent mean $\pm$ S.E.M. of ten rats per group. * $P < 0.05$ versus control (C); # $P < 0.05$ versus nicotine (N).

tissue was homogenized in ice-cold lysis buffer (50 mM HEPES, 1 mM MgCl₂, 10 mM EDTA, Triton X-100 1%, pH 6.4) containing the following protease inhibitors: 10 mg/ml aprotinin, 10 mg/ml leupeptin, 2 mg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride (Sigma–Aldrich) and centrifuged at 4 °C, 1120 g for 15 min.

Because tyrosine hydroxylase (TH) is an essential enzyme for catecholamine synthesizing pathway, its expression was measured in adrenal glands (Trevanzoli *et al.* 2007). Briefly, glands were homogenized in 1 ml PBS, pH 7.4, containing 1 μ l protease inhibitors cocktail (aprotinin 1 mg/ml, leupeptin 1 mg/ml, and SBTI 1 mg/ml) and centrifuged at 4 °C, 1120 g for 15 min.

Total protein content in hypothalamus and adrenal homogenates was determined by the BCA protein kit assay (Thermo Scientific, Rockford, IL, USA), and cell lysates were denatured in sample buffer (50 mM Tris–HCl, pH 6.8, 1% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.001% bromophenol blue) and heated at 95 °C for 5 min. Samples (hypothalamus: 30 mg total protein; adrenal: 20 μ g total protein) were run in 10% SDS–PAGE and electroblotted in a nitrocellulose membrane (Hybond P ECL membrane, Amersham Biosciences). Membranes were incubated with

TBS containing 5% nonfat dry milk for 90 min to block nonspecific binding sites. Then, membranes were washed with TBS and incubated with primary antibody (polyclonal goat anti-leptin receptor (OB-R); Santa Cruz Biotechnology, Santa Cruz, CA, USA; monoclonal mouse anti-TH; Sigma–Aldrich) overnight at 4 °C (0.5% nonfat dry milk TBS diluted, 1:2000). Then, membranes were washed and incubated with secondary antibody (goat anti-mouse; Santa Cruz Biotechnology) conjugated with HRP (0.5% nonfat dry milk TBS diluted, 1:2000) for 1 h at room temperature. Finally, OB-R and TH bands were visualized by chemiluminescent method (Kit ECL plus, Amersham Biosciences) followed by exposure to autoradiographic film (Hyperfilm ECL, Amersham Biosciences) for 5 s. Results were quantified with actin. Area and density of the bands were quantified by Image J program (Media Cybernetics, Bethesda, Maryland, USA).

Leptin and SIRT1 expressions: reverse transcription-PCR analysis

Total RNA was isolated from white inguinal and epididymal adipose tissue explants and liver using commercially available kit and standard methodology (RNeasy lipid tissue mini kit, Qiagen and TRIZOL reagent, Invitrogen) respectively. For real-time PCR analysis, total RNA was reverse transcribed

Figure 2 Body weight (A) and food intake (B) at 120 (first day of calcium supplementation) and 180 days old (killed) of C and N offspring treated with dietary calcium supplementation for 2 months (CCa and NCa). Values represent mean $\pm$ S.E.M. of ten rats per group. * $P < 0.05$ versus control (C).

Figure 3 Visceral fat (A), total body fat (B), and subcutaneous fat (C) masses of adult offspring whose mothers were exposed to nicotine or saline during lactation that were treated with dietary calcium supplement for 2 months. Values represent mean $\pm$ S.E.M. of ten rats per group. * $P < 0.05$ versus control (C).

using 1 μ g RNA for hepatic tissue and 500 ng for adipose tissue using SuperScript III kit (Invitrogen).

The products were amplified on Applied Biosystems 7500 Real-Time PCR System (Life Technologies Co.) using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) according to the recommendations of the manufacturer.

Leptin, SIRT1, and 36B4 cycle parameters were: 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 30 s, and 70 °C for 45 s. This final setup of the assay was defined after checking for product purity by analyses of melting curves and visualization of a single band of expected size in nusieve-agarose gel (4%). In addition, appropriate cDNA serial curves were performed in order to confirm that the efficiency of the reaction was ~ 1 . Changes in mRNA expression were calculated from the cycle threshold (C_t), after correcting for 36B4, a ribosomal protein used as control and that did not show variations with the treatments (Akamine *et al.* 2007). Data are expressed as fold induction over control group, which was set to 1.

The primer sequences were as follows: leptin forward 5'-CAT CTG CTG GCC TTC TCC AA-3' and reverse 5'-ATC CAG GCT CTC TGG CTT CTG-3', SIRT1 forward 5'-CAG GTT GCA GGA ATC CAA A-3' and reverse 5'-CAA ATC AGG CAA GAT GCT GT-3' and 36B4 forward 5'-CCG AGG CAA CAG TTG GGT A-3' and reverse 5'-TGT TTG ACA ACG GCA GCA TTT-3' (Rodgers *et al.* 2005, Machado *et al.* 2009, Paula *et al.* 2010).

Mitochondrial α -glycerol-3-phosphate dehydrogenase activity

Liver α -glycerol-3-phosphate dehydrogenase (GPD) activity, a TH-dependent enzyme considered a marker of thyroid status, was measured in the mitochondrial fraction using phenazine methosulfate (PMS) as an electron transporter

between the reduced enzyme and the iodonitrotetrazolium chloride violet (INT; Oliveira *et al.* 2007). Assay was performed in the presence of 0.1 M DL- α glycerophosphate diluted in KCN/KPB and a solution of 7.9 mM INT to 0.12 mM PMS. Samples were analyzed at 500 nm in a spectrophotometer (TU-180, UV-VIS, Beijing Purkinje General Instrument, Haidian, Beijing, China) and the values were expressed as absorbance (O.D)/mg of mitochondrial protein. Protein was measured using the Bradford method (1976).

Statistical analysis

Results are reported as mean $\pm$ S.E.M. The GraphPad Prism 5 was used for statistical analyses and graphics (GraphPad Software, Inc., La Jolla, CA, USA). The experimental data were analyzed by two-way ANOVA and Newman-Keuls multiple comparison tests. 25-Hydroxyvitamin D₃ data were log transformed due to variance heterogeneity. The significance level was set at $P < 0.05$.

Results

Calcium supplementation for 2 months did not alter any of the parameters evaluated in the adult C offspring, such as BW, adiposity, serum leptin, Ob-R, serum insulin, and blood glucose. In addition, calcemia was not different among all groups (Fig. 1A), but serum concentration of 25-hydroxyvitamin D₃ was higher (+76%, $P < 0.05$; Fig. 1B)

Figure 4 Serum leptin (A), leptin mRNA expression in subcutaneous (B) and visceral (C) adipose tissue, and hypothalamic Ob-R protein expression (D) of adult offspring whose mothers were exposed to nicotine or saline during lactation that were treated with dietary calcium supplement for 2 months. Values represent mean $\pm$ S.E.M. of ten rats per group. * $P < 0.05$ versus control (C); # $P < 0.05$ versus nicotine (N).

Figure 5 Glycemia (A), insulinemia (B), IRI (C), adiponectinemia (D), and adiponectin/visceral fat ratio (E) of adult offspring whose mothers were exposed to nicotine or saline during lactation that were treated with dietary calcium supplement for 2 months. Values represent mean $\pm$ S.E.M. of ten rats per group. * $P < 0.05$ versus control (C); # $P < 0.05$ versus nicotine (N). IRI, Insulin resistance index.

in nicotine group and it was reduced by calcium supplementation (-55% , $P < 0.05$; Fig. 1A).

There was no significant difference in food intake before or after calcium supplementation between groups. N offspring presented higher BW at P180 ($+7\%$, $P < 0.05$; Fig. 2A). As expected, P180 N offspring showed higher visceral and total body fat content ($+53$ and $+45\%$ respectively, $P < 0.05$; Fig. 3A and B), hyperleptinemia ($+54\%$, $P < 0.05$; Fig. 4A), and lower hypothalamic Ob-R content (-45% , $P < 0.05$; Fig. 4D). However, no difference in subcutaneous fat (Fig. 3C) was observed. As NCa offspring showed no difference from controls (C and CCa), calcium supplementation for 2 months normalized BW, visceral obesity, leptinemia, and hypothalamic Ob-R content in N offspring. Leptin mRNA expression was not significantly changed in the subcutaneous adipose tissue in all groups (Fig. 4B). However, NCa offspring had lower leptin mRNA expression in the visceral adipose tissue (-55% , $P < 0.05$; Fig. 4C) compared with the N group.

N offspring displayed normoglycemia (Fig. 5A), hyperinsulinemia ($+92\%$; Fig. 5B; $P < 0.05$), higher IRI (twofold increase; Fig. 5C; $P < 0.05$), and lower adiponectin/VFM ratio (-39% ; $P < 0.05$; Fig. 5E), suggesting impairment in glucose homeostasis. All changes observed in the N group were normalized by dietary calcium supplementation for 2 months.

In the subcutaneous adipose tissue, calcium supplementation did not affect *Sirt1* mRNA expression; however, N offspring showed lower *Sirt1* mRNA levels in the visceral fat (-37% , $P < 0.05$; Fig. 6A) and higher *SIRT1* mRNA levels in the liver of NCa offspring ($+42\%$; $P < 0.05$; Fig. 6B).

Absolute adrenal catecholamine content presented no significant difference between groups (Fig. 7A). N offspring showed higher relative adrenal catecholamine content ($+37\%$; Fig. 7B; $P < 0.05$) and higher TH expression ($+51\%$; Fig. 7C; $P < 0.05$). In NCa offspring, these changes were normalized.

As expected, maternal nicotine exposure programmed for lower serum tT_3 and fT_4 levels when compared with C offspring (-76 and -74% ; Fig. 8A and B respectively; $P < 0.05$), accompanied by lower liver mGPD activity (-54% ; Fig. 8C; $P < 0.05$), an enzyme considered an important marker of thyroid status. These thyroid function parameters were not normalized in NCa offspring.

Lipid profile evaluation (TC, HDL, LDL, VLDL, TG, and Castelli indexes I and II) showed no significant difference between groups (Table 1).

Discussion

We have recently reported maternal nicotine exposure programs for higher central obesity and IR, two important parameters of the metabolic syndrome, in the adult progeny (Oliveira *et al.* 2010). The most remarkable finding of this study is that calcium supplementation for 2 months reverts, in adult rats, some of the metabolic disorders that were programmed by nicotine exposure in early postnatal life.

Calcium carbonate was used instead of dairy products so as to isolate the effect of calcium, because dairy products contain

Figure 6 Sirtuin 1 mRNA expression in subcutaneous (A) and visceral (B) adipose tissue and in the liver (C) of adult offspring whose mothers were exposed to nicotine or saline during lactation that were treated with dietary calcium supplement for 2 months. Values represent mean $\pm$ S.E.M. of ten rats per group. * $P < 0.05$ versus control (C), # $P < 0.05$ versus nicotine (N).

Figure 7 Total (A) and relative (B) catecholamine content and tyrosine hydroxylase (TH) expression (C) of adult offspring whose mothers were exposed to nicotine or saline during lactation that were treated with dietary calcium supplement for 2 months. Values represent mean $\pm$ S.E.M. of ten rats per group. * $P < 0.05$ versus control (C), # $P < 0.05$ versus nicotine (N).

other substances, such as magnesium and leucine, which could have affected the interpretation of the present findings. Calcium dietary supplementation caused no increase in serum calcium levels but decreased serum 25-hydroxyvitamin D₃ in the nicotine offspring to normal levels. The unchanged calcium serum levels could be expected due to very tight homeostatic mechanisms associated with the maintenance of adequate calcium concentration in the extracellular liquid, which are mainly performed by PTH and calcitriol (Potts 2005, Heaney 2006). Surprisingly, serum 25-hydroxyvitamin D₃ was very high in N offspring, which were overweight, suggesting a role for excessive calcitriol in adipogenesis. The inhibition of these higher calcitriol levels probably reversed the higher adiposity. Also, this result shows that the calcium dose we used for obesity management was not toxic to the animals.

Our current data regarding higher visceral and total body fat contents, hyperleptinemia, hypothyroidism, IR, and leptin resistance in P180 offspring whose mothers were treated with nicotine during lactation corroborate our previous data (de Oliveira *et al.* 2010, Santos-Silva *et al.* 2010). Dietary calcium supplementation of adult N offspring was capable of normalizing central obesity, leptinemia, and insulin sensitivity. Obese transgenic rats expressing agouti protein that received a calcium-rich diet (1.2% calcium carbonate during 6 weeks) had lower weight gain and those who had a diet rich in dairy products had an even smaller gain. In humans, a significant decrease in abdominal obesity was observed with diets rich in calcium or dairy products (Zemel 2004). However, in another study using 1500 mg calcium carbonate during 2 years, obese individuals did not show significant differences in BW gain (Yanovski *et al.* 2009).

The mechanisms induced by calcium supplementation that could explain abdominal obesity reduction remain unclear. It is possible that calcitriol plays a role in energy metabolism by regulating the deposition and expansion of local fat in adipose tissue. Besides, excessive deposition of central fat in obesity may be the result of a greater capacity for the regeneration of glucocorticoids in visceral fat depots (Zemel 2004, Zemel & Sun 2008). Glucocorticoid levels in abdominal adipose tissue and the availability of intracellular glucocorticoids are controlled by the 11 β -hydroxysteroid dehydrogenase-1 (11 β -HSD-1) activity, which generates local active cortisol (or corticosterone, in rats) from cortisone. Obese individuals have increased mRNA of this enzyme in both subcutaneous and visceral fat tissues (Desbriere *et al.* 2006). Experimental studies (Masuzaki *et al.* 2001) have shown higher 11 β -HSD-1 gene expression in adipose tissue associated with features of metabolic syndrome such as increased waist circumference and IR. Calcitriol directly regulates local 11 β -HSD-1 expression and release of cortisol, indicating a potential role of calcitriol in visceral adiposity (Zemel 2003, Zemel & Sun 2008) and calcium-rich diet inhibits calcitriol in rats, resulting in the inhibition of 11 β -HSD-1 expression. In the adipocyte, calcitriol decreases mitochondrial UCP2 activity, apoptosis, and lipolysis and increases lipogenesis. So, the lower central obesity of N offspring induced by calcium supplementation can be attributed, at least in part, to the inhibition of calcitriol levels and cortisol generation by visceral adipocytes (Zemel & Sun 2008). Another hypothesis for the anti-obesity effect of dietary calcium is related to its capacity to form an insoluble complex with lipids in the intestine, increasing lipid fecal excretion and consequently reducing absorption (Zemel 2002, Teegarden *et al.* 2008).

Calcium supplementation reduced leptin mRNA expression in visceral tissue and normalized serum leptin. These effects can be explained by the higher lipolysis and

Figure 8 Serum total T₃ (A), serum-free T₄ (B), and liver mGPD activity (C) of adult offspring whose mothers were exposed to nicotine or saline during lactation that were treated with dietary calcium supplement for 2 months. Values represent mean $\pm$ S.E.M. of ten rats per group. * $P < 0.05$ versus control (C).

Table 1 Lipid profile of adult offspring whose mothers were exposed to nicotine (N) or saline (C) during lactation that were treated with dietary calcium supplement for 2 months (CCa and NCa)

	C	CCa	N	NCa
Total cholesterol (mg/dl)	63.00±6.03	75.25±3.13	65.70±2.10	69.33±3.33
HDL cholesterol (mg/dl)	35.43±3.94	40.38±2.04	32.20±2.42	30.56±2.96
LDL cholesterol (mg/dl)	13.00±12.00	11.00±3.75	15.67±3.89	19.57±5.41
VLDL cholesterol (mg/dl)	29.00±5.93	30.08±3.63	27.92±2.34	27.67±4.38
Triglycerides (mg/dl)	147.00±29.66	150.40±18.14	139.60±11.69	138.30±21.90
Castelli index I	1.85±0.20	1.90±0.14	2.23±0.29	2.48±0.33
Castelli index II	0.61±0.59	0.29±0.12	0.85±0.29	0.87±0.31

HDL cholesterol, high-density lipoprotein cholesterol; LDL cholesterol, low-density lipoprotein cholesterol; VLDL cholesterol, very low-density lipoprotein cholesterol.

adipocyte apoptosis possibly induced by higher calcium intake. N offspring presented resistance to the anorexigenic effect of leptin caused by lower Ob-R expression in hypothalamus, as already published (de Oliveira *et al.* 2010), which was reversed by the calcium supplementation. As lower Ob-R could be explained by a downregulatory effect of hyperleptinemia, the normalization of leptin levels with calcium also corrects hypothalamic Ob-R.

Concerning insulin sensitivity, the present finding confirms the impairment of glucose homeostasis previously observed in N offspring (de Oliveira *et al.* 2010) and showed that dietary calcium was successful in recovering this parameter. In fact, some epidemiological studies showed a negative association between calcium intake and glucose levels and insulin and IR (Sánchez *et al.* 1997, Davies *et al.* 2000, Pittas *et al.* 2007, Villegas *et al.* 2009).

Mechanisms that explain the role of calcium in IR are also not completely elucidated. Some studies suggest that the increase in intracellular calcium concentration can affect the transport of glucose mediated by insulin and insulin secretion (Zemel 1998, Tremblay & Gilbert 2009). In rat and human adipocytes, high concentrations of intracellular calcium reduce insulin-mediated glucose transport (Draznin *et al.* 1988). Changes in intracellular calcium in target insulin tissues may contribute to changes in insulin action (Ojuka 2004, Pittas *et al.* 2007). The increase in intracellular calcium caused by hyperinsulinemia leads to IR (Begum *et al.* 1993). Calcitriol can increase intracellular calcium in these tissues and higher calcium intake can block this influx by inhibition of calcitriol. Thus, normal concentration of intracellular calcium is essential for insulin secretion by pancreatic β-cells as well as for insulin-mediated intracellular processes in tissues such as skeletal muscle and adipose tissue.

Low-calcium diet increases serum calcitriol, increasing intracellular calcium and, in part, this could result in IR in adipocytes and other insulin target cells mainly by phosphorylation of the glucose transporter type 4 (GLUT4), making insulin-mediated glucose uptake less efficient and promoting systemic IR (Reusch *et al.* 1991, Begum *et al.* 1993, Zemel *et al.* 1995, McCarty *et al.* 2002). In fact, N offspring presented lower serum 25-hydroxyvitamin D₃ and IR.

The simplest mechanism that could explain the increase in insulin sensitivity with calcium therapy is related to the decrease in BW and adiposity. However, other more complex mechanisms may be involved. Increased SIRT1 activity has been associated with lower adiposity and protection from diet-induced metabolic disorders (Banks *et al.* 2008, Pfluger *et al.* 2008). In this sense, another possible mechanism that could correct the impairment in glucose homeostasis in N offspring with calcium supplementation is the change in expression of genes that regulate the metabolism, such as SIRT1. SIRT1 stimulates a glucose-dependent insulin secretion from pancreatic β-cells and directly stimulates insulin-signaling pathways in insulin-sensitive organs. Furthermore, SIRT1 regulates adiponectin secretion, gluconeogenesis, and reactive oxygen species levels, which together contribute to the development of IR. Moreover, SIRT1 overexpression and several SIRT1 activators have beneficial effects on glucose homeostasis and insulin sensitivity in obese mice models. We showed a higher liver SIRT1 expression in NCa offspring that may be potentially associated with the favorable metabolic phenotype observed in this group, such as the reduction in central obesity and improvement in insulin sensitivity. Surprisingly, NCa offspring displayed lower SIRT1 expression in visceral fat tissue. Perhaps lower leptin levels in NCa offspring are responsible for the lower SIRT1 expression in adipocyte, because leptin treatment in *ob/ob* mice increases SIRT1 expression in white adipose tissue in a dose-dependent manner (Zhang *et al.* 2009).

This study, for the first time, shows that postnatal nicotine exposure programmed for higher catecholamine levels and adrenal TH expression in adulthood, suggesting that N offspring developed higher catecholamine synthesis. Notably, leptin stimulates catecholamine synthesis and secretion (Trevenzoli *et al.* 2007), so the present findings may be due to the hyperleptinemia of N offspring. Calcium supplementation prevents adrenal medulla dysfunction in N offspring, possibly through the normalization of leptin levels. This result does not allow us to reach a conclusion about the effect of calcium supplementation on catecholamine action, because we did not measure serum catecholamine levels or its tissue action.

We have previously shown that maternal nicotine exposure during lactation programs for secondary hypothyroidism in adult offspring (Oliveira *et al.* 2009). In this study, we also detected lower serum thyroid hormone levels as well as lower liver mGPD activity in N group, confirming our previous data. However, calcium supplementation did not correct the thyroid hypofunction observed in the N group. Thus, the normalization of visceral adiposity cannot be attributed to the normalization of the thyroid function.

It is important to consider that nicotine may have a priming effect in calcium metabolism and regulation during lactation with possible programming consequences in calcium hormonal regulation in adulthood, because nicotine administration in female rats results in lower serum calcitriol with no differences in serum Ca^{2+} or PTH (Iwaniec *et al.* 2002) and because serum cotinine (a nicotine metabolite) has a significant inverse relationship with bone mineral content in a clinical study (Benson & Shulman 2005).

In conclusion, although the role of calcium on body fat is still controversial, calcium supplementation for 2 months reversed most of the alterations observed in adult obese rats programed by maternal nicotine exposure, such as increased visceral fat, which is a risk factor for IR, but did not correct the hypothyroidism development. Calcitropic hormones and lower intestinal fat absorption could be involved in the anti-obesity effect of calcium. Thus, a possible sequence of events after higher calcium intake is the inhibition of calcitriol with increases in lipolysis and apoptosis of adipocytes, which explain the lower VFM. The normalization of leptin and the increase in adiponectin are a result of less adipose tissue and both contribute to better insulin sensitivity. A calcium-rich diet seems to be an effective anti-obesity strategy, and perhaps, the reduction in fat mass *per se*, induced by calcium, is the main mechanism that leads to increased insulin action.

Declaration of interest

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

Funding

This research was supported by the 'National Council for Scientific and Technological Development' (Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq), the 'Carlos Chagas Filho Research Foundation of the State of Rio de Janeiro' (Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro-FAPERJ), and Coordination for the Enhancement of Higher Education Personnel (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES). E O and J L N were recipients of the CAPES fellowship, A P S-S was recipient of a CNPq fellowship, and N S L was recipient of a FAPERJ fellowship.

Acknowledgements

All the authors are grateful to Antonio C M de Sá, Vania Pinto, and Ana Maria B Coutinho from Laboratory of Lipids (LabLip, UERJ) for lipid profile determination. We also thank Miss Monica Moura and Luciano Santos for technical assistance.

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Received in final form 6 June 2011

Accepted 16 June 2011

Made available online as an Accepted Preprint
16 June 2011