Neonatal nicotine exposure causes insulin and leptin resistance and inhibits hypothalamic leptin signaling in adult rat offspring

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Abstract

Maternal nicotine (NIC) exposure during lactation leads to overweight, hyperleptinemia, and hypothyroidism in adult rat offspring. In this model, we analyzed adipocyte morphology, glucose homeostasis (serum insulin and adiponectin; liver and muscle glycogen), serum lipid, and the leptin signaling pathway. After birth, osmotic minipumps were implanted in lactating rats, which were divided into the groups NIC (6 mg/kg per day s.c. for 14 days) and control (C, saline). NIC and C offspring were killed at the age of 180 days. Adult NIC rats showed higher total body fat (+10%, $P < 0.05$), visceral fat mass (+12%, $P < 0.05$), and cross-sectional area of adipocytes (epididymal: +12% and inguinal: +43%, $P < 0.05$). Serum lipid profile showed no alteration except for apolipoprotein AI, which was lower. We detected a lower adiponectin:fat mass ratio (−24%, $P < 0.05$) and higher

insulinemia (+56%, $P < 0.05$), insulin resistance index (+43%, $P < 0.05$), leptinemia (+113%, $P < 0.05$), and leptin:adiponectin ratio (+98%, $P < 0.05$) in the adult NIC group. These rats presented lower hypothalamic contents of the proteins of the leptin signaling pathway (leptin receptor (OB-R): −61%, janus tyrosine kinase 2: −41%, and p-signal transducer and activator of transcription 3: −56%, $P < 0.05$), but higher suppressor of cytokine signaling 3 (+81%, $P < 0.05$). Therefore, NIC exposure only during lactation programs rats for adipocyte hypertrophy in adult life, as well as for leptin and insulin resistance. Through the effects of NIC, perinatal maternal cigarette smoking may be responsible for the future development of some components of the metabolic syndrome in the offspring.

Journal of Endocrinology (2010) **206**, 55–63

Introduction

Epidemiological and experimental data have shown that nutritional disorders and hormonal changes during critical periods of development and early life are associated with future changes in the structure and physiology of body tissues and systems of the progeny. This association has been named programming, which is defined as the basic biological phenomenon that putatively underlies the relationships among nutritional experiences in early life and diseases in adulthood (Barker 2003, Moura & Passos 2005, De Moura *et al.* 2008). Also, the risk of developing chronic diseases in adulthood is influenced by environmental and dietary chemicals that can mimic or interfere with hormone action, known as endocrine disruptors. In fact, some of them may act as chemical ‘obesogens’ and promote obesity (Grun & Blumberg 2006, Tabb & Blumberg 2006).

Epidemiological studies show that maternal smoking during pregnancy might be a risk factor for childhood obesity and hypertension (Vik *et al.* 1996, Blake *et al.* 2000). Although the mechanisms to explain the development of

obesity under these circumstances are still unclear, some experimental data suggest that nicotine (NIC), the main addictive compound of tobacco smoke, in gestational and lactation periods, is responsible for increased adiposity in the offspring (Von Kries *et al.* 2002, Chen & Kelly 2005, Gao *et al.* 2005, Goldani *et al.* 2007). In addition, we have recently shown that lactation is a crucial period for the programming of later obesity by NIC, with thyroid dysfunction being a possible contributing factor, since maternal NIC exposure only during lactation leads to overweight, higher central adiposity, hyperleptinemia, and secondary hypothyroidism in adult rat offspring (Oliveira *et al.* 2009).

Adipose tissue has a crucial role in metabolic disorders associated with obesity (Rasouli & Kern 2008). Beyond secreting free fatty acids, the adipocytes release several proteins and hormones, such as leptin and adiponectin, with autocrine, paracrine, and endocrine functions (Matsuzawa 2006). Adiponectin production is inversely proportional to whole-body adipose mass, and experimental studies suggest that adiponectin increases insulin sensitivity in peripheral tissues (Matsuzawa 2006). Leptin, mainly produced by adipose tissue, reduces food

intake and increases energetic expenditure (Friedman & Halaas 1998), by signaling through the leptin receptor (OB-R or LEPR listed in the MGI Database). Six alternatively spliced isoforms of OB-R (a, b, c, d, e, and f) with different lengths of C-termini have been identified in mice. The long form (OB-Rb) is capable of active intracellular signaling (Lee *et al.* 1996). Leptin binding to OB-Rb initiates tyrosine phosphorylation by janus tyrosine kinase 2 (JAK2). Phosphorylated JAK2 recruits and phosphorylates signal transducer and activator of transcription 3 (STAT3). The activated STAT3 dimerizes and translocates to the nucleus, stimulating gene transcription. The JAK2/STAT3 pathway stimulates suppressor of cytokine signaling 3 (SOCS3) transcription, a leptin-inducible inhibitor of leptin signaling (Vaisse *et al.* 1996). In obesity, leptin resistance has been associated with changes in the JAK2/STAT3 pathway in the hypothalamus, with a decrease in *OB-R*, *JAK2*, and *STAT3* expression and an increase in *SOCS3* content (Myers *et al.* 2008).

Because there is a high rate of smoking relapse among women who stopped smoking during pregnancy (McBride & Pirie 1990), and because we have previously demonstrated that early NIC exposure during lactation is capable of affecting the future development of the offspring (Oliveira *et al.* 2009), possibly acting as an endocrine disruptor and an obesogen factor, the present study was designed to investigate the effects of perinatal NIC on intermediary metabolism. We evaluated adipocyte morphology, serum lipid, and protein levels, parameters related to glucose homeostasis (tissue glycogen, serum glucose, insulin, adiponectin, and corticosterone), and the expression of proteins of the leptin signaling pathway in the hypothalamus in the adult rat.

Materials and Methods

The use of the animals according to our experimental design was 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) that based its analysis on the principles adopted and promulgated by Brazilian Law (Law no. 11.794/2008). Experiments were conducted to minimize the number of animals and the suffering caused by the procedures following the ethical doctrine of the three 'Rs' – reduction, refinement, and replacement (Drummond 2009, Marques *et al.* 2009). Wistar rats were kept in a temperature-controlled room ($25 \pm 1^\circ\text{C}$) with artificial dark–light cycles (lights on 0700 h, lights off 1900 h). Three-month-old, virgin female rats were caged with male rats at the ratio of 3:1. After mating, each female was placed in an individual cage with free access to water and food until delivery.

Experimental model of neonatal NIC exposure

Two days after birth, 12 lactating rats were randomly assigned to one of the following groups.

NIC ($n=6$) Dams were lightly anesthetized with thiopental, a 3×6 cm area on the back was shaved, and an incision was made to permit s.c. insertion of osmotic minipumps (Alzet, 2ML2, Los Angeles, CA, USA). To avoid the adverse effects of NIC peaks, we chose to perform the NIC exposure by using s.c. osmotic mini pump infusion. Pumps were filled with NIC-free base diluted in 0.9% NaCl, to deliver an initial dose rate of 6 mg/kg of NIC per day (during 14 days of lactation), as previously described (Oliveira *et al.* 2009). At this rate, this paradigm produces plasma NIC levels similar to those observed in typical smokers, ~ 25 ng/ml (Lichtensteiger *et al.* 1988). The incision was closed, and dams were permitted to recover in their home cages.

Control ($n=6$) Dams were implanted with osmotic minipumps containing only saline solution, used for the same period as the minipumps with NIC.

In general, pregnant rats produced 10–12 pups, and 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 the lactation performance, litters were adjusted to six male pups per NIC or control (C) dam. After weaning (21 days of lactation) until 180 days, body weight (BW) of the offspring was monitored every 4 days, as well as relative food intake (g/100 g BW) and body length were recorded every 15 days. Lee's index of obesity was determined in adult animals according to the following calculation: $\sqrt[3]{\text{body weight (g)/nasoanal length (cm)}}$. We used two offspring from each mother, which were killed at the age of 180 days after 12 h of fasting. The killing occurred by quick decapitation, with no prior anesthesia (because anesthesia affects hormone and lipid metabolism), to collect blood, hypothalamus, liver, muscle, and visceral and subcutaneous fat.

Detection of NIC metabolite in blood and milk

Serum and milk cotinine levels were determined using a cotinine assay kit from Orasure Technologies (Bethlehem, PA, USA) in accordance with the manufacturer's recommendations. On the 15th day of lactation, dams were separated from their litters, and 2 h later, milk was collected as previously described (Bonomo *et al.* 2005) and stored at -20°C until assayed. After that, dams (NIC, $n=6$; C, $n=6$) and pups (NIC, $n=6$; C, $n=6$) were killed, and blood was collected. Blood was centrifuged (2000 g, 20 min), and supernatant was stored (-20°C) until the time of assay.

Computed tomography

To study the adipose distribution, we performed computed tomography (GE – helicoidal HiSpeed – CUCC/HUPE /UERJ). Three-millimeter axial sections were obtained to evaluate the adipose tissue content (one section per rat, $n=12$ rats per group). Adult NIC and C offspring were lightly anesthetized with thiopental, and put in dorsal

decubitus for the computed tomography. Analyses of adipocyte cross-sectional area (cm^2) were determined using the program DicomWorks v1.3.5 Software (<http://dicom.online.fr/>).

Morphometric analysis of adipocytes

Visceral (epididymal) and subcutaneous (inguinal) white adipose tissues were fixed in a paraformaldehyde solution and embedded in paraffin, and 5- μm sections were stained with hematoxylin/eosin. Morphometric measurements were performed on 12 serial sections taken every 100 μm for each animal. Four adipocytes were measured in each section (48 cells analyzed per rat, $n=6$ rats per group), and the cross-sectional area of each adipocyte was determined using Image J 1.34s software (Wayne Rasband National Institute of Health, Bethesda, MA, USA).

Tissue glycogen content

Soleus muscles were weighed and homogenized in Turrax with 1 ml buffer (50 mM Tris-HCl; 5 mM NaF; 5 mM EGTA; and 1 mM dithiothreitol; pH 7.2). After centrifugation (2000 g at 4 °C for 20 min), 600 μl of supernatant was removed and frozen. On the next day, 100 μl of supernatant was incubated with 2 U of amyloglucosidase from *Aspergillus niger* (Sigma A7420), suspended in 0.2 M Na acetate, pH 4.8, in a final volume of 200 μl , for 4 h at 40 °C. A standard curve with increasing concentrations of glycogen was constructed using the same experimental conditions. The glucose produced by glycogen hydrolysis was measured using a commercial kit (Glucos, Doles, Goiás, Brazil). To ensure that glucose originated only from glycogen, blanks without enzyme were carried through the same procedure and subtracted from the experimental samples (Casimiro-Lopes *et al.* 2008).

Livers were weighed and homogenized in Turrax with 4 ml of trichloroacetic acid (TCA; 10%). After centrifugation (1000 g at 4 °C for 10 min), 2 ml of supernatant was added to 5 ml of absolute ethanol and frozen. After 24 h, the mixture was centrifuged (1000 g at 4 °C for 10 min), and the supernatant was discarded. Glycogen was hydrolyzed by boiling the pellet for 30 min with 1 M HCl. After addition of 1 ml of 1 M NaOH to neutralize the mixture, glucose was measured in 200 μl of supernatant as described above (Casimiro-Lopes *et al.* 2008).

Biochemical analysis

Serum levels of total cholesterol (TC), triglycerides, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and very low-density lipoprotein cholesterol (VLDL-C) were analyzed using Biosystem commercial test kits with an automated A15 spectrophotometer (Biosystems S.A., Barcelona, Spain). LDL-C and VLDL-C were calculated according to the

equation of Friedwald:

$$\text{VLDL-C} = \text{triglycerides}/5$$

$$\text{LDL-C} = (\text{TC} - \text{HDL-C} - \text{triglycerides})/5$$

Apo AI and Apo B concentrations were measured by immunoturbidimetric assay using an automated A15 BioSystems Analyzer.

A biuret reaction was performed for total plasma protein measurement, and the absorbance of products was determined on spectrophotometer (automated A15 BioSystems Analyzer). Plasma albumin was determined by the bromocresol green method, and globulin fraction was measured by the difference between total protein and albumin. Values are expressed as mg/dl.

Hormonal determination by RIA

Blood samples were centrifuged (1500 g /20 min per 4 °C) to obtain serum, which was frozen (−20 °C) until assaying. All measurements were performed in one assay; all samples were analyzed in duplicate. Insulin concentration was determined using an 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 with a specific RIA kit (Linco Research, St Charles, MO, USA) with an assay sensitivity of 0.5 ng/ml and an intra-assay variation of 7.1%. Leptin was measured with a specific RIA kit (Linco Research, Inc.), which measures both rat and mouse leptin with a range of detection from 0.5 to 50 ng/ml; the intra-assay variation was 2.9%. Corticosterone was measured using a specific RIA kit (ICN Biomedicals Inc., Aurora, OH, USA) with an assay sensitivity of 50 ng/ml and an intra-assay variation coefficient of 7%.

Insulin sensitivity

Fasting blood glucose was determined from the tail vein of fasting rats using a glucometer (ACCU-CHEK Advantage; Roche Diagnostics). To determine the insulin sensitivity of adult animals, we used the insulin resistance index (IRI): fasting insulin ($\mu\text{IU}/\text{ml}$) $\times$ fasting glucose (mmol/l). As hypertrophic adipocytes secrete more leptin and less adiponectin, the serum leptin:adiponectin ratio (LAR) was used to evaluate insulin resistance and vascular risk (Finucane *et al.* 2009). Also, plasma adiponectin levels were normalized to whole-body fat mass (Park *et al.* 2005).

Western blotting analysis

The hypothalamus was isolated using the coordinates established by the Atlas of Neuroanatomy: with Systems Organization and Case Correlations (Warner 2001). To obtain cell extracts, hypothalamic tissues were homogenized in ice-cold lysis buffer (50 mM HEPES, 1 mM MgCl_2 , 10 mM EDTA, Triton X-100 1%, pH 6.4) containing the

following protease inhibitors: 10 µg/µl aprotinin, 10 µg/µl leupeptin, 2 µg/µl pepstatin, and 1 mM phenylmethylsulfonyl fluoride (Sigma–Aldrich). Ob-R, JAK2, STAT3, and SOCS3 content were analyzed by western blotting as described below, using actin as internal control.

Total protein content in the hypothalamus homogenate was determined by the BCA protein kit assay (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 (30 µg total protein) were run in 10% SDS–PAGE and transferred to polyvinylidene filters (PVDF Hybond-P, Amersham Pharmacia Biotech). Rainbow markers (Amersham Biosciences) were run in parallel to estimate molecular weights. Membranes were blocked with 5% nonfat milk in Tween–TBS (20 mM Tris–HCl, pH 7.5, 500 mM NaCl, and 0.1% Tween-20). Primary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) used were anti-OB-R (1:500), anti-JAK2 (1:500), anti-STAT3 (1:500), anti-p-STAT3 (1:500), anti-SOCS3 (1:500), and anti-actin (1:1000). PVDF filters were washed three times with Tween–TBS (0.1%), followed by incubating 1 h with the appropriate secondary antibody conjugated to biotin (Santa Cruz Biotechnology, Inc). Then, filters were incubated with streptavidin-conjugated HRP (Caltag Laboratories, Burlingame, CA, USA). All western blots were allowed to react with HRP substrate (ECL-plus; Amersham Pharmacia Biotech), and then exposed to X-ray film for 10 s to 30 min. In most cases, the membranes were stripped (Restore Western Blot Stripping buffer; Pierce, Rockford, IL, USA) at 37 °C for 15 min, washed with Tween–TBS (0.1%) three times, and reprobed with specific primary antibody, following all steps above. Images were scanned, and the bands were quantified by densitometry, using Image J 1.34s software (Wayne Rasband National Institute of Health).

Statistical analysis

Results are reported as mean ± s.e.m. The GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analyses and graphics. Data were analyzed by unpaired Student’s *t*-test, and differences were considered significant at *P* < 0.05.

Results

Milk and serum cotinine levels

NIC treatment affected cotinine milk and plasma levels. In the NIC dams, milk and serum cotinine concentrations were similar (milk: 225.8 ± 7.1 ng/ml; serum: 239.7 ± 25.2 ng/ml). NIC pups at the age of 15 days had serum cotinine levels of 20.4 ± 1.5 ng/ml. Control dams and pups had cotinine levels below the detection limit of the technique (< 8 ng/ml).

Figure 1 Absolute body weight (A) and body weight gain (B) after weaning of offspring whose mothers were nicotine (NIC) or saline (C) exposed during lactation (*n* = 12 animals per group), *P* < 0.05.

Nutritional evaluation

We found higher BW gain for NIC offspring between 75 and 100 days of life (around 10%, *P* < 0.05) as well as after the age of 165 days, reaching 10% (*P* < 0.05) at the age of 180 days (Fig. 1). At adulthood, NIC offspring showed higher Lee’s index scores (+7%, *P* < 0.05) compared with controls; however, they exhibited no differences concerning body length and food intake (Table 1).

Central and total adiposity were higher in adult NIC rats (+10 and +12% respectively, *P* < 0.05; Fig. 2C and D). Subcutaneous fat content did not show any significant change (Fig. 2E). To determine whether the increase in fat mass in NIC offspring was associated with an increase in the size of adipocytes (hypertrophy), we performed morphometric analysis on histological sections of visceral and subcutaneous adipose tissue (Fig. 3A and B). Quantitative analysis of

Table 1 Nutritional parameters and glycogen content of adult offspring from control and nicotine-exposed dams

	C	NIC
Body length (cm)	25.4 ± 0.2	24.8 ± 0.2
Body weight (g)	414.9 ± 14.8	455.9 ± 8.9*
Lee’s index (g 1/3/cm)	0.30 ± 0.002	0.32 ± 0.004*
Food intake (g)	31.44 ± 0.6	33.83 ± 0.2
Liver glycogen (mM/g)	1.32 ± 0.03	1.22 ± 0.02*
Muscle glycogen (µM/ml g)	22.16 ± 4.2	48.81 ± 5.6*

C, control offspring; NIC, nicotine offspring. Values represent mean ± s.e.m. of 12 rats per group (**P* < 0.05).

Figure 2 Computed tomography of abdominal sections in rats, in dorsal decubitus, to measurement of visceral adipose tissue. Figures A and B represent tomography images of central (A) and total (B) adiposity from individual NIC and C offspring. Mean of visceral (C), total (D), and subcutaneous (E) adipose tissue areas was expressed in cm² ($n=12$ animals per group), $P<0.05$.

these sections showed that both epididymal and inguinal adipocytes were larger in NIC rats compared with controls (Fig. 3C: +12% and Fig. 3D: +43% respectively; $P<0.05$). NIC offspring exhibited higher serum leptin concentrations (+113%, $P<0.05$; Fig. 4G).

As depicted in Table 2, no significant difference was detected in serum TC, HDL-C, LDL-C, VLDL-C, or triglycerides, nor for serum globulins, albumin, or total protein levels. However, Apolipoprotein (Apo) AI protein was lower in the NIC animals at the age of 180 days (−66%, $P<0.05$).

Glucose homeostasis

NIC rats presented lower liver glycogen (−8%, $P<0.05$) and higher muscle glycogen (+120%, $P<0.05$) in adulthood (Table 1).

Adult rats whose mothers were exposed to NIC during lactation had no significant difference in fasting blood glucose (Fig. 4A), corticosterone (Fig. 4D), or adiponectin (Fig. 4E), in spite of a lower adiponectin/VFM ratio (−24%, $P<0.05$; Fig. 4F), higher blood insulin (+56%, $P<0.05$; Fig. 4B), higher IRI (+43%, $P<0.05$; Fig. 4C), and a higher LAR (+98%, $P<0.05$; Fig. 4H) when compared with control rats.

Hypothalamic leptin signaling

The NIC group showed lower OB-R (−61%, Fig. 5A), JAK2 (−41%, Fig. 5B), p-STAT3 (−56%, Fig. 5D), and higher SOCS3 expression (+81%, Fig. 5E) in the hypothalamus at the age of 180 days ($P<0.05$ for all). Additionally, a lower ratio of p-STAT3 to total STAT3 was observed in the NIC group compared with the C group (−40%, $P<0.05$; data not shown).

Discussion

Some studies have found an increased risk of obesity in children whose mothers smoked during pregnancy (Vik *et al.* 1996, Von Kries *et al.* 2002). Our group has been studying several imprinting factors during lactation, such as nutritional and hormonal status, which are capable of programming body composition and endocrine function in adult life (Toste *et al.* 2006, De Moura *et al.* 2007, Passos *et al.* 2007, Lisboa *et al.* 2008). Also, we have recently shown that NIC exposure during lactation programs the offspring to exhibit overweight, higher serum leptin levels, and hypothyroidism in adulthood (Oliveira *et al.* 2009). In fact, our findings regarding obesity corroborate those of other researchers (Newman *et al.* 1999, Chen & Kelly 2005, Gao *et al.* 2005), who have studied the effects of prenatal and postnatal

Figure 3 Histological sections of epididymal (A) and subcutaneous (B) white adipose tissue at 180-day-old offspring whose mothers were nicotine or saline exposed during lactation. Mean area of visceral (C) and subcutaneous (D) adipocyte was expressed in μm² ($n=6$ animals per group), $P<0.05$. Objective 20×. Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-10-0104>.

Figure 4 Serum glucose (A), insulin (B), IRI (C), corticosterone (D), adiponectin (E), and ratio adiponectin/visceral fat mass (F), leptin (G), and LAR (H) at 180-day-old offspring whose mothers were nicotine or saline exposed during lactation. Values represent mean $\pm$ S.E.M. of 12 rats per group; * $P < 0.05$.

NIC exposure and have suggested that NIC can be an endocrine disruptor and obesogen.

It is important to consider that there are developmental differences between rodents and humans. Some structures that are immature in rat at birth, mature only in postnatal period (in the first week of life), whilst in humans occur since the second trimester of gestation, for example, lactation in rats is a critical period to NIC exposure, corresponding roughly, at least concerning the neural development in the second trimester of gestation in humans (Vinay *et al.* 2005).

An infusion rate of 6 mg/kg per day NIC in rats produces serum NIC levels similar to those observed in typical smokers, ~25 ng/ml (Lichtensteiger *et al.* 1988). To simulate this situation during lactation, rat dams were implanted with osmotic minipumps releasing 6 mg/kg per day continuously (Oliveira *et al.* 2009). We chose the period of lactation because this period exhibits a high rate of smoking relapse among women who stopped smoking during pregnancy (McBride & Pirie 1990). Cotinine is the main NIC metabolite, and the serum NIC:cotinine ratio is 1:5 to 1:10 (Trauth *et al.* 2000). In the present study, we found high levels of serum cotinine in NIC mothers (239 ng/ml) as well as in

their milk (225.8 ng/ml), which translated to a serum cotinine concentration of 20.4 ng/ml in NIC pups. We then exposed NIC dams to NIC at levels comparable to those of heavy smoking mothers. We detected low cotinine concentrations in the NIC pups. Because NIC is transferred through the milk (Luck & Nau 1987), perhaps this observation can be explained by the separation of the pups from their mothers for 2 h, which could underestimate the real impact of maternal NIC exposure.

Lee's index (an obesity indicator) and central and total adiposity, as evaluated by computed tomography, were higher in adult NIC offspring, which corroborates our previous finding of overweight (Oliveira *et al.* 2009) and confirms the reproducibility of our experimental model. Morphological analyses of the adipose tissue revealed hypertrophy of the epididymal (visceral) and inguinal (subcutaneous) adipocytes in adult NIC rats. This indicates that the obesity in offspring induced by maternal NIC exposure may be due to adipocyte hypertrophy. Although the number of fat cells has not been measured in this study, we cannot rule out that hyperplasia also occurs in this model. Overexpression of *PparG*, a key transcription factor in adipocyte differentiation and adipose physiology, has been detected in weaned rats that were prenatally exposed to NIC (Somm *et al.* 2008). The incubation of rat adipocytes with NIC caused a dose-dependent increase of tumor necrosis factor, adiponectin, and free fatty acid secretion into the medium (Liu *et al.* 2004). Therefore, it is possible that NIC acts directly on adipocytes, affecting gene expression and/or hormonal secretion, which could be the initial link between early NIC exposure and later obesity.

It has been reported that NIC treatment alters the lipid profile of adult female rats (Abd el Mohsen *et al.* 1997). In the present study, despite their overweight and higher adiposity, NIC offspring showed no change in triglycerides, TC, LDL-C, HDL-C, or VLDL-C levels, which is in agreement with our previous data (Oliveira *et al.* 2009). Apo AI is present in HDL-C, which is considered an antiatherogenic factor

Table 2 Serum biochemical parameters of offspring from control and nicotine-exposed dams

	C	NIC
Total cholesterol (mg/dl)	59.1 $\pm$ 1.9	53.3 $\pm$ 3.1
HDL cholesterol (mg/dl)	12.7 $\pm$ 0.8	13.8 $\pm$ 1.1
LDL cholesterol (mg/dl)	33.6 $\pm$ 1.9	32.5 $\pm$ 3.4
VLDL cholesterol (mg/dl)	12.8 $\pm$ 1.1	14.8 $\pm$ 4.8
Triglycerides (mg/dl)	64.1 $\pm$ 5.6	50.6 $\pm$ 2.9
Apo AI (mg/dl)	29.5 $\pm$ 6.96	10.0 $\pm$ 2.55*
Apo BI (mg/dl)	16.1 $\pm$ 2.67	12.8 $\pm$ 2.31
Total protein (mg/dl)	7.9 $\pm$ 0.18	7.4 $\pm$ 0.28
Albumin (mg/dl)	3.1 $\pm$ 0.10	2.8 $\pm$ 0.14
Globulin (mg/dl)	4.9 $\pm$ 0.10	4.6 $\pm$ 0.19

HDL cholesterol, high-density lipoprotein cholesterol; LDL cholesterol, low-density lipoprotein cholesterol; VLDL cholesterol, very low-density lipoprotein cholesterol; Apo, apolipoprotein; C, control offspring; NIC, nicotine offspring. Values represent mean $\pm$ S.E.M. of 12 rats per group (* $P < 0.05$).

Figure 5 Effect of maternal nicotine exposure on protein expression of leptin signaling pathway in hypothalamus of adult rats (180 days old). Homogenates of hypothalamus of C and NIC groups were obtained, and OB-R (A), JAK2 (B), STAT3 (C), p-STAT3 (D), and SOCS3 (E) detections were performed by western blotting. Bands on the top figure represent the protein expression in hypothalamus homogenate from individual C and NIC offspring. OB-R, JAK2, STAT3, p-STAT3, and SOCS3 content were quantified by scanning densitometry of the bands (AU, arbitrary units). Actin content was used as control loading. Results are expressed as mean $\pm$ S.E.M. ($n=6$ rats per group), * $P<0.05$. A representative experiment is shown from three independent experiments.

(Onat *et al.* 2009). In the present study, we also detected lower serum Apo A1 concentrations in NIC rats. This finding may indicate a chronic subclinical inflammation. We also evaluated total serum protein, albumin, and globulin concentrations, which are important to the overall nutritional assessment. Despite their overweight condition, NIC rats presented no alteration of these parameters at the age of 180 days.

Some studies have shown that fetal and postnatal NIC exposure causes impaired glucose tolerance in neonatal offspring (Somm *et al.* 2008). According to Bruin *et al.* (2007), NIC exposure from conception until lactation results in permanent β -cell depletion and subsequent impaired glucose tolerance. Therefore, the development of diabetes requires that NIC exposure occurs in both gestation and lactation periods. In fact, in our experimental model, no changes in fasting blood glucose or adiponectin were observed in adult NIC rats. Serum corticosterone, which is

also involved in the control of glucose homeostasis, was unchanged. However, we detected hyperinsulinemia and a higher IRI in adult NIC offspring. Moreover, their LAR was higher, and when we calculated the ratio of adiponectin to white adipose tissue mass, we observed lower adiponectin production per gram of adipose tissue. Taken together, these findings suggest that our programmed animals developed insulin resistance. Insulin has well-known stimulatory effects on glycogen synthesis, and this helps to explain the higher glycogen content in skeletal muscle in adulthood. The lower liver glycogen content may indicate the early steps of selective insulin resistance induced by maternal NIC exposure during lactation. In addition, adult NIC rats are hyperleptinemic (Oliveira *et al.* 2009), and since leptin also stimulates glycogen synthesis (Aiston & Agius 1999), it is possible that the programmed rats developed leptin resistance in liver but not in muscle.

As already mentioned, neonatal NIC exposure programs higher adiposity and hyperleptinemia but does not affect food intake (Oliveira *et al.* 2009). Thus, we evaluated the leptin signaling pathway in the hypothalamus. We observed lower OB-R, JAK2, and p-STAT3 expression as well as higher SOCS3 expression in the hypothalami of adult rats submitted to neonatal NIC exposure. These data suggest resistance to the anorexigenic effect of leptin, which could explain why the higher serum leptin levels in adult NIC group did not change the food intake.

We cannot discern whether the present data is caused by a direct or an indirect NIC effect. Recently, we observed that NIC-treated dams presented hyperprolactinemia and, consequently, higher milk production with higher lactose concentration and energy content. However, in the weaning period (after NIC withdrawal), these dams showed only hyperleptinemia (Oliveira *et al.* 2010). So, at least three hypotheses are possible to explain the NIC action in our model of programming by neonatal NIC exposure. First, NIC transfer through milk (Luck & Nau 1987, Narayanan *et al.* 2002) may alter some factor(s) in offspring. Secondly, maternal changes caused by NIC treatment can be transferred to the pups through the milk. And thirdly, the programming by neonatal NIC exposure may be due to biochemical, hormonal, or behavior changes in either the mothers or pups. In general terms, epigenetic mechanisms, such as DNA methylation or histone acetylation/deacetylation, induced by neonatal environmental factors (nutrition, hormones, or endocrine disruptors) may lead to an increased risk of metabolic disorders in the adult progeny (De Moura *et al.* 2008). Thus, this explanation could help to explain the mechanism involved in the permanent changes to BW regulation induced by early NIC exposure. Whether NIC can make children exposed to cigarette smoke during the postnatal period more susceptible to obesity and other metabolic and endocrine disorders in adulthood warrants epidemiological and prospective studies.

In summary, we have shown that maternal NIC exposure during lactation programs offspring for adipocyte

hypertrophy and central leptin resistance in adult life, which may explain their adulthood overweight. We conclude that through the effects of NIC, maternal smoking in the critical period of lactation can be responsible for the future development of some components of the metabolic syndrome in progeny.

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).

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 Mr Carlos Roberto, Miss Monica Moura, and Mr Luciano Santos for technical assistance.

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Received in final form 29 April 2010

Accepted 6 May 2010

Made available online as an Accepted Preprint
7 May 2010