

# Nicotine exposure affects mother's and pup's nutritional, biochemical, and hormonal profiles during lactation in rats

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## Abstract

We have shown that maternal nicotine exposure during lactation has long-lasting effects on body adiposity and hormonal status of rat offspring. Here, we studied the nutritional and hormonal profiles in this experimental model. Two days after birth, osmotic minipumps were implanted in lactating rats divided into two groups: NIC – continuous s.c. infusions of nicotine (6 mg/kg per day) for 14 days and C – saline. Dams and pups were killed at 15 and 21 days of lactation. Body weight and food intake were evaluated. Milk, blood, visceral fat, carcass, and adrenal gland were collected. All the significant data were  $P < 0.05$ . At the end of nicotine exposure (15 days), dams presented higher milk production, hyperprolactinemia, and higher serum high-density lipoprotein cholesterol (HDL-C). Milk from NIC

dams had higher lactose concentration and energy content. After nicotine withdrawal (21 days), dams showed lower food intake and hyperleptinemia. The 15-day-old NIC pups presented higher total body fat, higher HDL-C, serum leptin, serum corticosterone, and adrenal catecholamine content, but lower tyrosine hydroxylase protein levels. The 21-day-old NIC pups had higher body protein content and serum globulin. Thus, maternal nicotine exposure during lactation results in important changes in nutritional, biochemical, and hormonal parameters in dams and offspring. The pattern of these effects is clearly distinct when comparing the nicotine-exposed group to the withdrawal group, which could be important for the programming effects observed previously.

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## Introduction

It is well known that tobacco contains numerous compounds that are potentially cytotoxic, such as nicotine, thiocyanate, carcinogens, carbon, and certain gases (Stellman & Djordjevic 2009). Tobacco smoke affects numerous biological processes including the secretion of hormones, such as ADH, GH, ACTH, cortisol, catecholamines, and leptin (Robinson 1977, Yeh & Barbieri 1989, Grassi *et al.* 1994, Walker *et al.* 1999). In normal men, smoking causes an increase in heart rate and blood pressure as a result of blood vessel constriction (Kapoor & Jones 2005). Nicotine, an important addictive compound of tobacco smoke, is an exogenous acetylcholine agonist that activates nicotine receptors, including those in the adrenal medulla (Wakade & Wakade 1983). After smoking, plasma levels of adrenaline and noradrenaline rise (Grassi *et al.* 1994, Walker *et al.* 1999, Reselandn *et al.* 2005); these factors play an important role in the development of hypertension.

During pregnancy, cigarette smoke is considered a risk factor for low birth weight and neurological abnormalities

(Butler & Goldstein 1973, Navarro *et al.* 1989, DiFranza & Lew 1995). In humans, smoking during pregnancy is related to lower leptin concentration in the cord blood of newborns (Mantzoros *et al.* 1997). Maternal smoking also causes an increase in the levels of catecholamines in the amniotic fluid, suggesting that fetal adrenergic activation results from fetal hypoxia and/or from a direct effect of nicotine on the fetal adrenergic system (Divers *et al.* 1981). Research has also shown an increased risk of hypertension in children whose mothers smoked during pregnancy (Blake *et al.* 2000).

While it has been established that many women quit smoking during pregnancy (Giglia *et al.* 2006, Polańska *et al.* 2006), little is known about *postpartum* maintenance of smoking cessation and relapse. Some studies reveal that most women who stop smoking during gestation relapse during lactation (McBride & Pirie 1990, O'Campo *et al.* 1992, Hannöver *et al.* 2008). Lactation is a critical period of life once important cognitive and neurological developments occur. Mother's milk represents the primary source of nutrition (Golding *et al.* 1997). Nicotine is transferred

through maternal milk and causes tachycardia in the offspring due to higher adrenergic activity. Cotinine, the main metabolite of nicotine, can be measured in blood, urine, saliva, and milk (Luck & Nau 1987, Dahlstrom *et al.* 1990, Nel & Morgan 1996, Narayanan *et al.* 2002). Recently, it has been demonstrated that tobacco smoke alters cytokine levels in maternal milk. Smoking mothers present lower IL-1 in the colostrum than non-smoking mothers (Zanardo *et al.* 2005). Because nicotine is present in the breast milk, it can affect other milk cytokines, such as leptin.

We recently showed that in rats, maternal nicotine exposure during lactation only leads to long-term effects on body weight (BW) regulation, leptin concentration, and thyroid function in adult offspring (Oliveira *et al.* 2009). In this study, we used nicotine osmotic minipumps at an infusion rate of 6 mg/kg per day in the mothers to produce nicotine levels quite similar to those achieved in heavy smokers (Murrin *et al.* 1987, Lichtensteiger *et al.* 1988). To our knowledge, there are a few experimental studies focusing on the effects of nicotine exposure exclusively during the early postnatal period. This may be of particular interest because there is a high rate of smoking relapse among lactating women (McBride & Pirie 1990), and tobacco has well-known effects on metabolic diseases and cardiovascular dysfunction. Thus, our aim was to evaluate the short-term consequences of maternal nicotine exposure, during lactation only, on the hormonal, biochemical, and nutritional profiles of mothers and suckling pups during nicotine exposure and after its withdrawal.

## 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 the 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 ( $25 \pm 1$  °C) with artificial light–darkness cycles (lights on at 0700 h and lights off at 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.

### Model of neonatal nicotine exposure

Two days after birth, 20 lactating rats were randomly assigned to one of the following groups:

Nicotine (NIC,  $n=10$ ) – dams were lightly anesthetized with thiopental, a  $3 \times 6$  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 nicotine peaks, we chose to perform the nicotine exposure using s.c. osmotic minipump infusion. Pumps were prepared with nicotine-free base diluted in a saline solution (NaCl 0.9%) to deliver an initial dose rate of 6 mg/kg of nicotine per day (during 14 days of lactation) as described previously (Abreu-Villaca *et al.* 2004). At this rate, this paradigm produces plasma nicotine levels similar to those in typical smokers,  $\sim 25$  ng/ml (Lichtensteiger *et al.* 1988). The incision was closed, and the mothers were permitted to recover in their home cages.

Control (C,  $n=10$ ) – dams were implanted with osmotic minipumps containing only saline solution, released for the same period as in rats in the experimental group.

At birth, litter adjustment was performed, and six male pups were kept per NIC or C dam to maximize lactation performance. During all lactation periods, BW (mothers and pups) and relative food intake (g/100 g BW) of the mothers were monitored daily. Dams and offspring were killed at 15 (end of NIC exposure) and 21 (6 days post NIC withdrawal) days of lactation by rapid decapitation, with no prior anesthesia, because anesthesia affects hormone and lipid metabolism. Blood, visceral fat mass (VFM), carcass, and adrenal gland were collected.

### Milk collection

Milk samples were collected at 15 and 21 days of lactation. For this, dams were separated from litters for a period of 2 h before milking (Bonomo *et al.* 2005). After i.p. injection of oxytocin (5 UI) under pentobarbital anesthesia (30 mg/kg BW), milk was manually collected from all teats. We obtained 0.5–1.0 ml from each lactating rat, and the samples were frozen at  $-20$  °C for further analysis.

### Estimation of milk production

This experiment was performed as described previously (Bonomo *et al.* 2005). Briefly, NIC and C pups were divided into two subgroups: a) breast-feeding or b) fasting, and the pups were separated from the dams for 24 h and used as controls to estimate milk production. Both groups were weighed before (W1) and after 24 h (W2), and milk production was estimated according to the formula: Milk yield (g/day) =  $W2a - W1a(1 - K)$ , where the correction factor  $K$  is the relative loss of weight in the fasted pups ( $K = W1b - W2b/W1b$ ).

### Detection of cotinine in serum and milk

Serum and milk cotinine levels were determined at 15 days of lactation using a cotinine assay kit obtained from Orasure Technologies (Bethlehem, PA, USA) in accordance with the manufacturer's recommendations. Dams were separated from

their litters, and 2 h later, milk was collected as described previously. After that, dams and pups were killed for blood collection. Samples were stored at  $-20^{\circ}\text{C}$  until analysis.

#### *Analysis of milk biochemical composition*

Total milk protein was measured according to the Peterson method (1977), using BSA as the standard. Protein concentration was determined based on the Stauffer formula (1975), and the results were expressed in mg/ml.

Total lipids were measured in milk samples diluted in distilled water (1:25) by colorimetric assay, using a Bioclin commercial kit. Results were expressed in mg/dl.

Milk lactose was measured by a colorimetric method using picric acid (Khrumov *et al.* 2008), using commercial lactose as the standard (Sigma). Results were expressed in mg/ml.

Milk energy was calculated from milk production in 24 h using the isolated macronutrients.

#### *Body composition evaluation*

On the day of killing, VFM was excised (mesenteric, epididymal, and retroperitoneal white adipose tissue) and immediately weighed for evaluation of central adiposity (Toste *et al.* 2006). Total body fat and protein levels were determined by carcass analysis (Fagundes *et al.* 2007). Pups were eviscerated; the carcasses were weighed, autoclaved for 1 h, and homogenized in distilled water (1:1). Homogenates were stored at  $4^{\circ}\text{C}$  for analysis.

Three grams of homogenate were used to gravimetrically determine fat content. Samples were hydrolyzed in a shaking water bath at  $70^{\circ}\text{C}$  for 2 h with 30% KOH and ethanol. Total fatty acids and free cholesterol were removed with three successive washes of petroleum ether. After drying overnight in vacuum, tubes were weighed and data were expressed as grams of fat per 100 g of carcass.

Protein content was determined in 1 g of homogenate. Tubes were centrifuged at 2000 g for 10 min. The total protein concentrations were determined by the Lowry method (Lowry *et al.* 1951). Data were expressed as grams of protein per 100 g of carcass.

#### *Serum biochemical evaluation*

Total cholesterol (TC), triglycerides, high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were analyzed using Biosystem commercial test kits with an automated A15 spectrophotometer (Biosystems S.A., Barcelona, Spain). LDL-C and very low-density lipoprotein cholesterol (VLDL-C) were calculated according to the Friedewald equation (Friedewald *et al.* 1972):

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

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

Castelli indices I and II, which correlate to atherogenesis, were obtained using the formulas:

$$1) \text{ Castelli index I} = \text{Total cholesterol}/\text{HDL-C}$$

$$2) \text{ Castelli index II} = \text{LDL-C}/\text{HDL-C}.$$

The Biuret reaction (Gornall *et al.* 1949) was performed for total serum protein measurement, and absorbance of products was determined on a spectrophotometer (automated A15 BioSystems analyzer). Serum albumin was determined by bromocresol green method (Doumas *et al.* 1971), and globulin fraction was measured as the difference between total protein and albumin. Values were expressed as mg/dl.

#### *Hormone determination by RIA*

Blood samples were centrifuged (1500 g/20 min per  $4^{\circ}\text{C}$ ) to obtain sera, which were kept at  $-20^{\circ}\text{C}$  until the assay. All measurements were performed in one assay.

Serum prolactin (PRL) concentrations were measured by a specific RIA using reagents supplied by the National Institute of Diabetes and Digestive and Kidney Diseases (NIH, Bethesda, MD, USA). Data were reported in nanogram from the reference preparation RP-3 with an assay sensitivity of 0.3 ng/ml and an intra-assay coefficient of 8%.

Serum and milk leptin concentrations were measured by a specific RIA kit (Linco Research, Inc., St Louis, MO, USA) that measures both rat and mouse leptin (range of detection: 0.5–50 ng/ml; intra-assay variation 2.9%).

Serum corticosterone was measured using a specific commercial RIA kit (ICN Biomedicals Inc., Aurora, OH, USA) with an assay sensitivity of 50 ng/ml and an intra-assay coefficient of variation of 7%.

#### *Catecholamine level quantification*

Adrenal glands were used for the quantification of total catecholamines (epinephrine and norepinephrine) using the trihydroxyindole fluorescence method (Trevenzoli *et al.* 2007). Left adrenal glands were homogenized in 500  $\mu\text{l}$  of 10% acetic acid and centrifuged (10 000 g for 1 min). For the assay, 50  $\mu\text{l}$  of the supernatant/epinephrine standards were mixed with 250  $\mu\text{l}$  of phosphate buffer (0.5 M, pH 7.0) and 25  $\mu\text{l}$  of potassium ferricyanate (0.5%) followed by incubation (20 min; ice bath). The reaction was stopped with 500  $\mu\text{l}$  ascorbic acid/10 M NaOH (1:19). The parameters used in the fluorimeter (Victor2, PerkinElmer, Waltham, MA, USA) were 420 nm excitation and 510 nm emission. Results were obtained by plotting the values as a linear regression of the standard epinephrine curve. Data were expressed as micrometer catecholamines.

#### *Western blotting analysis*

In order to evaluate adrenal function on the last day of nicotine exposure when adrenal catecholamine levels were high, tyrosine hydroxylase (TH) levels in the adrenal medulla were

quantified by western blotting. TH, an essential enzyme in the catecholamine synthesis pathway, was measured in 15-day-old pups. For this, adrenal glands were processed for western blotting as reported previously (Trevenzoli *et al.* 2007, Fagundes *et al.* 2009). Briefly, glands were homogenized in 1 ml phosphate buffer (pH 7.4), containing 1  $\mu$ l protease inhibitor cocktail (1 mg/ml each of aprotinin, leupeptin, and trypsin inhibitor), and centrifuged at 1120 g for 5 min at 4 °C. Protein concentration in the supernatants was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, San Jose, CA, USA). The supernatants were analyzed by SDS-PAGE using 10  $\mu$ g total protein. Samples were electroblotted onto nitrocellulose membranes (Hybond P ECL membrane, Amersham Biosciences). Membranes were incubated with Tris-buffered saline (TBS) containing 5% non-fat dry milk for 90 min to block non-specific binding sites. Then, membranes were washed with TBS and incubated with a specific primary antibody (monoclonal mouse anti-TH; Sigma-Aldrich) overnight at 4 °C (diluted 1:2000 in 0.5% non-fat dry milk-containing TBS). Anti-actin (119 sc, goat polyclonal 1616; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at a 1:500 dilution was used as the internal control. Membranes were washed and incubated with secondary antibody (goat anti-mouse; Santa Cruz Biotechnology) conjugated with HRP (diluted 1:2000 in 0.5% non-fat dry milk-containing TBS) for 1 h at room temperature. Finally, TH bands were visualized by chemiluminescence (Kit ECL plus, Amersham Biosciences) followed by exposure to autoradiographic film (Hyperfilm ECL, Amersham Biosciences) for 5 s. Area and density of the bands were quantified by Image J software (Media Cybernetics, Bethesda, MD, USA). Results were expressed as relative (%) to the control group.

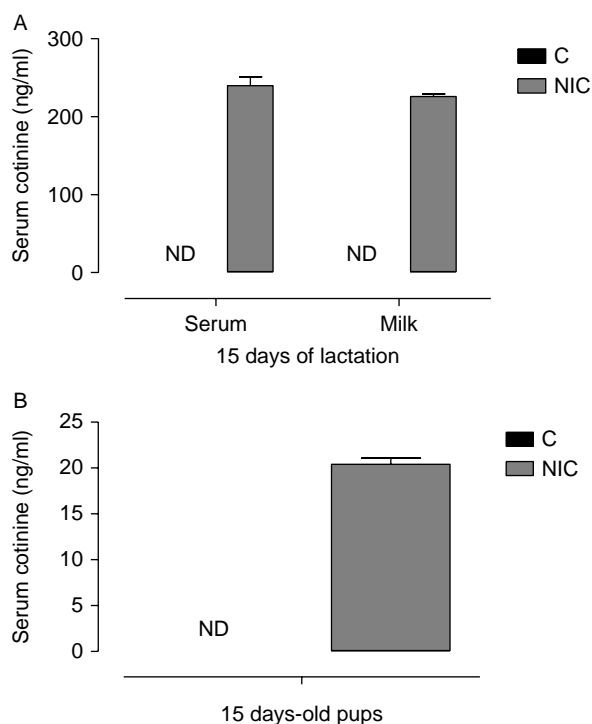
### Statistical analysis

Results were reported as mean  $\pm$  s.e.m. GraphPad Prism 5 was used for statistical analyses and graphics (GraphPad Software, Inc., La Jolla, CA, USA). Changes in BW and food intake were analyzed by two-way ANOVA and Newman-Keuls multiple comparison tests. TH expression was analyzed by the non-parametric Mann-Whitney *U* test. The other experimental data were analyzed by unpaired Student's *t*-test with significance level set at  $P < 0.05$ . We studied two offspring from each mother at each time point (ten pups per group). However, for the analyses, litter was used as the experimental unit so we considered the average of values from animals of the same litter instead of using individual animal values.

## Results

### Cotinine levels

Cotinine levels of control dams and pups were below the technique's detection limit ( $< 8$  ng/ml). Nicotine treatment during 14 days of lactation affected cotinine milk and serum levels. In NIC-treated dams, milk and serum levels of cotinine



**Figure 1** Milk and serum cotinine in NIC-treated (NIC) or saline-treated (C) lactating rats at 15 days of lactation (A). Serum cotinine in NIC or C pups (B). Note that nicotine metabolic levels are non-detectable (ND) in control dams and pups. Values represent mean  $\pm$  s.e.m. of five to ten rats per group.

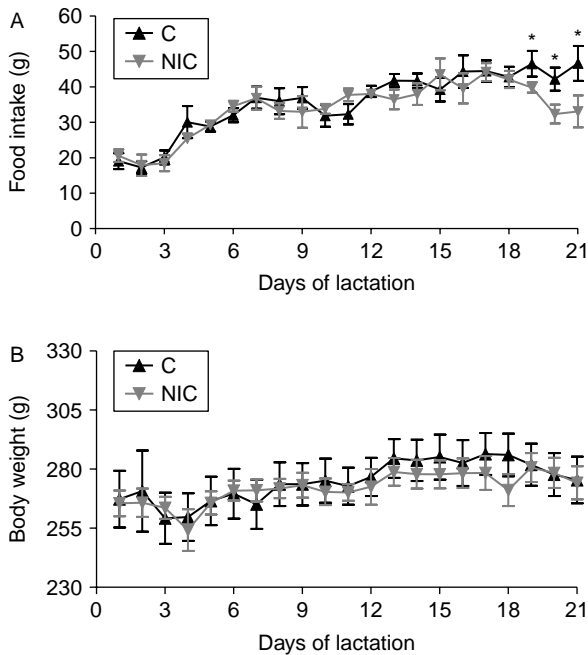
were similar (Fig. 1A). The 15-day-old NIC pups presented lower serum cotinine (Fig. 1B) compared with maternal levels, probably as a result of separation from their mothers before killing.

### Mothers

During nicotine exposure, NIC dams exhibited no change in BW or food intake in comparison to the C group (Fig. 2). However, NIC dams showed lower food intake during the last 3 days of lactation (day 19:  $-19\%$ , day 20:  $-22\%$ , and day 21:  $-36\%$ ; Fig. 2A,  $P < 0.05$ ). At weaning, NIC dams showed higher serum and milk leptin levels ( $+68\%$  and  $+3$  times respectively, Fig. 3A and B,  $P < 0.05$ ).

At 15 days of lactation, NIC dams showed higher serum PRL ( $+60\%$ , Fig. 4,  $P < 0.05$ ) as well as higher milk production and energy ( $+45$  and  $+36\%$  respectively, Table 1,  $P < 0.05$ ). Analysis of milk biochemistry showed that NIC dams had higher lactose content ( $+29\%$ , Table 1,  $P < 0.05$ ) only at 15 days of lactation, with no changes in milk protein or lipids during either of the periods studied.

As shown in Table 2, we observed higher levels of HDL-C ( $+16\%$ ,  $P < 0.05$ ) and lower Castelli index I values ( $-9\%$ ,  $P < 0.05$ ) in NIC dams at 15 days of lactation. At weaning, no alterations in serum biochemical parameters were detected in NIC dams.



**Figure 2** Food intake (A) and body weight (B) in NIC-treated (NIC) or saline-treated (C) lactating rats during lactation. Values represent mean  $\pm$  S.E.M. of five dams per group. \* $P < 0.05$  versus C.

NIC dams presented no change in serum corticosterone (Fig. 5A), adrenal total catecholamine content (Fig. 5B), or adrenal gland mass (data not shown) during both lactation periods.

#### Progeny

Maternal NIC exposure did not alter BW gain in offspring during lactation (Fig. 6A). We observed hyperleptinemia (+35%,  $P < 0.05$ ) in 15-day-old NIC pups, as depicted in Fig. 6B.

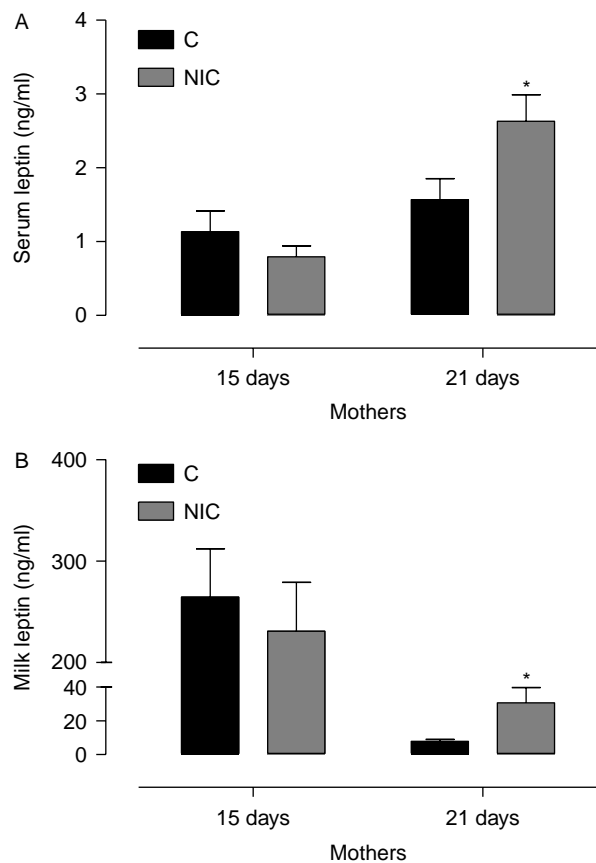
As shown in Table 3, the 15-day-old NIC pups showed higher total fat mass (+30%,  $P < 0.05$ ) and a trend toward a significant increase for VFM (+73%,  $P = 0.08$ ). At weaning, these pups presented only higher total body protein (+33%,  $P < 0.05$ ).

Fifteen-day-old NIC pups presented higher serum HDL-C (+24%; Table 2,  $P < 0.05$ ), but this difference was not maintained at weaning. At weaning, this group showed higher serum globulin (+34%,  $P < 0.05$ ).

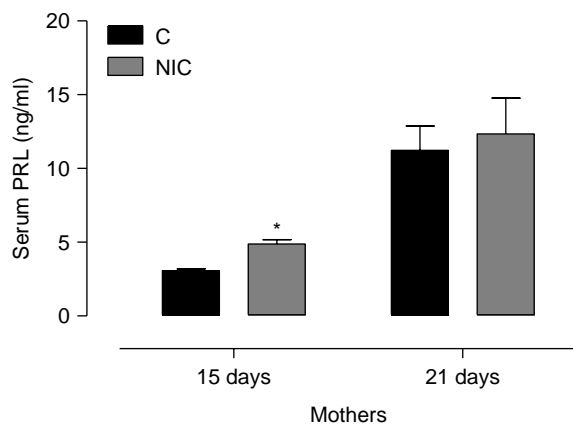
Serum corticosterone levels were higher in 15-day-old NIC pups compared with the control group (Fig. 7A). Adrenal catecholamine content (+69%, Fig. 7B,  $P < 0.05$ ) as well as adrenal gland mass (C:  $2.50 \pm 0.17$ , NIC:  $3.49 \pm 0.20$  mg,  $P < 0.05$ ) was higher in 15-day-old NIC pups, but these parameters were unchanged at weaning. Adrenal TH protein expression was lower (−33%,  $P < 0.05$ ) in NIC pups at the end of the period of maternal exposure to nicotine (Fig. 8).

## Discussion

There is a high rate of smoking relapse among women who quit smoking during pregnancy (McBride & Pirie 1990, O'Campo *et al.* 1992, Hannöver *et al.* 2008). Nonetheless, most epidemiological and experimental studies on maternal smoking or nicotine exposure were performed during pregnancy or pregnancy and lactation. In the present study, for the first time, we used an experimental model of postnatal nicotine treatment to show that maternal nicotine exposure during lactation leads to important changes in the nutritional, biochemical, and hormonal profiles in both mother and offspring. After nicotine withdrawal, some alterations revert to baseline, despite the emergence of other metabolic changes. Accordingly, we have identified lactation as a period that is sensitive to the isolated effects of nicotine. Future studies are necessary to verify whether similar alterations occur in smoking mothers and lactating neonates. Developmental differences occur in rats when compared with humans. Some structures that are immature in rats at birth mature only in postnatal period (in the first week of life), whilst in humans,



**Figure 3** Serum (A) and milk (B) leptin content in NIC-treated (NIC) or saline-treated (C) lactating rats at 15 and 21 days of lactation. Values represent mean  $\pm$  S.E.M. of five dams per group. \* $P < 0.05$  versus C.



**Figure 4** Serum PRL in NIC-treated (NIC) or saline-treated (C) lactating rats at 15 and 21 days of lactation. Values represent mean  $\pm$  S.E.M. of five dams per group. \* $P < 0.05$  versus C.

this happens in gestation. Lactation in rats is a critical period to nicotine exposure, corresponding roughly, at least concerning to the neural development to the second trimester of gestation in humans (Vinay *et al.* 2005).

#### Changes in lactating mothers

The goal of this study was to characterize the effect of nicotine alone during lactation, which is the main cigarette compound that is addictive. We believe that this could represent the maternal smoking effect upon the progeny. Serum nicotine levels of 25 ng/ml are characteristic of heavy smokers (Lichtensteiger *et al.* 1988). To simulate this situation during lactation, lactating rats were implanted with s.c. osmotic minipumps releasing 6 mg/kg per day nicotine continuously (Oliveira *et al.* 2009). The serum nicotine:cotinine ratio was 1:5 to 1:10 (Trauth *et al.* 2000). In the present study, we detected high levels of serum cotinine in NIC dams (239 ng/ml) as well as in the milk (226 ng/ml). Then, in our experimental model, NIC dams were exposed to nicotine at levels that mimicked those in mothers who smoke heavily. Regarding the pups, maternal treatment with nicotine resulted in very low cotinine levels (20.4 ng/ml). Because nicotine is transferred through the milk (Narayanan *et al.* 2002),

separation of the pups from their mothers for 2 h to obtain milk samples before killing may have led to underestimations of the real impact of maternal nicotine exposure.

Adult rats treated with nicotine lose weight and are hypophagic (Li *et al.* 2000); however, treatment with nicotine during pregnancy does not affect maternal BW gain or food intake (Chen *et al.* 2005). In our study, lactating rats had no change in BW and food intake during nicotine exposure. Both pregnancy and lactation period are peculiar in several aspects. Particularly during lactation, many mechanisms are activated in order to supply the high energy requirement, including hyperphagia, basal metabolic rate reduction, and preferential nutrient flux for lactogenesis (Dewey 1998). Accordingly, it is possible that during this particular period, these events gain importance and counterbalance the well-known effects of nicotine in non-lactating animals. However, after nicotine withdrawal, lactating dams displayed lower food intake. The hyperleptinemia in NIC dams observed at weaning could be responsible for this lower food intake. The high leptin levels in the milk of NIC dams at weaning may be due to the higher levels of leptin in maternal serum.

At the end of nicotine exposure (15 days of lactation), dams showed higher serum PRL that corresponded to higher levels of milk production. In fact, previous studies showed that smoking acutely increases plasma PRL (Wilkins *et al.* 1982, Gossain *et al.* 1986). At weaning, in the absence of nicotine, these parameters were normalized. Again, only under the effects of nicotine, we detected changes in milk composition, i.e. higher lactose content. This alteration may be responsible for the higher energy contained in the milk of NIC dams at 15 days of lactation. Also, PRL is orexigenic during lactation (Roy *et al.* 2007). Thus, PRL can be the counterbalancing factor that normalizes food intake due to the probable anorexigenic effect mediated by nicotine.

Cigarette smoking contributes to cardiovascular disease through alterations in the lipid profile, and in particular, the impact on HDL-C, because smoking cessation leads to an increase in HDL-C (Oeser *et al.* 1999, Maeda *et al.* 2003). Mixed results have been reported for nicotine replacement therapy (chewing gum, patches, and nasal spray). Certain studies described an increase in HDL-C concentrations (Allen *et al.* 1994), while other studies showed no lipoprotein

**Table 1** Milk changes in NIC-treated dams during lactation. Values represent mean  $\pm$  S.E.M. of five lactating rats per group

	15th lactation day		21st lactation day	
	C	NIC	C	NIC
<b>Milk nutrients</b>				
Lactose (mg/ml)	27.02 $\pm$ 3.01	38.31 $\pm$ 3.13 <sup>a</sup>	23.78 $\pm$ 2.55	28.93 $\pm$ 3.10
Milk total protein (mg/ml)	72.42 $\pm$ 4.10	64.11 $\pm$ 8.12	63.10 $\pm$ 4.78	66.76 $\pm$ 3.26
Cholesterol (mg/dl)	320.4 $\pm$ 15.86	369.2 $\pm$ 24.11	383.0 $\pm$ 30.21	379.0 $\pm$ 35.85
Triglycerides (g/dl)	5.29 $\pm$ 0.34	7.03 $\pm$ 0.94	7.42 $\pm$ 0.58	8.36 $\pm$ 0.84
Milk energy concentration (Cal/ml)	1.35 $\pm$ 0.97	1.84 $\pm$ 0.21 <sup>a</sup>	1.98 $\pm$ 0.19	2.14 $\pm$ 0.28
Milk production (g)	4.43 $\pm$ 0.41	6.46 $\pm$ 0.80 <sup>a</sup>	8.83 $\pm$ 1.90	7.35 $\pm$ 1.04

<sup>a</sup>Significant differences between groups.

**Table 2** Serum biochemical parameters in NIC-treated dams and offspring during lactation. Values represent mean  $\pm$  S.E.M. of five to ten dams per group. For pups, the average of values from animals of the same litter was used so that  $n=5$  per group

	15 days		21 days	
	C	NIC	C	NIC
<b>Mothers</b>				
Total cholesterol (mg/dl)	71.20 $\pm$ 3.16	74.90 $\pm$ 2.56	55.60 $\pm$ 4.48	58.30 $\pm$ 4.12
HDL-C (mg/dl)	27.60 $\pm$ 1.03	32.00 $\pm$ 1.07*	21.11 $\pm$ 2.7	22.67 $\pm$ 4.7
LDL-C (mg/dl)	25.40 $\pm$ 3.20	28.00 $\pm$ 3.03	27.29 $\pm$ 6.30	23.60 $\pm$ 4.47
VLDL-C (mg/dl)	18.20 $\pm$ 2.31	14.90 $\pm$ 2.37	16.90 $\pm$ 2.17	13.80 $\pm$ 1.90
Triglycerides (mg/dl)	90.30 $\pm$ 11.53	75.20 $\pm$ 11.83	84.60 $\pm$ 10.89	69.40 $\pm$ 9.52
Castelli index I	2.58 $\pm$ 0.07	2.35 $\pm$ 0.06*	3.41 $\pm$ 0.58	3.89 $\pm$ 0.67
Castelli index II	0.93 $\pm$ 0.12	0.87 $\pm$ 0.09	1.84 $\pm$ 0.61	1.71 $\pm$ 0.45
Total protein (mg/dl)	6.11 $\pm$ 0.09	6.03 $\pm$ 0.07	5.76 $\pm$ 0.23	5.71 $\pm$ 0.24
Albumin (mg/dl)	2.73 $\pm$ 0.24	3.16 $\pm$ 0.26	2.33 $\pm$ 0.13	2.15 $\pm$ 0.17
Globulin (mg/dl)	3.38 $\pm$ 0.23	2.87 $\pm$ 0.26	3.43 $\pm$ 0.13	3.56 $\pm$ 0.10
<b>Pups</b>				
Total cholesterol (mg/dl)	146.9 $\pm$ 5.36	159.6 $\pm$ 6.99	104.7 $\pm$ 4.12	126.7 $\pm$ 7.02
HDL-C (mg/dl)	28.70 $\pm$ 0.92	35.70 $\pm$ 1.84*	30.50 $\pm$ 2.73	41.70 $\pm$ 3.15
LDL-C (mg/dl)	91.20 $\pm$ 5.94	97.20 $\pm$ 8.64	52.40 $\pm$ 4.66	59.10 $\pm$ 5.31
VLDL-C (mg/dl)	26.88 $\pm$ 3.09	26.70 $\pm$ 3.35	21.80 $\pm$ 3.02	25.90 $\pm$ 2.53
Triglycerides (mg/dl)	134.4 $\pm$ 15.38	133.7 $\pm$ 16.96	109.1 $\pm$ 15.12	129.7 $\pm$ 12.50
Castelli index I	5.15 $\pm$ 0.21	4.55 $\pm$ 0.26	3.63 $\pm$ 0.29	3.125 $\pm$ 0.19
Castelli index II	3.22 $\pm$ 0.24	2.78 $\pm$ 0.28	1.92 $\pm$ 0.29	1.50 $\pm$ 0.19
Total protein (mg/dl)	4.82 $\pm$ 0.12	4.92 $\pm$ 0.09	5.44 $\pm$ 0.19	6.14 $\pm$ 0.14
Albumin (mg/dl)	2.34 $\pm$ 0.07	2.48 $\pm$ 0.10	3.02 $\pm$ 0.17	2.89 $\pm$ 0.08
Globulin (mg/dl)	2.48 $\pm$ 0.10	2.44 $\pm$ 0.09	2.42 $\pm$ 0.19	3.25 $\pm$ 0.23*

HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; VLDL-C, very low-density lipoprotein cholesterol. \* $P < 0.05$ .

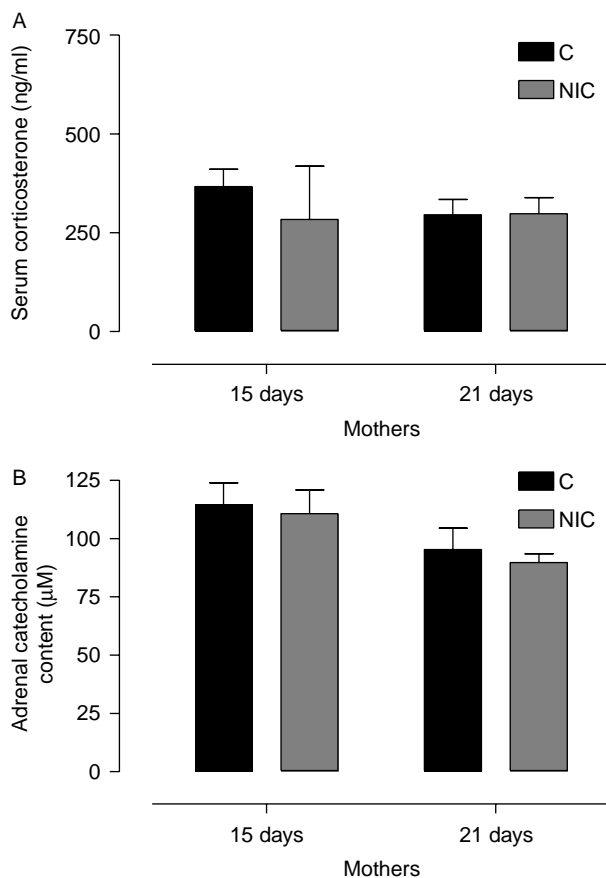
increase until nicotine withdrawal (Quensel *et al.* 1989, Thomas *et al.* 1995). In adult female rats, nicotine treatment increases TC and non-esterified fatty acids (Abd el Mohsen *et al.* 1997). However, to date, there are no data concerning the short-term effect of maternal nicotine exposure during lactation upon serum lipid profile. In our study, for the first time, we found higher levels of HDL-C in NIC dams at the end of nicotine exposure, similar to the findings published by Allen *et al.* (1994). This result is interesting, and certainly deserves more study. We suggest that these higher levels result from a compensatory mechanism typical of the lactation period, which was not observed in non-lactating female rats. Moreover, our results suggest that the deleterious effect of smoking on HDL cannot be attributed to nicotine.

It is well known that nicotine and other cholinergic agonists act directly through nicotine receptors in chromaffin cells of the adrenal medulla, increasing catecholamine production and release (Sala *et al.* 2008). Furthermore, nicotine can stimulate the hypothalamic-pituitary-adrenal axis, increasing glucocorticoid levels (Pauly *et al.* 1992). Nonetheless, with regard to the evaluation of maternal adrenal hormones, we did not observe significant changes in the incidence of corticosteronemia or adrenal catecholamine content in lactating rats during treatment with nicotine or after its withdrawal. Our results signal that these mothers are not stressed in our experimental model of neonatal nicotine exposure.

#### Changes in suckling pups

In previous experimental studies, pre- and postnatal nicotine exposure failed to cause changes in BW during the exposure period (Chen & Kelly 2005, Gao *et al.* 2005). As demonstrated previously by our group (Oliveira *et al.* 2009), maternal nicotine exposure did not affect BW gain of NIC offspring during lactation. Interestingly, pups showed higher fat mass during nicotine exposure, but after nicotine withdrawal, weaned pups did not display changes in adiposity. At 15 days, NIC pups showed hyperleptinemia that may have been caused by higher adiposity since this hormone is mainly produced by adipose tissue (Ahima 2005) or the result of higher leptin transfer through maternal milk.

At weaning, in the absence of nicotine, NIC pups displayed higher body protein content and elevated serum globulin. Because leptin stimulates GH secretion (Tannenbaum *et al.* 1998), it is possible that the high leptin levels of 15-day-old NIC pups stimulate muscle protein synthesis through GH action, resulting in higher levels of total body protein. Hyperleptinemia is also associated with higher protein content in the carcass (Toste *et al.* 2006). Also, some hormones, such as leptin, insulin, GH, sex steroids, and thyroid hormones, are capable of altering liver production of proteins such as globulin (Gómez 2007, Szymeczko *et al.* 2009). Maybe, the increase in serum globulin levels of NIC pups at weaning is a consequence of changes, such as hyperleptinemia.



**Figure 5** Serum corticosterone (A) and adrenal catecholamine content in NIC-treated (NIC) or saline-treated (C) lactating rats at 15 and 21 days of lactation. Values represent mean  $\pm$  S.E.M. of five dams per group.

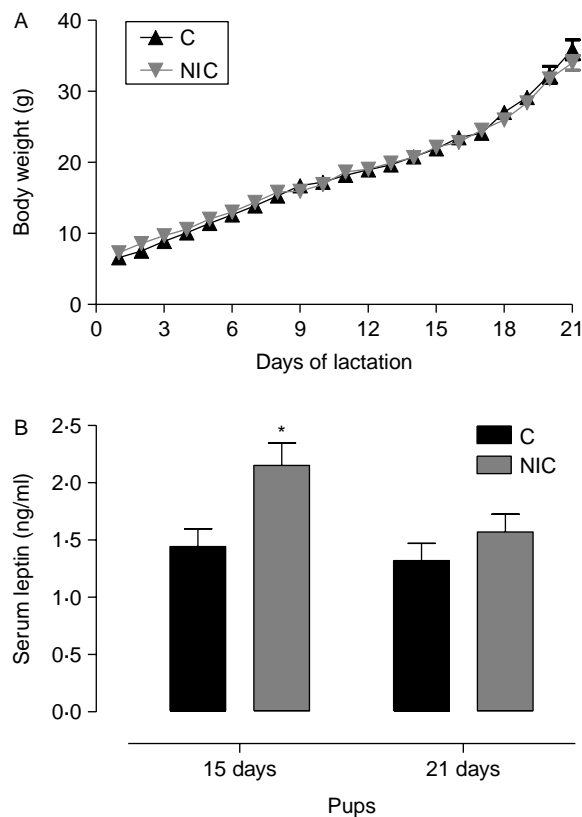
As observed in mothers, we detected higher serum HDL-C in 15-day-old NIC pups. Therefore, as explained for the mothers, nicotine exposure during lactation could exert a protector effect against the development of dyslipidemia in offspring, despite the higher TC observed in weaned NIC pups.

Adrenal medulla stimulation occurs in smokers due to nicotine-stimulated catecholamine release (Aziz *et al.* 1978, Hansen *et al.* 1997). Maternal nicotine treatment for 14 days of lactation yielded higher adrenal gland mass and total catecholamine levels in 15-day-old pups; these changes may have resulted from a direct effect of nicotine on adrenal chromaffin cells (Sala *et al.* 2008). Notably, leptin stimulates catecholamine synthesis and secretion (Trevenzoli *et al.* 2007), so the present findings may be due to higher serum leptin in NIC pups.

On the other hand, we detected lower levels of adrenal TH protein, a key enzyme in the catecholamine synthesis pathway, in 15-day-old NIC pups. Thus, the observation of elevated catecholamine content in combination with lower TH expression in suckling pups at the end of nicotine

exposure suggests that these neonates present dysfunctional catecholamine synthesis and reduced catecholamine secretion. It is also possible that the excess of catecholamines inhibits TH expression and activity as a mechanism for feedback control of the biosynthetic pathway (La Gamma & Black 1989). However, we cannot rule out desensitization of the nicotine receptor induced by long-term exposure to nicotine. This phenomenon would explain lower TH expression as well as higher levels of catecholamines in association with lower levels of cholinergically stimulated release. In contrast, other studies have shown higher TH expression and activity in the adrenal medulla of rats exposed acutely (Tank *et al.* 1998, Sterling & Tank 2001) or chronically to nicotine (Sun *et al.* 2003). Cheng *et al.* (2005) observed no changes in *Th* mRNA levels in rats that were chronically exposed. Distinct outcomes may be related to the period of exposure. The present study is the only one in which exposure was restricted to the lactation period.

In weaned offspring whose mothers received nicotine from day 2 until day 14 of lactation, there were no changes in total catecholamine content, suggesting that after nicotine withdrawal, the alteration in adrenal medulla is normalized.



**Figure 6** Body weight during lactation (A) and serum leptin (B) of pups whose mothers were nicotine (NIC) or saline (C) exposed during lactation. For body weight, values represent mean  $\pm$  S.E.M. of ten pups per group. For leptin, the average of values from animals of the same litter was used so that  $n=5$  per group. \* $P<0.05$  versus C.



**Table 3** Body composition of pups from NIC-treated dams during lactation. Values represent mean  $\pm$  S.E.M. The average of values from animals of the same litter was used so that  $n=5$  per group

Parameters	15 days old		21 days old	
	C	NIC	C	NIC
Visceral fat mass (%)	0.38 $\pm$ 0.04	0.65 $\pm$ 0.09	0.94 $\pm$ 0.07	0.94 $\pm$ 0.15
Total fat content (%)	4.28 $\pm$ 0.27	5.53 $\pm$ 0.15 <sup>a</sup>	6.37 $\pm$ 0.56	5.51 $\pm$ 0.52
Total protein content (%)	12.49 $\pm$ 1.28	13.21 $\pm$ 1.70	9.11 $\pm$ 0.54	11.90 $\pm$ 0.90 <sup>a</sup>

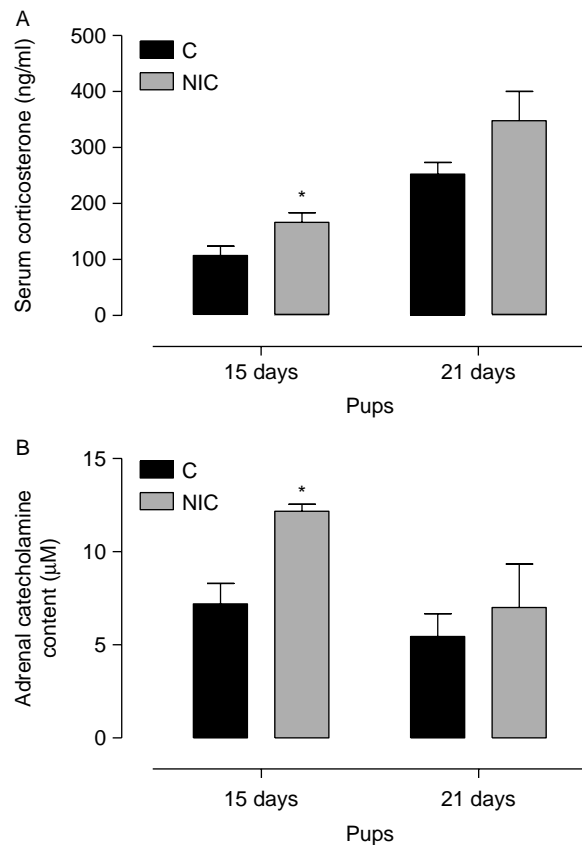
<sup>a</sup>Significant differences between groups.

We have already shown that maternal nicotine exposure causes early hypothyroidism during lactation. This effect is normalized at weaning when the animals are no longer under the influence of nicotine. However, at adulthood, these animals develop secondary thyroid hypofunction (Oliveira *et al.* 2009). Then, even with the normalization of catecholamine content at weaning, NIC offspring may present adrenal medullary dysfunction at adulthood, as that occurring for thyroid status.

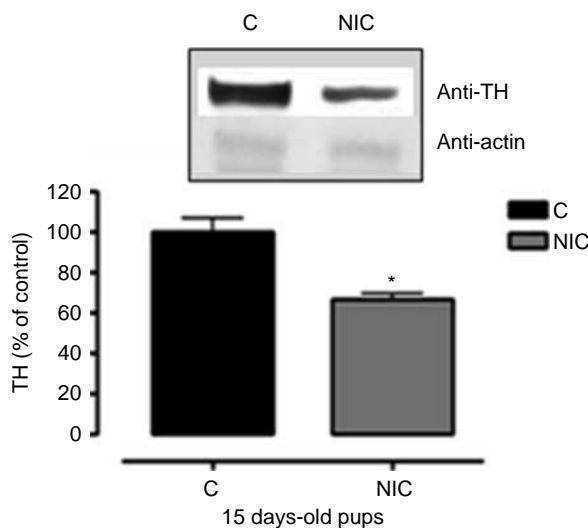
Cigarette smoking acutely raises serum cortisol levels (Yeh & Barbieri 1989), and nicotine increases ADH. These factors may cause higher ACTH and cortisol release (Robinson 1977). In our experimental model, serum corticosterone concentrations were higher in NIC pups at 15 days of lactation. It is well known that intra-adrenal portal vascular system provides the medulla with uniquely high concentrations of glucocorticoids. These high concentrations are needed to induce the medullary enzyme, phenylethanolamine-N-methyltransferase, which controls the synthesis of epinephrine from norepinephrine (Wurtman 2002). Then, the hypercorticosteronemia detected could be responsible for the elevated catecholamine production in NIC pups at the end of the period of maternal exposure to nicotine. Previously, in the model of neonatal hyperleptinemia, we also observed the association between higher serum corticosterone and higher adrenal total catecholamine contents in adult life (Fraga-Marques *et al.* 2009). Data on the relationship between nicotine and corticosterone are divergent; rats that are chronically nicotine treated may have higher serum corticosterone (Davis *et al.* 2005) or no alteration (Cruz *et al.* 2007). Chen *et al.* (2007) showed that during pregnancy, nicotine exposure leads to an increase in maternal corticosterone that results in higher corticosterone levels in the fetus. However, we did not observe changes in maternal corticosterone during nicotine exposure or after its removal. This may explain, at least in part, the lack of any alteration in the corticosteronemia observed in NIC pups at 21 days of lactation. We can therefore conclude that stress does not play a role in these events at weaning. On the other hand, as evidenced for thyroid function in a previous study (Oliveira *et al.* 2009) and already mentioned for catecholamine in the present data, even with the normalization of glucocorticoid levels at weaning, NIC offspring may present long-term consequences of stress mechanism.

### Final considerations

In the present study, at least, there are three ways to understand the effects of nicotine. First, nicotine transfer through maternal milk (Dahlstrom *et al.* 1990, Narayanan *et al.* 2002) may change some factor(s) in offspring. Secondly, maternal alterations caused by nicotine treatment, for example nutritional and/or hormonal changes, may be transferred through the milk to the pups. Thirdly, our findings may result from functional changes in both mothers and pups.



**Figure 7** Serum corticosterone (A) and adrenal catecholamine content (B) of 15- and 21-day-old pups whose mothers were nicotine (NIC) or saline (C) exposed during lactation. Values represent mean  $\pm$  S.E.M. The average of values from animals of the same litter was used so that  $n=5$  per group. \* $P<0.05$  versus C.



**Figure 8** Tyrosine hydroxylase (TH) content of 15-day-old pups whose mothers were nicotine (NIC) or saline (C) exposed during lactation. Representative blots and density of protein were evaluated by western blotting method in adrenal medullae. Results were expressed as relative (%) to the control group. Actin content was used as control loading. Values are expressed as mean  $\pm$  S.E.M. of ten pups per group. \* $P < 0.05$ .

In conclusion, we have demonstrated that maternal nicotine exposure exclusively during lactation induces changes in mothers, milk and pups. The patterns of these effects are clearly distinct during exposure compared with withdrawal. As epidemiological studies show that maternal tobacco smoke during gestation and lactation can lead to subsequent hypertension, it is possible that the transient early adrenal medullary dysfunction caused by nicotine exposure may have a later impact on cardiovascular parameters in adult progeny. Despite the fact that generalizations to the human population should be carried out with care due to inherent differences between species, our results indicate that exposure to nicotine during the lactation period involves deleterious nutritional, biochemical, and hormonal alterations in both mothers and neonates. Other tobacco components could also have an effect, and we are developing new experiments using the total cigarette smoke, with or without nicotine.

#### 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.

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