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Transgenerational effects of fetal and neonatal exposure to nicotine

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Abstract A wide variety of in utero insults are associated with an increased incidence of metabolic disorders in the offspring and in subsequent generations. We have shown that fetal and neonatal exposure to nicotine results in endocrine and metabolic changes in the offspring that are consistent with those observed in type 2 diabetes. This study examines whether fetal and neonatal exposure to nicotine has transgenerational effects in the F2 offspring. Female Wistar rats were given either saline or nicotine (1 mg/kg/d) during pregnancy and lactation to create saline- and nicotine-exposed female F1 progeny. These F1 females were then bred to produce F2 offspring. We examined glucose homeostasis, serum lipids and fat pad weights, mitochondrial enzyme activity in skeletal muscle and blood pressure in these F2 offspring between 13 and 15 weeks of age. Offspring of nicotine- versus saline-exposed mothers had elevated fasting serum insulin concentrations and an enhanced total insulin response to the glucose challenge. This apparent insulin resistance was unrelated to changes in skeletal muscle mitochondrial volume or activity. The offspring of nicotine-exposed mothers also had elevated blood pressure. These data demonstrate that adverse effects of fetal and neonatal exposure to nicotine can influence aspects of metabolic risk in subsequent generations.

Keywords Intrauterine exposure · Glucose homeostasis · Mitochondrial enzyme activity · Nicotine · Skeletal muscle · Transgenerational effects

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Introduction

There is compelling evidence from human epidemiological studies and animal experiments that exposure of the fetus to certain hormonal, nutritional, metabolic, and environmental stressors may result in physiological alterations that persist into adulthood. In animal models and human epidemiological studies, in utero insults such as undernutrition, glucocorticoid exposure, uteroplacental insufficiency, and maternal smoking are associated with an increased incidence of adult onset diseases including obesity, type 2 diabetes, hypertension, coronary heart disease, dyslipidemia, and stroke in the offspring [1–5]. Furthermore, there is increasing evidence to suggest that the effects of in utero insults are not confined to those exposed in fetal life, but may be passed onto subsequent generations.

In animal studies, many of the reported transgenerational effects have been a result of germ line mutations due to chemical insults or exposure to radiation [6–9]. However, more recent studies have suggested that the consequences of non-chemical in utero insults, including maternal nutrient restriction and placental insufficiency, can also be passed transgenerationally from mother (F_0) to daughter (F_1) to the F_2 progeny [10, 11], likely via altered mitochondrial DNA and/or epigenetic modifications to DNA [12]. We have previously demonstrated that fetal and neonatal exposure to nicotine, the major addictive

constituent of cigarette smoke, results in offspring with metabolic and endocrine changes associated with type 2 diabetes [13] similar to what is seen in children born to women who smoke [14]. The goals of this study were to determine whether nicotine exposure also has transgenerational effects that could manifest as metabolic abnormalities in the F_2 offspring, and whether any metabolic changes in the F2 offspring could be attributed to impaired mitochondrial dysfunction in skeletal muscle.

Results

Birth weight, litter size, sex ratio, and live birth index were not significantly different between F2 offspring born to saline or nicotine-exposed dams (data not shown). The offspring of nicotine-exposed dams had reduced body weight at weaning (saline 55.8 ± 1.1 g; nicotine 52.4 ± 0.8 g, $P < 0.05$) due to impaired weight gain during lactation. This difference disappeared by 4 weeks of age due to catch-up growth in the offspring of nicotine-exposed dams following weaning. Following this period of catch-up growth in the pups born to nicotine-exposed dams there were no further differences in postnatal growth (data not shown).

Body fat, serum lipid, and adipokine levels

At 15 weeks of age, offspring of nicotine-exposed dams tended ($P = 0.065$) to have an increased percentage of body fat, and had elevated serum leptin concentrations with no difference in serum adiponectin concentrations (Table 1). Furthermore, the F2 offspring of nicotine-exposed dams had significantly higher levels of total cholesterol (saline: 121.6 ± 3.9 mg/dl; nicotine: 133.9 ± 3.5 mg/dl; $P < 0.05$), but there was no significant difference in serum triglycerides or non-esterified free fatty acids (Table 1).

Table 1 Body fat, serum lipid, and adipokine levels at 15 weeks of age

Outcome measure	F2 Saline	F2 Nicotine
Body weight (g)	496.2 ± 12.4	514.8 ± 11.4
Fat pad weight (% of body weight)	2.8 ± 0.2	3.3 ± 0.1
Leptin (ng/ml)	3.3 ± 0.4	4.4 ± 0.3*
Adiponectin (ng/ml)	3765.5 ± 343.1	3398.9 ± 221.5
Total cholesterol (mg/dl)	121.6 ± 3.9	133.9 ± 3.5*
Triglycerides (mmol/l)	1.3 ± 0.1	1.5 ± 0.1
NEFA (mEq/l)	0.48 ± 0.02	0.46 ± 0.03

Data are presented as mean ± SEM. Values with an asterisk (*) are significantly different ($P < 0.05$) than the saline controls

Glucose homeostasis

Serum glucose and insulin levels were measured at 15 weeks of age after an overnight fast and following glucose challenge. Although there was no difference in fasting serum glucose (Fig. 1), basal fasting insulin concentrations were ($P < 0.005$) higher in pups born to nicotine-exposed dams (Fig. 2). The F2 offspring of nicotine-exposed dams tended ($P = 0.062$) to have a reduced ability to clear the glucose load as evidenced by elevated serum glucose concentrations at 180 min (Fig. 1), however the total glucose response (area under the curve: AUC) to the glucose challenge was not different between the treatment groups (AUC glucose saline F2: 16867 ± 671; AUC glucose nicotine F2: 17712 ± 947; $P > 0.05$). The offspring of nicotine-exposed dams had an enhanced ($P < 0.05$) total insulin response (AUC) to the glucose challenge and elevated insulin to glucose ratio ($P < 0.05$) (Table 2).

Blood pressure

Offspring of nicotine-exposed dams had significantly higher systolic, diastolic, and mean arterial pressure (MAP) compared to offspring of saline-exposed dams (Table 3).

Mitochondrial enzyme activity

The maximal activity of citrate synthase, complex I + III and complex IV were not different between the offspring of

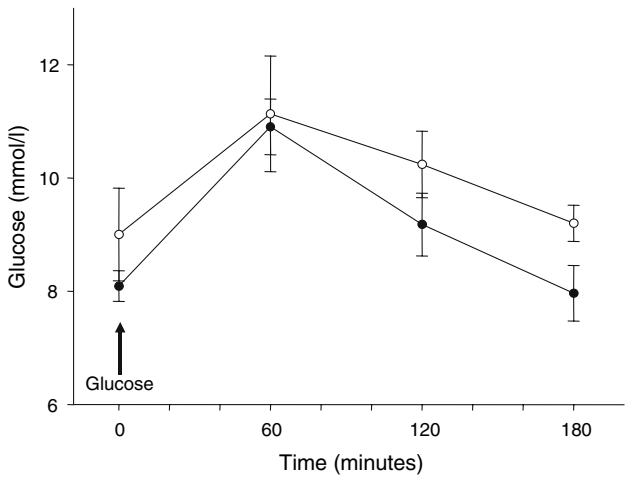

Fig. 1 Serum glucose concentrations (mmol/l) following administration of an i.p. glucose load (2 g/kg body weight) at 15 weeks of age for the offspring of dams who were exposed to saline (closed circles) or nicotine (1 mg kg^{-1} d^{-1}) (open circles). Data are presented as mean ± SEM

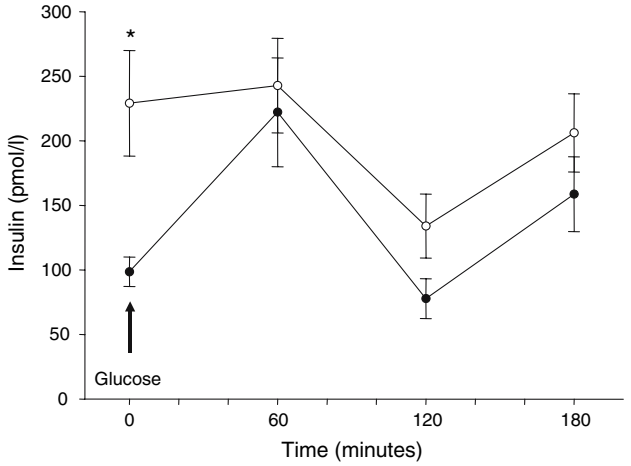

Fig. 2 Serum insulin concentrations (pmol/l) following administration of an i.p. glucose load (2 g/kg body weight) at 15 weeks of age for the offspring of dams who were exposed to saline (closed circles) or nicotine (1 mg kg^{-1} d^{-1}) (open circles). Data are presented as mean ± SEM. Values with an asterisk (*) are significantly ($P < 0.05$) different than the saline controls

saline- and nicotine-exposed mothers (data not shown). Moreover there were no differences in the ratio of complex I + III relative to citrate synthase or the ratio of complex IV to citrate synthase between the two groups of animals (Fig. 3).

Discussion

We have previously demonstrated that fetal and neonatal exposure to nicotine results in adverse postnatal metabolic outcomes in male offspring [13]. This study has expanded those findings and shown that the male offspring, who were themselves not exposed to nicotine, but whose mothers were exposed to nicotine in utero and during lactation also have impaired metabolic function suggesting that nicotine exposure via maternal cigarette smoking may effect future generations, an effect which has not previously been fully explored. The extent to which these differences are a result of the transgenerational inheritance of epigenetic changes induced by nicotine versus an indirect effect of nicotine exposure resulting in metabolic perturbations in the F1 mother that affect the fetal development of the F2 offspring is unknown.

F2 animals born to mothers who were exposed to nicotine in utero and during lactation had increased fasting insulin levels and the total insulin response (AUC) to the glucose challenge was increased relative to offspring of saline-exposed mothers. This phenotype is similar to what is seen in

Table 2 Glucose homeostasis at 15 weeks of age

Outcome measure	F2 Saline	F2 Nicotine
Fasting glucose (mmol/l)	8.1 ± 0.3	9.0 ± 0.8
Fasting insulin (pmol/l)	98.6 ± 11.4	229.1 ± 40.8*
AUC glucose	16866.9 ± 670.5	17711.8 ± 946.9
AUC insulin	23272.4 ± 3126.7	35317.4 ± 3610.9*

Data are presented as mean ± SEM. Values with an asterisk (*) are significantly different ($P < 0.05$) than the saline controls

Table 3 Blood pressure at 13 weeks of age

Outcome measure	F2 Saline	F2 Nicotine
Systolic BP (mmHg)	135.7 ± 8.1	158.4 ± 4.6*
Diastolic BP (mmHg)	101.4 ± 6.3	131.3 ± 5.1*
Mean arterial pressure (mmHg)	112.6 ± 6.9	132.5 ± 4.8*

Data are presented as mean ± SEM. Values with an asterisk (*) are significantly different ($P < 0.05$) than the saline controls

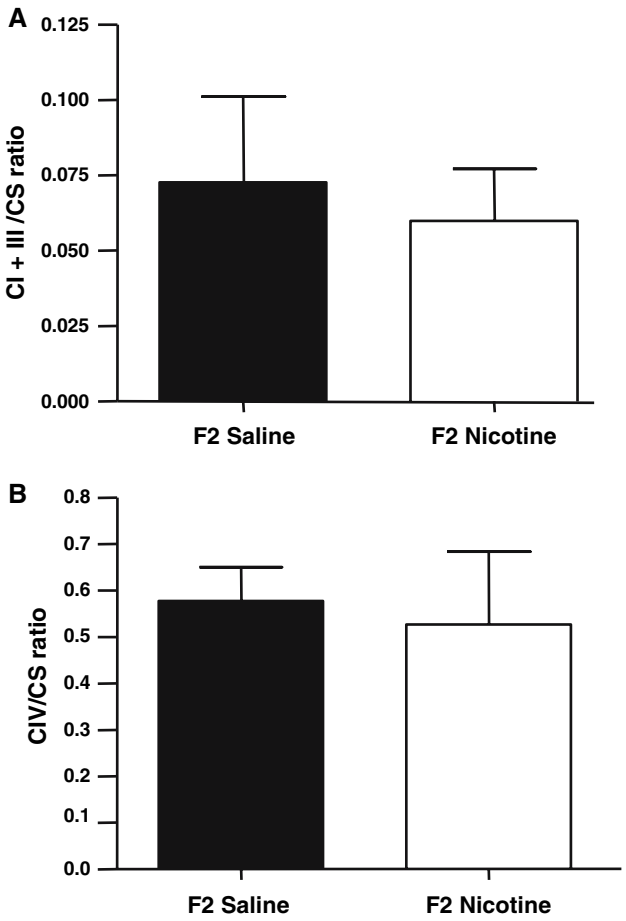

Fig. 3 Mitochondrial electron transport chain enzyme activities (**A**) CI + III (NADH—cytochrome c oxidoreductase) and (**B**) CIV = complex IV activity (cytochrome c oxidase) in gastrocnemius muscle at 15 weeks of age for the offspring of dams who were exposed to saline or nicotine (1 mg kg^{-1} d^{-1}). Data are presented as mean ± SEM and are expressed relative to total mitochondrial mass (citrate synthase: CS)

F1 males who were themselves exposed to nicotine during pregnancy and lactation [13]. These data suggest that the offspring of the nicotine-exposed mothers were insulin-resistant. Insulin resistance has also been seen in the offspring in animal studies of mothers with dysglycemia and type 2 diabetes [10, 11, 15]. Insulin resistance is associated with lower skeletal muscle mitochondrial capacity [16–20], and insulin-resistant offspring of parents with type 2 diabetes have impaired mitochondrial function [16]. However, in this study we did not identify differences in mitochondrial volume or activity in the insulin-resistant offspring of nicotine-exposed mothers. Furthermore, the fact that the offspring of nicotine-exposed animals do not have increased birth weight is not consistent with the predicted or observed phenotype for the offspring of a diabetic rat [10, 21]. Therefore, these results are difficult to reconcile with the hypothesis that the transgenerational effects of nicotine exposure are due *solely* to an abnormal diabetogenic intrauterine environment in the nicotine-exposed mothers and suggest that alternative mechanisms are involved.

In other animal studies, chemical insults [6, 7] can exert transgenerational effects via epigenetic mechanisms. It is therefore possible that nicotine acts via a similar mechanism. Indeed, one study has demonstrated that in utero exposure to nicotine can increase DNA methylation and acetylation in the fetus [22] and nicotine has been shown to alter gene methylation in cultured human esophageal squamous epithelial cells [23]. Moreover, since insulin resistance in the current animal model is rapidly induced by exposing the F1 animals to nicotine in utero and during lactation, it is likely that this reflects an acute epigentic response and not a genetic predisposition, as suggested for the human condition [24]. However, further studies are required to determine how fetal and neonatal exposure to nicotine can cause epigenetic changes.

This study has demonstrated that the male offspring (F2) of mothers who were exposed to nicotine during fetal and neonatal development have elevated fasting serum insulin concentrations, an exaggerated insulin response to a glucose challenge, and elevated blood pressure. The fact that these are risk factors for future diabetes, obesity, and stroke suggests that nicotine exposure either via maternal cigarette smoking or nicotine replacement therapy use during pregnancy may have long term, transgenerational health consequences which have not been fully explored.

Materials and methods

All animal experiments were approved by the Animal Research Ethics Board at McMaster University, in accordance with the guidelines of the Canadian Council for Animal Care.

Generation of F1 dams

Details of the nicotine administration and generation of the F1 pups have been fully described previously [13]. Briefly, nulliparous 200–250 g female Wistar rats (F0) (Harlan, Indianapolis, IN) were maintained under controlled lighting (12:12 L:D) and temperature (22°C) with *ad libitum* access to food and water. Two weeks prior to mating the dams were randomly assigned to receive either saline (vehicle) or nicotine. Dams were injected s.c. with 1 mg kg^{-1} day^{-1} nicotine bitartrate (Sigma Aldrich, St. Louis, MO) or saline for 14 days prior to mating, and during pregnancy until weaning. The maternal steady-state levels of serum cotinine (the major metabolite of nicotine) resulting from this exposure of 135.9 ± 7.86 ng/ml [26] are within the range of cotinine concentrations reported in pregnant smokers during both early and late pregnancy [27]. Pups were weighed after birth (postnatal day 1; PND1) and litter size was culled to eight at birth to assure uniformity of litter size between treated and control litters. At weaning, female offspring (F1) were caged as sibling pairs.

Generation of F2 progeny

At 24–26 weeks of age, F1 females that had been exposed to saline or nicotine ($n = 6$ per group) during fetal and neonatal development were bred to proven males from outside the experiment to produce the F2 offspring. All F1 females were allowed to deliver normally. Pups were sexed and weighed after birth and litter size was culled to eight pups per dam maintaining as close to a 1:1 sex ratio as possible. Pups were weighed weekly until weaning. At weaning, F2 pups were divided by sex and caged as sibling pairs. To eliminate any confounding effects of the female reproductive cycle, only male F2 were studied. Body weight and food consumption were recorded weekly. At 15 weeks of age, the rats were euthanized by CO_2 inhalation, and perirenal, mesenteric, and epididymal fat pads were removed from the animals and weighed. Body fat percentage was calculated as the total fat pad weight/body weight × 100%.

Serum lipid and hormone measurements

Serum samples collected at necropsy (15 weeks of age) following an overnight (16 h) fast were analyzed for triglycerides, total cholesterol (Pointe Scientific, Canton, MI), non-esterified free fatty acids (NEFA: Wako Chemicals GmbH, Richmond, VA), leptin (Linco Research, St Charles, MO), and adiponectin (Phoenix Pharmaceuticals, Inc. Belmont, CA).

Glucose tolerance test in the F2 offspring

F2 offspring (n = 8 per group) were randomly selected for a glucose tolerance test at 15 weeks of age. Serum concentrations of insulin and glucose were measured in saphenous vein samples obtained after 16 h of food deprivation at 0 (0900 h), 60, 120, and 180 min after rats were given 2 g kg^{-1} glucose (Sigma-Aldrich, St. Louis MO) in water i.p. Blood samples were allowed to clot at 4°C, centrifuged and stored at −80°C until needed for measurement of serum glucose and insulin concentrations. Serum glucose concentrations were measured using a commercially available kit using the glucose oxidase method (Pointe Scientific Inc., Canton, MI), and insulin levels were measured using an ultra sensitive rat insulin ELISA (Crystal Chem Inc., Downers Grove, IL).

Blood pressure

Blood pressure was measured at 13 weeks of age (n = 6 per group) after a 5 day acclimatization period, using the CODA 6 non-invasive computerized acquisition system (Kent Scientific Corporation, Torrington, CT). Blood pressure was collected using a VPR sensor and occlusion cuff to determine systolic, diastolic, and mean arterial pressure based on the average of ten cycles per animal.

Mitochondrial enzyme activity

At the time of sacrifice, skeletal muscle samples were obtained by removing the entire gastrocnemius muscle (white and red sections), freezing on dry ice and storing at −70 until analysis. Muscle biopsy samples (n = 7 per group) were homogenized to measure citrate synthase activity (a marker of total mitochondrial volume), complex I + III activity (NADH-cytochrome c oxidoreductase), and complex IV (cytochrome c oxidase) activity in whole muscle homogenates using UV-spectrophotometry (Agilent, Mississauga, ON) as described by our group [26]. To account for the total amount of mitochondrial volume in the samples we expressed complex I + III and IV relative to citrate synthase activity (relative activity).

Statistical analysis

All statistical analyses were performed using SigmaStat (v.3.1, SPSS, Chicago, IL). The results are expressed as mean ± SEM. Data were checked for normality and equal variance and were tested using unpaired Student's t-tests (α = 0.05). Where data failed normality or equal variance test, data was reanalyzed using Mann–Whitney rank sum test. Area under the curve for serum glucose and insulin levels during the GTT and postnatal growth was determined using the trapezoidal rule.

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