Fetal and neonatal nicotine exposure and postnatal glucose homeostasis: identifying critical windows of exposure

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Abstract

Fetal and lactational exposure to nicotine at concentrations comparable with those in women who smoke causes impaired glucose tolerance in male offspring in postnatal life. It remains unknown whether there are critical windows of susceptibility to nicotine exposure. Female nulliparous Wistar rats were given saline vehicle or nicotine bitartrate (1 mg/kg per day) prior to pregnancy, which was then: A) discontinued during pregnancy and lactation; B) continued until parturition; C) continued until weaning; and D) discontinued during pregnancy and restarted from lactation until weaning. At 26 weeks of age, offspring in each group were challenged with an oral glucose load. β–Cell mass, apoptosis, and proliferation were measured at birth, and at 4 and 26 weeks of age. The animals in group C (exposed to nicotine throughout pregnancy and lactation) had reduced β–cell mass from birth through 26 weeks of age and impaired glucose homeostasis at 26 weeks of age. β–Cell mass was also reduced at birth and at 4 weeks of age in animals exposed to nicotine during pregnancy alone (group B). However, enhanced proliferation following weaning led to recovery of this defect to 98% of control levels by week 26. The response to the glucose load in groups A, B, and D did not differ from controls. Continued exposure to nicotine from conception through lactation results in permanent β–cell loss and subsequent impaired glucose tolerance. This model of type 2 diabetes requires that nicotine exposure occurs both *in utero* and during lactation.

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Introduction

Approximately 15–20% of all women smoke while pregnant (Andres & Day 2000, Bergmann *et al.* 2003), despite intentions to refrain from smoking during that period (Okuyemi *et al.* 2000). Cigarette smoking during pregnancy remains one of the most important modifiable risk factors for adverse fetal, obstetrical, and developmental outcomes (Andres & Day 2000, Hofhuis *et al.* 2003). Moreover, epidemiological studies have demonstrated that fetal exposure to maternal smoking during pregnancy is associated with adverse postnatal health outcomes, including obesity, hypertension, and type 2 diabetes (Morley *et al.* 1995, Vik *et al.* 1996, Blake *et al.* 2000, Montgomery & Ekbom 2002, Toschke *et al.* 2002, Von *et al.* 2002, Bergmann *et al.* 2003, Wideroe *et al.* 2003). In pregnant women who smoke or use nicotine-replacement therapy, nicotine crosses the placenta, concentrates in fetal blood and amniotic fluid, and is detectable in breast milk during lactation (Lambers & Clark 1996). Therefore, maternal nicotine exposure results in both fetal and neonatal exposure.

Animal studies have demonstrated that fetal and neonatal exposure to nicotine alone, at levels that are representative of women who smoke or use nicotine replacement therapy, results in low birth weight, and postnatal impaired glucose homeostasis, hyperinsulinemia, increased body weight and dyslipidemia (Williams & Kanagasabai 1984, Newman *et al.* 1999, Pausova *et al.* 2003, Gao *et al.* 2005, Holloway *et al.* 2005). The β-cell loss, impaired glucose tolerance, and hyperinsulinemia observed in the nicotine-exposed offspring from this animal model (Holloway *et al.* 2005) closely represent symptoms associated with type 2 diabetes in humans (Pratley & Weyer 2001, Leahy 2005). However, the heterogeneous nature of smoking behavior and nicotine replacement therapy use in the pregnant and breastfeeding population (McBride & Pirie 1990, Castrucci *et al.* 2006, Thyrian *et al.* 2006) results in nicotine exposures during different windows of development. Epidemiological evidence strongly suggests that cessation or at least reduction of cigarette smoking during pregnancy will ameliorate the damage to a developing fetus (Lindley *et al.* 2000, Pickett *et al.* 2003); however, the effect of smoking cessation on metabolic disturbances in the offspring has not yet been determined. Furthermore, the developmental stages when nicotine exposure can result in an irreversible impact on glucose homeostasis in the offspring have not yet been identified. This study was designed to identify critical windows of fetal and neonatal exposure to maternal nicotine

on the development of the pancreatic β-cell and glucose intolerance in the offspring.

Materials and Methods

Maintenance and treatment of animals

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. Nulliparous, 200–250 g female Wistar rats (Harlan, Indianapolis, IN, USA) were maintained under controlled lighting (12 h light:12 h darkness) and temperature (22 °C) with food and water available *ad libitum*. Dams were randomly assigned ($n=5$ per group) to receive saline (vehicle) or nicotine bitartrate (1 mg/kg per day; Sigma–Aldrich) via s.c. injection daily: A) for 2 weeks prior to mating only; B) for 2 weeks prior to mating until parturition (fetal exposure); C) for 2 weeks prior to mating until weaning (fetal and neonatal exposure); and D) for 2 weeks prior to mating and after parturition until weaning (neonatal exposure).

At postnatal day (PND) 1, litters were culled to eight, retaining males in preference to assure uniformity of litter size between treated and control litters. To eliminate any confounding effects of the female reproductive cycle, only male offspring were used in this study. After weaning at PND21, male offspring were selected randomly and caged as sibling pairs.

Oral glucose tolerance

Glucose homeostasis was investigated in nicotine-exposed and control rats at 26 weeks of age ($n=12$ per group) using an oral glucose tolerance test (OGTT). To avoid litter effects, no more than three animals from a single litter were tested. After an overnight fast, serum concentrations of insulin and glucose were measured in saphenous vein samples, collected by repeated puncture, at baseline (0900 h), 30 and 120 min after rats were given 2 g/kg glucose (Sigma–Aldrich) in water by gavage as previously described (Cleasby *et al.* 2003). Blood samples were allowed to clot at 4 °C, centrifuged, and stored at −80 °C until assayed. Serum glucose concentrations were measured by a commercially available kit using the glucose oxidase method (Pointe Scientific Inc., Canton, MI, USA), and insulin levels were measured by an ultra-sensitive rat insulin ELISA (Crystal Chem Inc., Downers Grove, IL, USA).

β-Cell mass

To assess whether nicotine exposure during pregnancy and lactation alters prenatal and postnatal development of pancreas, pancreatic tissue was collected from a subset of pups at birth (PND1), and at 4 and 26 weeks of age. Animals at PND1 were killed by decapitation, and those at 4 and 26 weeks of age were killed by isofluorane overdose. The pancreas from each animal was weighed and then fixed by immersion in 10% (v/v) neutral buffered formalin (EM Science, Gibbstown, NJ, USA) at 4 °C overnight, washed in water, and embedded in paraffin. Immunohistochemical detection of insulin was performed on 5 μm serial sections, separated by an average of 30 μm, of PND1, week 4 and week 26 pancreatic tissues from saline-exposed (five sections per animal; five animals randomly selected to include animals from each saline exposure group) and nicotine-exposed (five sections per animal; five animals per group) offspring. These age groups were selected to examine the effects of nicotine exposure on fetal pancreatic development (PND1), neonatal pancreatic development (week 4), and adult pancreatic development (week 26). Tissue sections were deparaffinized in xylene, rehydrated, and washed in PBS. Endogenous peroxidase activity was quenched in methanol, followed by antigen retrieval in 10 mmol/l citrate buffer (pH 3·0) and blocking with 10% (v/v) normal goat serum and 1% (w/v) BSA. Sections were then incubated with the primary antibody, a polyclonal, guinea pig anti-swine insulin antibody (1:150 dilution; DakoCytomation, Carpinteria, CA, USA), which has been shown by the manufacturer to cross-react with rat insulin, overnight at 4 °C. Sections were then washed in PBS, and immunostaining was identified using the Vectastain kit (Vector Laboratories, Burlinghame, CA, USA), with diaminobenzidine as the chromogen. Tissue sections were counterstained with Harris' hematoxylin, destained with acid alcohol, dehydrated and mounted with Permount (Fisher Scientific, Fair Lawn, NJ, USA). Control sections were incubated with 1% (w/v) BSA in PBS in place of the primary antibody. In all sections, the whole pancreas was analyzed by combining measurements from up to 90 fields per section. Immunopositive cells were identified using Image Pro Plus v. 5.1 software (Media Cybernetics Inc., Silver Spring, MD, USA) for automated cell counting and the calculation of β-cell area and total pancreas area. The percent β-cell area was calculated as a ratio of the β-cell area (immunopositive staining only) to the total pancreas area (immunopositive staining and pancreas counterstaining)×100. The β-cell mass was calculated as the product of the percent β-cell area and the corresponding total pancreas weight in milligrams.

Islet apoptosis and proliferation

Detection of apoptotic cells in pancreatic islets from saline- and nicotine-treated rats was performed using a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay according to manufacturer's instructions (Roche Applied Science) with insulin colocalization. Briefly, tissue sections were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol to PBS. Next, tissues were subjected to antigen retrieval in 10 mmol/l citrate buffer (pH 3·0) and blocking with 10% (v/v) normal goat serum and 1% (w/v) BSA. Sections were then incubated with the primary antibody, a polyclonal, guinea pig anti-swine insulin antibody (1:150 dilution; DakoCytomation), overnight at 4 °C followed by anti-rabbit Alexa Fluor 594 secondary antibody (1:400 dilution; Molecular Probes Inc.,

Eugene, OR, USA) for 2 h at room temperature. Following the immunofluorescence immunostaining for insulin, tissues were subjected to the TUNEL assay. Following a PBS wash, tissues were permeabilized in 0·5% (v/v) Triton X-100 for 30 min at room temperature and then incubated with the fluorescein isothiocyanate(FITC)-conjugated TUNEL enzyme for 60 min to detect DNA fragmentation. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI) (Sigma–Aldrich) and tissue sections were imaged with an Olympus BX-61 microscope and analyzed with Image Pro Plus v. 5.1 software (Media Cybernetics Inc.). For analysis, three islets per section (three sections per animal; five animals per group) were quantified for apoptosis and reported as the percentage of TUNEL$^+$ β-cells.

To evaluate islet cell proliferation, tissues were processed as above and incubated with a mouse anti-proliferating cell nuclear antigen (PCNA) antibody (1:2000 dilution; Sigma–Aldrich) overnight at 4 °C. PCNA has been demonstrated to be a useful marker of islet cell proliferation, as it is present in the cell nuclei within the later part of G1, S, and G2 phases of the cell cycle (Petrik *et al.* 1999). Immunostaining was identified using the Vectastain kit (Vector Laboratories), with diaminobenzidine as the chromogen. Tissue sections were counterstained with Harris' hematoxylin, destained with acid alcohol, and dehydrated and mounted with Permount (Fisher Scientific). For analysis, three islets per section (three sections per animal; five animals per group) were quantified. Islet cell proliferation was assessed as the percentage of total islet cells that were PCNA$^+$.

Statistical analysis

All statistical analyses were performed using SigmaStat (v. 2.03; SPSS, Chicago, IL, USA) and one-way ANOVA followed by *post hoc* multiple comparisons when significance was indicated by ANOVA ($\alpha = 0·05$). When significance was indicated by ANOVA, OGTT results for each treatment group were compared with the saline controls (Bonferroni's *t*-test, $\alpha = 0·05$). There was no difference in the glucose or insulin response to the OGTT at baseline, 30, or 120 min among the four saline groups (one-way ANOVA; all $P > 0·90$), so the data were pooled for comparison with the nicotine-exposed offspring. Similarly, there were no differences in β-cell mass, apoptosis, or proliferation among

the four saline groups (one-way ANOVA; all $P > 0·90$), so the results for the saline-exposed groups were pooled for comparison with the nicotine-exposed offspring. When significance was indicated by ANOVA ($P < 0·05$), the results for β-cell mass, proliferation, and apoptosis among each treatment group were compared with each other using the Student–Neuman–Keuls test. The data were tested for normality as well as equal variance, and when normality or variance tests failed, data were analyzed using Kruskal–Wallis one-way ANOVA on ranks. The area under the curve (AUC) for the total glucose response during the OGTT was assessed using the trapezoidal rule.

Results

Pregnancy outcome and birth phenotypes

Nicotine administration had no effect on maternal food consumption during the 2-week period prior to mating (saline $8·5 \pm 0·24$ g food/100 g body weight versus nicotine $8·9 \pm 0·30$ g food/100 g body weight; $P = 0·30$) or during pregnancy (Table 1). In addition, nicotine exposure did not affect mating success (100% in all groups), maternal weight gain during pregnancy, litter size, or birth weight in any treatment group (Table 1).

Glucose homeostasis

At 26 weeks of age, nicotine exposure had no effect on fasting serum glucose concentrations ($P > 0·05$) or insulin concentrations (Kruskal–Wallis one-way ANOVA on ranks; $P > 0·05$) in any treatment group (Table 2). Following an oral glucose challenge, animals in group C (fetal and neonatal nicotine exposure) had a higher total glucose response (AUC) to the glucose load relative to the saline controls ($P < 0·01$), an effect that was not observed in any other group (Fig. 1). Furthermore, the peak glucose concentration at 30 min was higher in this group (saline $9·1 \pm 0·29$ mmol/l; group C $10·9 \pm 1·15$ mmol/l; $P < 0·05$) and the ability to clear the glucose load, determined by serum glucose concentrations at 120 min following the glucose challenge, was impaired (saline $8·2 \pm 0·33$ mmol/l; group C $11·4 \pm 1·21$ mmol/l; $P < 0·05$; Fig. 1). The total insulin response (AUC) to the glucose challenge was also elevated ($P < 0·05$) in the offspring in

Table 1 Pregnancy outcome and birth phenotype. Values are presented as mean$\pm$S.E.M.

	Maternal food consumption (g food/100 g body weight)	**Maternal weight gain during pregnancy** (g)	**Birth weight** (g)	**Litter size**
Treatment				
Saline	7·9±0·10	138·4±7·30	6·0±0·06	13·8±0·97
Nicotine group A/D	7·9±0·14	160·6±12·66	5·9±0·04	15·1±0·88
Nicotine group B/C	8·1±0·24	130·4±10·05	6·2±0·09	12·0±1·07

Results for groups A and D and groups B and C were combined as pups in these groups had the same exposure to nicotine (preconceptual only and preconceptual+fetal exposure respectively).

Table 2 Effect of developmental exposure to nicotine in Wistar rats. Values are presented as mean ± S.E.M.

	Fasting glucose (mmol/l)	Fasting insulin (ng/ml)	Insulin:glucose	AUC insulin
Treatment				
Saline	7·4 ± 0·27	1·9 ± 0·68	0·27 ± 0·063	221·8 ± 27·23
Nicotine group A	7·1 ± 0·68	2·0 ± 0·54	0·24 ± 0·050	300·2 ± 50·02
Nicotine group B	7·6 ± 0·30	1·4 ± 0·24	0·19 ± 0·030	250·2 ± 38·70
Nicotine group C	8·3 ± 0·36	1·8 ± 0·18	0·26 ± 0·014	342·2 ± 40·32*
Nicotine group D	7·4 ± 0·19	1·5 ± 0·31	0·21 ± 0·048	255·9 ± 31·88

Values with an asterisk are significantly different from the saline controls ($P<0.05$).

group C relative to the control (saline-exposed) offspring (Table 2).

β-Cell mass

At birth (PND1), results for groups A and D and groups B and C were combined as pups in these groups had the same exposure to nicotine (preconceptual only and preconceptual + fetal exposure respectively). Fetal exposure to nicotine (groups B and C) resulted in reduced β-cell mass ($P<0.05$) relative to saline-exposed offspring (Fig. 2A), an effect that was still present at 4 weeks of age (Fig. 2B). By 26 weeks, animals exposed to nicotine during pregnancy alone (group B) had recovered their β-cell mass relative to saline controls (Fig. 2C). The β-cell mass at 26 weeks of offspring exposed to nicotine during both fetal and neonatal development (group C) continued to be lower than the β-cell mass of any other group (Fig. 2C). Offspring of dams exposed to nicotine prior to pregnancy or prior to pregnancy and during lactation (groups A and D respectively) did not exhibit a loss of β-cell mass at any age examined (Fig. 2A–C). At all the three ages, changes in β-cell mass were a reflection of changes in the β-cell area, not a reduction in pancreas weight (Table 3).
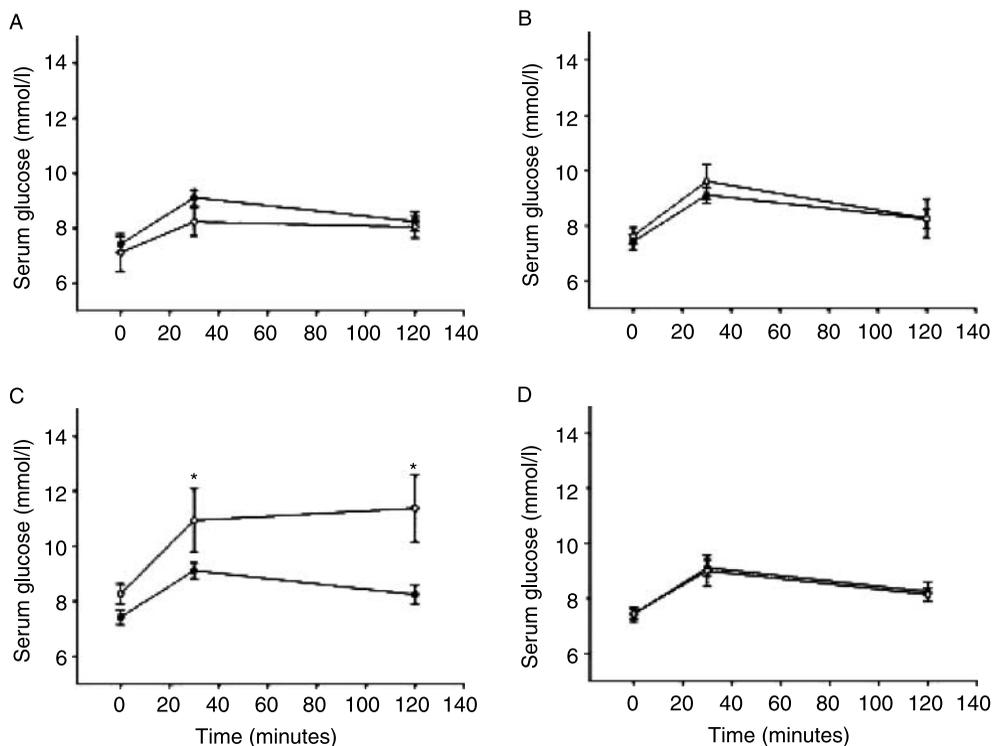

Figure 1 Serum glucose concentrations (mmol/l) following administration of an oral glucose load (2 g/kg body weight) at 26 weeks of age for the offspring of female Wistar rats given saline (control, closed circles) or nicotine (1 mg/kg per day, open circles): (A) prior to pregnancy (group A); (B) prior to pregnancy and during pregnancy (group B); (C) prior to pregnancy, during pregnancy and during lactation (group C); and (D) prior to pregnancy and during lactation (group D); $n=12$ per group. Data are presented as mean ± S.E.M. Values with an asterisk are significantly different from the saline controls ($P<0.05$).
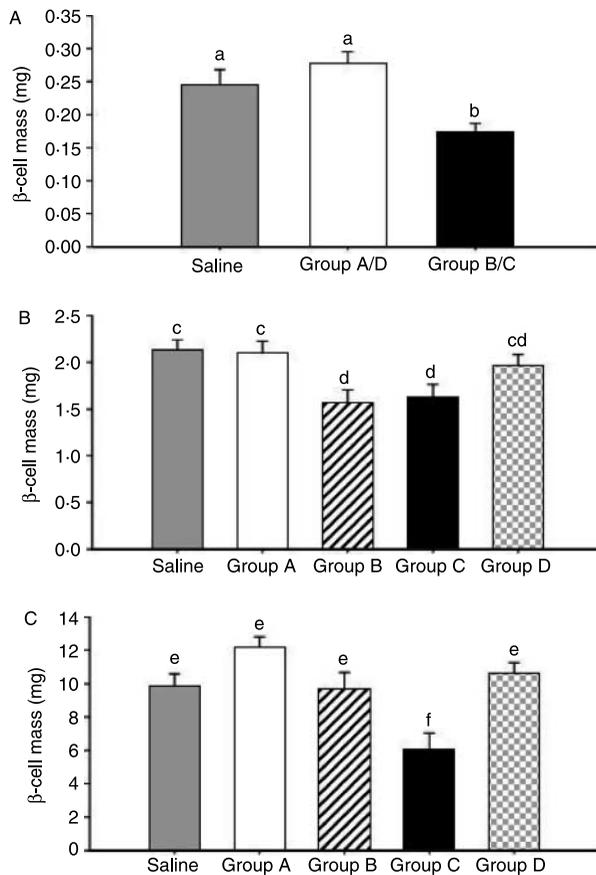
Figure 2 Effect of fetal and neonatal exposure to nicotine bitartrate (1 mg/kg per day) on pancreatic β-cell mass (mg) of rats at: (A) PND1; (B) 4 weeks of age; and (C) 26 weeks of age. β-Cell mass was calculated using $n=5$ sections per animal, and $n=5$ animals per treatment group. Data are presented as mean±s.e.m. Values with different superscripts are significantly different from each other ($P<0·05$).

β-Cell proliferation and apoptosis

Although β-cell mass at 4 weeks of age was reduced in animals with fetal exposure alone or both fetal and neonatal exposure to nicotine, by 26 weeks of age the β-cell mass of animals without neonatal exposure (group B) was the same as saline controls, but remained suppressed in animals exposed during fetal and neonatal periods (group C). To determine whether this recovery was due to increased proliferation and/or decreased apoptosis in pancreatic islets, further studies were undertaken. At 4 weeks of age, animals in group B (fetal exposure only) had a higher percentage of PCNA$^+$ islet cells relative to the animals that had been exposed to nicotine during fetal and neonatal development (group C) and saline controls (Fig. 3A). Animals with fetal and neonatal exposure to nicotine (group C) had an increased level of β-cell apoptosis relative to those with fetal exposure alone or to saline controls (Fig. 3B).

Discussion

Our laboratory has previously demonstrated that nicotine exposure during pregnancy and lactation results in endocrine and metabolic changes in the offspring that are consistent with those observed in type 2 diabetes (Holloway et al. 2005). The dose of nicotine used in this study (1 mg/kg per day nicotine bitartrate) results in maternal serum cotinine concentrations of 136 ng/ml (Holloway et al. 2006), which is within the range of cotinine levels (80–163 ng/ml) reported in women who are considered as 'moderate smokers' (Eskenazi & Bergmann 1995). In addition, this dose of nicotine resulted in serum cotinine concentrations of 26 ng/ml in the nicotine-exposed offspring at birth (Holloway et al. 2006), which is also within the range (5–30 ng/ml) observed in infants nursed by smoking mothers (Luck & Nau 1985). Although 15–20% of pregnant women smoke (Andres & Day 2000, Okuyemi et al. 2000), many women attempt to stop smoking during pregnancy and then relapse following parturition (McBride & Pirie 1990, Castrucci et al. 2006, Thyrian et al. 2006), resulting in nicotine exposure at conception and during lactation only. The influence this pattern may have on offspring health is unknown, as the developmental stages of fetal and neonatal development, which are susceptible to nicotine exposure, have not been determined. The present study was designed to represent the various windows of exposure that children of average smokers would experience. Since nicotine is rapidly metabolized in rats (half life of 45 min), the nicotine from each daily injection is entirely cleared before the next injection (Matta et al. 2007), ensuring that each window of nicotine exposure remains separate. In this study, we have demonstrated, in an animal model, that postnatal glucose homeostasis is impaired only if nicotine exposure occurs during both pregnancy and lactation. Neither developmental stage alone leads to subsequent dysglycemia at 26 weeks of age in adult rat offspring. The impaired glucose homeostasis observed in this animal model is an early indicator of risk for the development of type 2 diabetes, a disease which is associated with numbers of comorbidities, including cardiovascular disease, nephropathy, retinopathy, and neuropathy (Clemens et al. 2004, Mlinar et al. 2007)

In humans, type 2 diabetes develops due to a progressive reduction in the ability of the pancreas to produce sufficient insulin to compensate for any underlying resistance to the action of insulin (Lindley et al. 2000, Kahn 2003, Leahy 2005). This defect in β-cell function is already observed in dysglycemic individuals with impaired fasting glucose or impaired glucose tolerance, long before the onset of frank type 2 diabetes (Kahn 2003). Recent studies suggest that this insulin insufficiency may be due, in part, to a reduction in β-cell mass (Leahy 2005, Rhodes 2005). Indeed, in humans, pancreatic β-cell mass is reduced by 40–60% in patients with type 2 diabetes (Lindley et al. 2000, Sakuraba et al. 2002, Yoon et al. 2003), and this reduction in β-cell mass precedes the

Table 3 Effect of developmental exposure to nicotine on β-cell mass in Wistar rats. Values are presented as mean ± S.E.M.

Age	Treatment	β-cell area (%)	Pancreas mass (mg)	β-cell mass (mg)
PND 1	Saline	2·0 ± 0·18	12·0 ± 0·90	0·24 ± 0·023
	Nicotine group A/D	2·0 ± 0·12	11·9 ± 0·11	0·28 ± 0·017
	Nicotine group B/C	1·4 ± 0·12*	14·4 ± 0·90	0·17 ± 0·013*
4 weeks	Saline	0·7 ± 0·04	306 ± 23·7	2·13 ± 0·11
	Nicotine group A	0·6 ± 0·04	327 ± 22·0	2·09 ± 0·14
	Nicotine group B	0·4 ± 0·04*	347 ± 27·9	1·57 ± 0·14*
	Nicotine group C	0·5 ± 0·04*	343 ± 12·0	1·62 ± 0·14*
	Nicotine group D	0·7 ± 0·04	293 ± 14·3	1·96 ± 0·12
26 weeks	Saline	1·2 ± 0·06	915 ± 81·1	9·83 ± 0·76
	Nicotine group A	1·3 ± 0·06	928 ± 44·0	12·17 ± 0·64
	Nicotine group B	0·90 ± 0·05	1072 ± 64·9	9·66 ± 1·02
	Nicotine group C	0·64 ± 0·03*	946 ± 118·0	6·09 ± 0·95*
	Nicotine group D	1·2 ± 0·07	964 ± 34·1	10·65 ± 0·61

At birth (PND1), results for groups A and D and groups B and C were combined, as pups in these groups had the same exposure to nicotine (preconceptual only and preconceptual + fetal exposure respectively). Values with an asterisk are significantly different from the saline controls ($P<0.05$).

diagnosis of diabetes (Lindley *et al.* 2000). Similarly, we have shown in an animal model that reduced β-cell mass preceded the loss of normal glycemic control, and that although insulin

Figure 3 Effect of fetal exposure only (group B), and fetal and lactational exposure (group C) to nicotine bitartrate (1 mg/kg per day) on: (A) percentage of islet cell proliferation (PCNA^{+} islet cells) and (B) percentage of β-cell apoptosis (TUNEL[+] β-cells) at 4 weeks of age. Both apoptosis and proliferation were calculated using $n=3$ islets per section, with $n=3$ sections per animal and $n=5$ animals per group. Data are presented as mean ± S.E.M. Values with different superscripts are significantly different from each other ($P<0.01$).

secretion in response to the OGTT was increased, it was insufficient to normalize glucose concentrations, an effect which is in accordance with our previous findings (Holloway *et al.* 2005).

We had previously suggested that nicotine-induced damage to the β-cell during fetal development may induce permanent changes in pancreatic structure and function evident as impaired glycemia in adults (Holloway *et al.* 2005). Similar findings have been reported for other *in utero* insults, including glucocorticoid administration, uteroplacental insufficiency, and fetal undernutrition (Petrik *et al.* 1999, Holness *et al.* 2000, Simmons *et al.* 2001, Shen *et al.* 2003). However, this work illustrates that *in utero* nicotine exposure alone transiently reduces β-cell mass without permanent metabolic defects. The full recovery of β-cell mass and function can be attributed to enhanced β-cell proliferation leading to increased expansion of β-cell mass between 1 and 6 months of age. Control animals experienced a 4·6-fold increase in β-cell mass between 1 and 6 months of age, whereas animals with fetal exposure only (group B) had lower absolute β-cell mass at 1 month of age but a 6·2-fold 'catch-up' in β-cell mass, such that by 26 weeks of age they had recovered to 98% of the saline control β-cell mass. There was no decrease in apoptosis found to explain this 'catch up' in β-cell mass. Continued nicotine exposure through lactation appears to prevent this pancreatic cell 'catch up'. The rats exposed to nicotine during both fetal and neonatal development had a β-cell mass that was only 62% of controls at 26 weeks of age. Furthermore, the inhibited growth of β-cell mass in these offspring was due to increased β-cell apoptosis and not due to decreased β-cell proliferation.

Other insults during fetal and neonatal development, such as maternal undernutrition, also cause a reduction in the β-cell mass at birth, an effect which was irreversible even though restoration of nutrition at the end of gestation resulted in normal β-cell proliferation during lactation (Garofano *et al.*
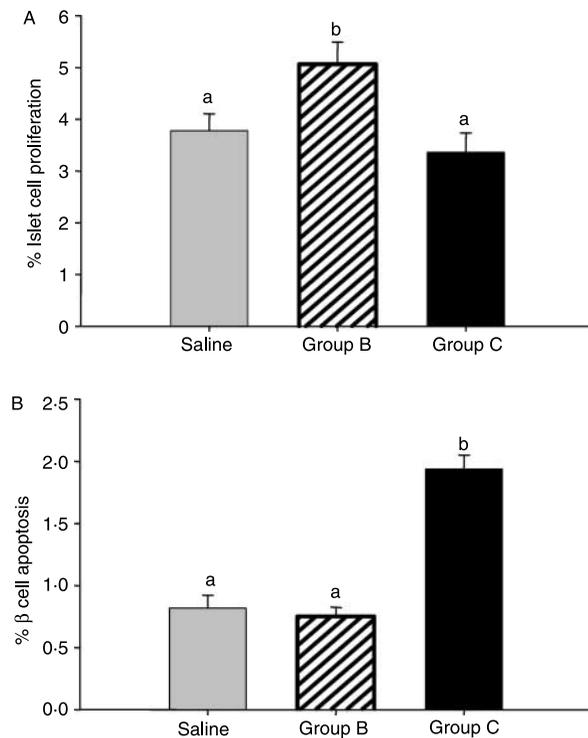
1997). In contrast, the results from this study have demonstrated that increased β–cell replication following a reduction in β–cell mass at birth was able to fully restore β–cell mass and function. However, recovery occurred only when nicotine exposure was stopped prior to neonatal pancreatic development. When nicotine exposure continued through neonatal pancreatic development via lactation (group C animals), the capacity for islet cell proliferation and, therefore, β–cell recovery appears to be lost. These results are also consistent with another rodent model, in which the primary mechanisms for an adaptive increase in β–cell mass were islet neogenesis and β–cell replication, while an adaptive reduction in β–cell mass was primarily due to increased β–cell apoptosis (Butler *et al*. 2003).

In conclusion, nicotine exposure during both pregnancy and lactation results in impaired glucose homeostasis in the offspring of this animal model. This effect is mediated by an irreversible reduction in pancreatic β–cell mass in early life. These results confirm the previous findings that recovery of β–cell mass occurs during critical developmental windows, otherwise the β–cell loss is permanent and will lead to metabolic defects in the offspring. In applying these results to the human population, it is essential to consider the developmental differences between species. In rats, pancreatic development occurs both prenatally and postnatally, whereas in humans, the majority of development is completed prenatally (Hill & Duvillie 2000). However, regardless of the differences in timing, the essential principle that impairment of early pancreatic development will result in permanent changes remains the same. Therefore, data from the present study imply that smoking cessation prior to the completion of pancreatic development may be beneficial in terms of protecting the future metabolic capacity of the offspring. This study also raises some concerns regarding the safety of continuous nicotine replacement therapy during pregnancy and lactation.

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