FETAL AND NEONATAL NICOTINE EXPOSURE:

EFFECTS ON PANCREATIC BETA CELLS.

FETAL AND NEONATAL NICOTINE EXPOSURE: EFFECTS ON PANCREATIC BETA CELLS.

By

JENNIFER E. BRUIN, B.Sc.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

DOCTOR OF PHILOSOPHY (2009) McMaster University

(Medical Sciences) Hamilton, Ontario

TITLE: Fetal and neonatal nicotine exposure: effects on pancreatic beta cells.

AUTHOR: Jennifer E. Bruin, B.Sc., (University of Guelph)

SUPERVISOR: Dr. Alison C. Holloway

SUPERVISORY COMMITTEE: Dr. Hertzel C. Gerstein

Dr. Warren G. Foster

NUMBER OF PAGES: 217

ABSTRACT

Fetal exposure to cigarette smoke is associated with an increased risk of adult-onset metabolic abnormalities. In Canada, nicotine replacement therapy (NRT) is recommended as a safe smoking cessation aid for pregnant women. However, our laboratory has demonstrated that fetal and neonatal nicotine exposure results in glucose intolerance in adult rats. The goal of this thesis was to determine the mechanism(s) underlying the observed dysglycemia following fetal and neonatal nicotine exposure, with a specific focus on the effects of nicotine on pancreatic development and postnatal beta cell function.

Nulliparous female Wistar rats received daily subcutaneous injections of either saline or nicotine bitartrate (1mg/kg/d) for 2 weeks prior to mating until weaning (postnatal day 21 – PND21). Pancreatic tissue was collected from male offspring at birth (PND1), 3, 7, 15 and 26 weeks of age. For the critical windows study, dams received nicotine or saline during different stages of pancreatic development, including: A) pre-mating only, B) pre-mating + pregnancy only, C) pre-mating, pregnancy and lactation, or D) pre-mating + lactation only. For the intervention study, nicotine-exposed dams received either normal chow or diet containing antioxidants (1000 IU/kg vitamin E, 0.25% w/w coenzyme Q10 and 0.05% w/w α-lipoic acid) during mating, pregnancy and lactation.

Results from this thesis demonstrate that exposure to nicotine during **both** fetal and neonatal development (but neither stage alone) causes a permenant

loss of beta cell mass beginning at birth, and adult-onset dysglycemia in rodents. Furthermore, nicotine exposure induces pancreatic oxidative stress and mitochondrial-mediated beta cell apoptosis in neonates, followed by a progressive decline in mitochondrial structure and function. Maternal treatment with a dietary antioxidant cocktail during pregnancy and lactation protected the developing beta cells from nicotine-induced apoptosis and mitochondrial swelling. These data indicate that the safety of NRT use during pregnancy should be re-evaluated.

ACKNOWLEDGEMENTS

There are so many people that need to be thanked for their support throughout my PhD. Grad school has been a wonderful experience due in large part to the amazing people who surrounded me. First, I would like to thank my supervisor, Dr Alison Holloway for her endless support, both professionally and personally; I antticipate seeking advice at many future coffee meetings. My PhD was also supported by my excellent committee memebers, Dr Hertzel Gerstein and Dr Warren Foster, whose doors were always open. Thank you to the entire roproductive biology lab group and specifically to Carolyn Cesta, Dana Anger, Anne Mulligan-Tuttle, Amanda Woynillowicz and Heather Cameronfor their support over the years. Finally, there are so many individuals who need to be thanked for their technical assistance with my thesis research. I would like to specifically mention three undergraduate students whose work was particularly instrumental in my thesis: Jillian Hyslop, Maria Petre and Lisa Kellenberger.

The support I recieved from my friends and family was also a powerful motivator for me throughout my PhD. Although I have many wonderful friends, a special thanks to my Hamilton friends: Erin and Jen Bassett, Eric and Christine Lootsma, Jason Tang, Rebecca Gannan, Krista Howarth and Jason Russell. Thank you to my parents and siblings for your constant interest and excitement about my research, and for being such an amazing, supportive family. Finally, to Bryan Oates, my fiancé, roommate and best friend; you are the reason that I have had so much fun in the past 6 years.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

FORMAT AND ORGANIZATION OF THESIS

This thesis was prepared in the "sandwich format" as outlined in the School of Graduate Studies' Guide for the Preparation of Thesis. This thesis is comprised of 5 original research papers (Chapters 2-6), preceded by a general introduction and followed by a general discussion. All papers have either been previously published (Chapters 2-5) or submitted for publication (Chapter 6) in peer reviewed journals with the candidate as first author.

CONTRIBUTION TO PAPERS WITH MULTIPLE AUTHORSHIP

Chapter 2

Publication

Bruin JE, Kellenberger LD, Gerstein HC, Morrison KM, Holloway AC. Fetal and neonatal nicotine exposure and postnatal glucose homeostasis: Identifying critical windows of exposure. *Journal of Endocrinology.* 2007. 194(1):171-8.

Contribution

This study was design by A.C. Holloway, H.C. Gerstein and K.M. Morrison. Animal experiments were coordinated and conducted by A.C. Holloway. Paraffin-embedded pancreas tissue was sectioned and analysed by J.E. Bruin, with the exception of the islet PCNA immunostaining which was performed by L.D. Kellenberger. Manuscript preparation was completed by J.E. Bruin.

Chapter 3

Publication

Bruin JE, Gerstein HC, Morrison KM and Holloway AC. Increased pancreatic beta cell apoptosis following fetal and neonatal exposure to nicotine is mediated via the mitochondria. *Toxicological Sciences.* 2008 Jun;103(2):362-70. Epub 2008 Jan 17.

Contribution

The animal experiments for this study were designed and coordinated by J.E. Bruin and A.C. Holloway. All laboratory experiments were designed and performed by J.E. Bruin. Manuscript preparation was completed by J.E. Bruin.

Chapter 4

Publication

Bruin JE, Petre MA, Lehman MA, Raha S, Gerstein HC, Morrison KM and Holloway AC. Maternal exposure to nicotine increases oxidative stress in the pancreas of the offspring. *Free Radical Biology and Medicine*. 2008 Jun 1;44(11):1919-25. Epub 2008 Mar 4.

Contribution

The animal experiments for this study were designed and coordinated by J.E. Bruin, M.A. Petre and A.C. Holloway. All laboratory experiments were designed and performed by J.E. Bruin, with the exception of the reverse-transcription PCR for the nAChR subunits (performed by M.A. Lehman). In addition, M.A. Petre assisted with the development of the islet isolation technique and experiments measuring reactive oxygen species production in isolated islets. Manuscript preparation was completed by J.E. Bruin.

Chapter 5

Publication

Bruin JE, Petre MA, Raha S, Morrison KM, Gerstein HC, Holloway AC. Fetal and neonatal nicotine exposure in Wistar rats causes a progression of pancreatic mitochondrial alterations and leads to beta cell dysfunction. 2008. *PLoS ONE* 3(10): e3371. doi:10.1371/journal.pone.0003371.

Contribution

The animal experiments for this study were designed and coordinated by J.E. Bruin and A.C. Holloway. All laboratory experiments were designed and performed by J.E. Bruin. M.A. Petre assisted with the experiments in isolated islets. Complex IV and citrate synthase enzyme activity assays were performed by J.E. Bruin with the guidance of S. Raha. Manuscript preparation was completed by J.E. Bruin.

Chapter 6

Publication

Bruin JE, Woynillowicz AK, Hettinga BP, Tarnopolsky MA, Gerstein HC, Holloway AC. Maternal dietary antioxidant supplementation prevents beta cell loss and mitochondrial defects following fetal and neonatal nicotine exposure. Submitted to *Diabetes.*

Contribution

The animal experiments for this study were designed by J.E. Bruin, M.A. Tarnopolsky, and A.C. Holloway. Animal experiments were coordinated by J.E. Bruin and conducted with the assistance of A.K. Woynillowicz, B.P. Hettinga and A.C. Holloway. All laboratory experiments were performed by J.E. Bruin. Manuscript preparation was completed by J.E. Bruin.

CHAPTER 1

GENERAL INTRODUCTION

1.1 TYPE 2 DIABETES

1.1.1 Impact

Type 2 diabetes has been classified as a global "epidemic", given the enormous human and economic costs of this disease and its associated health complications (1). Patients with type 2 diabetes are characterized primarily by hyperglycemia, and an increased risk of developing numerous comorbidities, including retinopathy, nephropathy, neuropathy and cardiovascular mortality (2). The prevalence of type 2 diabetes is rapidly increasing worldwide (1) and in Canada (3). It is estimated that the number of people with diabetes will rise globally from 171 million in 2000 to 366 million in 2030 (2.8% to 4.4% of the population) (1). However, a recent population-based study in Ontario suggests that these projected numbers may severely under-estimate the global diabetes epidemic (3). Lipscombe and Hux reported that in Ontario, the age- and sex-adjusted prevalence of diabetes increased from 5.2% in 1995 to 8.8% in 2005 (a 69% increase over 10 years), levels which already exceed the predicted rates for 2030 (3).

1.1.2 Pathology

Type 2 diabetes is diagnosed when fasting plasma glucose (FPG) levels rise higher than 7.0 mmol/l and/or the 2 hour post-load glucose (following a 75 g oral glucose challenge) is greater than 11.1 mmol/l (4). These glucose measures are high relative to individuals with "normal" FPG of 5.1 mmol/l and 2-hour glucose of 5.4 mmol/l. Furthermore, patients are identified as "pre-diabetic" if they have impaired glucose tolerance (IGT; 2 hour glucose 7.8-11.0 mmol/l) or impaired fasting glucose (IFG; FPG 6.1-6.9 mmol/l) (4). Glycemic control worsens during the transition from IFG / IGT towards type 2 diabetes, in part, because of a progressive decline in the ability of pancreatic beta cells to secrete sufficient insulin for maintenance of glucose homeostasis (5-8). Impaired beta cell function has been observed in pre-diabetic patients with IFG or IGT (5), likely due in part to declining beta cell mass, which has also been observed in patients prior to diagnosis of diabetes (7). Patients in the early stages of type 2 diabetes are generally hyperinsulinemic as a result of compensatory increases in beta cell secretion, but over time, chronic hyperglycemia results in deterioration of beta cell function and ultimately, hypoinsulinemia ensues (6;8).

1.1.3 Etiology

The etiology of type 2 diabetes is multi-factorial and involves a complex interaction of both environmental and genetic factors. Numerous modifiable lifestyle factors are strongly associated with the development of impaired glucose

homeostasis, including physical inactivity, dietary quality (in particular, dietary fat intake), and cigarette smoking (9). Smoking not only increases the risk of developing type 2 diabetes in a dose-dependant manner, but also impairs metabolic control in diabetic patients (reviewed in (10-12)). However, in addition to lifestyle choices of the individual with diabetes, maternal lifestyle has also been shown to substantially influence the metabolic phenotype of the offspring. Therefore, risk factors for type 2 diabetes can be traced back as far as fetal life.

1.2 FETAL PROGRAMMING OF ADULT DISEASE

1.2.1 Barker's Hypothesis: Epidemiological Evidence

Dr. David Barker's "developmental origins of adult disease" theory states that a fetus adapts to an adverse intrauterine environment by favoring the development of organs that ensure short term survival (13-15). This reprogramming is beneficial for the immediate survival of the fetus, but has harmful long-term effects as the metabolic demands of the individual increase with age (13-15).

Fetal programming of adult disease was first demonstrated in a landmark cohort study where maternal exposure to the Dutch famine of 1944-1945 during the first half of pregnancy was associated with significantly higher rates of obesity in 500,000 male offspring at age nineteen (16). A more recent cohort study followed 122,000 American women and showed a significant correlation between low birth weight and increased risk of coronary heart disease, stroke, and type 2

diabetes, even after adjusting for potential confounding lifestyle factors (17). This association between intrauterine growth restriction (IUGR) and the metabolic syndrome has since been documented around the world in studies from England (18-20), Sweden (21), Holland (22), India (23) and South Africa (24).

1.2.2 Animal Models

In normal human development there is little variation in fetal growth up to 16 weeks, but during mid to late gestation fetal growth becomes particularly sensitive to adverse intrauterine environments (25). The major determinants of normal fetal growth include: genetics, placentation, the materno-placento-fetal unit integrity, adequate nutrient and oxygen supply, and correct hormonal balance (26). Disruption of any of these elements, particularly in late gestation, may lead to impaired fetal growth and possibly IUGR.

To study the mechanisms underlying fetal programming of adult disease, numerous research groups have developed animal models of IUGR. The most common approaches to simulate fetal growth restriction include: 1) maternal undernutrition with calorie or protein deprivation (27-30), 2) placental insufficiency using bilateral uterine artery ligation to restrict blood flow (31;32), 3) glucocorticoid exposure via maternal dexamethasone treatment (33-35), inhibition of placental 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2) (36) or fetal adrenalectomy followed by exogenous cortisol infusion (37;38), and 4) maternal testosterone treatment (39). These animal models consistently produce

offspring with low birth weight and a variety of metabolic abnormalities during adulthood, including hypertension, hypercholesterolemia, obesity, glucose intolerance, and insulin resistance (40;41). Since cigarette smoking is one of the most common causes of IUGR clinically (42;43), animal models have been designed to examine cigarette smoke-induced intrauterine growth restriction (44;45). However there are no animal models designed to investigate the long term effects of maternal cigarette smoke exposure on metabolic outcomes in the offspring.

1.3 CIGARETTE SMOKING

1.3.1 Prevalence of Cigarette Smoking

In 1964, the US Surgeon General first reported that cigarette smoking is causally associated with lung cancer (46). Since this time, the link between smoking and the morbidity and mortality associated with numerous clinical conditions has been well-established (46). Consequently, smoking rates have drastically declined over the years; in 1965, 52% of males and 34% of females in the US were smokers compared with 21% of men and 19% of females currently (47). However, approximately 15-20% of all women smoke during pregnancy (48;49), despite intentions to refrain from smoking during that period (50).

1.3.2 Smoking During Pregnancy: Effects on the Fetus

Cigarette smoking during pregnancy is associated with a number of adverse obstetrical outcomes (48;51;52), many of which are thought to be attributed to the effects of maternal smoking on the placenta. For example, maternal smoking is associated with an increased risk of placental abruption (premature separation of a normally implanted placenta before delivery) (53) and placenta previa (implantation of the placenta over or near the cervix, causing partial detachment during labor) (54). Oxygen and nutrient delivery to the fetus is compromised with cigarette smoking as a result of uteroplacental vasoconstriction, placental infarcts and calcifications (55). Consequently, both maternal cigarette smoking and exposure to secondhand smoke are unequivocally associated with intrauterine growth restriction (51). Children of women who smoke during pregnancy weigh approximately 200 g less on average than children of non-smokers at birth (42;43;52;56;57), with a clear dose-dependent association between cigarette smoking and reduced birth weight (42;43). Finally, the risks of stillbirth and sudden infant death syndrome (SIDS) are also strongly increased with maternal smoking (51).

1.3.3 Smoking During Pregnancy: Fetal Programming

The risks associated with smoking during pregnancy are not limited to adverse fetal and neonatal outcomes, but are also clearly linked with adverse postnatal health consequences (51). For instance, prenatal exposure to tobacco

smoke has been associated with an increased risk of childhood cancers, including childhood brain tumors and leukemia/lymphoma (58). Prenatal tobacco exposure also predicts adverse postnatal neurobehavioural outcomes, such as attention deficit hyperactivity disorder (ADHD), learning disabilities, behavioral problems and increased risk of nicotine addiction (reviewed in (51;59;60)). Furthermore, recent epidemiological studies have shown a strong relationship between maternal smoking and subsequent obesity, hypertension and type 2 diabetes in the offspring (49;61-65). The British National Child Development Study reported that the offspring of women who were either "medium to heavy", or "heavy" smokers were over 4 times more likely to develop type 2 diabetes after 16 years of age than the offspring of non-smokers (64). However the mechanisms underlying these associations have not yet been determined.

1.3.4 Smoking Cessation: Nicotine Replacement Therapy

Epidemiological evidence demonstrates that cessation or at least reduction of cigarette smoking during pregnancy can ameliorate damage to a developing fetus (66;67). As such, women are strongly encouraged to quit smoking during this critical window of development. However due to the highly addictive nature of nicotine, quitting smoking is extremely difficult and often requires considerable support, as well as the use of pharmacological smoking cessation aids. Only 20-30% of female smokers successfully abstain from smoking during pregnancy and half of these women replapse within 6 months of

parturition (68).

Nicotine has been identified as the addictive component of cigarettes and the majority of adverse physiological symptoms associated with smoking cessation (cravings, irritability, restlessness, anxiety and increased appetite) have been attributed to nicotine withdrawal (47). Therefore, pharmacological smoking cessation therapies have been designed to replace nicotine, including nicotine replacement therapy (NRT), bupropion (Zyban®, a noncompetitive antagonist at the nicotinic receptor) and varenicline (CHANTIX®, a partial nicotinic receptor agonist) (47). Specifically, NRT is available in the form of nicotine chewing gum, transdermal patch, lozenges, nasal spray and inhaler (47).

Although NRT is highly effective for smoking cessation in non-pregnant smokers (reviewed in (47)), there is currently no evidence to suggest that NRT use is effective for smoking cessation in pregnant women (69;70). These findings may be attributed to the increased rate of nicotine metabolism in pregnant versus non-pregnant smokers (60% higher nicotine clearance and 140% higher cotinine clearance during pregnancy) (71). Despite the lack of evidence to support the efficacy of NRT use during pregnancy, the percentage of pregnancies in which NRT was prescribed has increased steadily between 1998 and 2004 (70).

Nicotine, in the form of gum, nasal spray and lozenges, is currently classified as a Pregnancy Category C drug, meaning that studies on animals show conclusive adverse effects on fetal development, but no adequate and well-

controlled studies have been performed on pregnant women. The transdermal nicotine patch is classified in Pregnancy Category D, meaning that there is positive evidence of human fetal risk, but the benefits from use in pregnant women may be acceptable despite the risk (60). Based on these US FDA classifications, it is generally agreed that the risk to the fetus of continued smoking outweighs any potential adverse effects of NRT (47;72). Furthermore, NRT is thought of as a safer alternative to smoking during pregnancy because the mother and fetus are exposed to one chemical instead of thousands (47;72). The Ontario Medical Association (OMA) currently advocates NRT as a safe alternative to cigarette smoking for pregnant women and has urged Health Canada to modify their labeling requirements to include NRT use during pregnancy (72). However, there are several issues with the OMA recommendations. First, NRT use compared to placebo does not appear to increase the probability of successful smoking cessation during pregnancy, and secondly, nicotine may not be the "safe" chemical in cigarettes as was previously assumed, particularly in light of recent studies examining the long term consequences of prenatal nicotine exposure. Indeed, concerns have been raised in a recent critical review by Ginzel and colleagues about the safety of nicotine replacement therapy use during pregnancy, based on evidence of fetotoxicity and neuroteratogenicity associated with maternal nicotine exposure (73).

1.4 TOXICOLOGY OF NICOTINE

1.4.1 Nicotine Exposure During Pregnancy: Epidemiology Evidence

In pregnant women who smoke or use NRT, nicotine crosses the placenta, concentrates in fetal blood and amniotic fluid, and is detectable in breast milk during lactation (74;75). Therefore, maternal nicotine exposure results in both fetal and neonatal exposure.

There are a limited number of trials examining the safety of NRT in humans, and all of these are focused on the short term toxicological effects on the fetus. One study showed an increased prevalence of specific malformations in pregnant NRT users compared to both nonsmokers and smokers (76), while another study on the same cohort data showed that NRT use during pregnancy was not associated with an increased risk of stillbirth (77). Nicotine gum and patches have been shown to increase maternal blood pressure and heart rate, as well as fetal heart rate, but to a lesser degree than cigarette smoking (78). Although smoking is clearly associated with intrauterine growth restriction (42;43), this effect does not appear to be attributed to nicotine, as birth weights were higher in women using NRT compared with the placebo group (79).

Currently there are no prospective epidemiological studies that examine NRT use during pregnancy and the incidence of disease in adult offspring. Therefore, animal models have been utilized to examine the long term effects of prenatal nicotine exposure, as well as to carefully evaluate the acute effects of maternal nicotine on the fetus and neonate in a controlled environment.

1.4.2 Nicotine Exposure During Pregnancy: Animal Models

Animal models that study the toxicity of nicotine during pregnancy have focused primarily on both the short and long term effects of nicotine on the central nervous system. Taken together, evidence from numerous animal studies has clearly established nicotine as a neuroteratogen that compromises the development of critical neural pathways in the developing brain (60;80). Short term, prenatal nicotine exposure is associated with a compromised neonatal response to hypoxia (81-83), suggesting that nicotine may play a key role in the increased incidence of SIDS in newborns of maternal cigarette smokers (51). Numerous long term neurological effects have also been documented following prenatal nicotine exposure, which are thought to explain many of the adverse neurobehavioural outcomes in the offspring of women who smoke during pregnancy (reviewed in (51;59;60)). For example, prenatal nicotine exposure in rodents causes postnatal hyperactivity, cognitive impairment, increased anxiety, somatosensory deficits, persistent neurochemical alterations, changes in sensitivity to nicotine, alterations in nicotine self administration and altered patterns of neural cell survival and synaptogenesis (reviewed in (59;60)).

Therefore, numerous adverse health consequences in the offspring of women who smoke during pregnancy have been attributed to the detrimental effects of nicotine alone. However, until recently the long term metabolic consequences of prenatal nicotine exposure had not been examined in an animal model, despite the strong relationship between maternal smoking and

subsequent obesity, hypertension and type 2 diabetes in the offspring (49;61-65). This topic has recently gained more attention, and was the subject of a 2009 review paper entitled: "Prenatal Nicotine Exposure and the Programming of Metabolic and Cardiovascular Disorders", which cited several of the studies from this thesis (84).

1.4.3 Fetal and Neonatal Nicotine Exposure: Metabolic Outcomes

Our lab has developed a rodent model to determine whether nicotine, the addictive component of cigarette smoke, is involved in fetal programming of adverse metabolic outcomes. In women who smoke or use NRT, both the fetus and neonate are exposed to nicotine, as this chemical easily crosses the placenta, concentrates in fetal blood and amniotic fluid, and is detectable in breast milk during lactation (74;75). Therefore, this animal model was designed to expose offspring to highly relevant concentrations of nicotine during critical windows of fetal and neonatal development. Female rats in this model are injected daily with either nicotine bitartrate (1.0 mg/kg/day) or saline (vehicle) for two weeks prior to mating, three weeks during gestation (fetal development) and three weeks during lactation (neonatal development). The dose of nicotine used in this animal model results in maternal serum cotinine concentrations of 136 ng/ml (85), which is within the range of cotinine levels reported in women who are considered "moderate smokers" (80 to 163 ng/mL) (86). In addition, this dose of nicotine results in serum cotinine concentrations of 26 ng/ml in the nicotine-

exposed offspring at birth (85), which is also within the range (5 to 30 ng/ml) observed in infants nursed by smoking mothers (87).

The long term metabolic consequences of fetal and neonatal nicotine exposure have been well characterized in this animal model. Maternal nicotine exposure during pregnancy and lactation in rats has been shown to cause increased adiposity and obesity (88;89), altered perivascular adipose tissue composition and function (90), elevated blood pressure (90) and impaired glucose homeostasis (89) in the adult male offspring at 26 weeks of age relative to saline controls. Similarly, other research groups have confirmed that offspring exposed to nicotine during fetal and neonatal development have altered adiposity (91-93) and impaired glucose homeostasis during adulthood (93). However, the mechanisms underlying the association between fetal and neonatal nicotine exposure and adult-onset metabolic abnormalities have not been investigated in this animal model. In addition, the mechanisms through which maternal cigarette smoking leads to an increased incidence of obesity, hypertension and type 2 diabetes in the offspring is also unknown (49;61-65). *Therefore, the general goal of this thesis was to determine the mechanism(s) underlying the observed dysglycemia following fetal and neonatal nicotine exposure. Specifically, this work will investigate the effects of nicotine on pancreatic development and determine how an early chemical insult to the pancreatic beta cell can lead to dysfunctional glucose homeostasis during adulthood.*

1.5 PANCREATIC DEVELOPMENT

1.5.1 Critical Windows of Pancreatic Development

In rats, pancreatic development occurs both prenatally and postnatally, whereas in humans the majority of development is completed prenatally (94). Since the critical windows for determining beta cell mass differ between rodents and humans, this section will focus on rodent pancreas development. The maximum rate of beta cell mass expansion occurs during late gestation, beginning at day 16 post-conception; the beta cell population doubles daily, mainly due to beta cell neogenesis from undifferentiated precursor cells (95). Growth of the beta cell population continues during the first week of postnatal development in rats via replication and neogenesis, although at a much slower rate than during late gestation (95;96). During the second week of neonatal life, a wave of beta cell remodeling occurs via apoptosis, but is not accompanied by changes in beta cell mass (95;96). During adult life (1 to 7 months), beta cell mass continues to expand slowly, and mainly through increased size of individual cells rather than an increase in the number of cells (95). Therefore, an adverse fetal and/or neonatal environment that affects the early development of beta cell mass may permanently alter the beta cell population due to the limited opportunity for proliferation and neogenesis after the first two weeks of neonatal life.

1.5.2 Beta Cell Mass and Function in Humans with Type 2 Diabetes

In healthy individuals, beta cells efficiently respond and adapt to metabolic challenges through hyperplasia, hypertrophy and increased insulin synthesis and secretion (97). Conversely, patients with type 2 diabetes are characterized by their inability to produce a sufficient amount of insulin to normalize blood glucose levels and compensate for peripheral insulin resistance (5-7;98). In other words, impaired beta cell function is a central defect in patients with type 2 diabetes (5;8). Furthermore, this insulin insufficiency is observed prior to the diagnosis of type 2 diabetes (5) and is thought to be attributed to a reduction in beta cell mass (6;99). Indeed, humans with type 2 diabetes exhibit a 40-60% reduction in beta cell mass prior to diagnosis compared to weight-matched controls (7;100;101). Therefore, impaired beta cell mass and function are crucial contributing factors to the progressive decline in glucose homeostasis control in patients with type 2 diabetes (5;8). The regulation of beta cell mass is determined by a balance of beta cell size, replication, neogenesis and apoptosis (95;99;102). The loss of beta cell mass in humans with type 2 diabetes appears to be due mainly to increased apoptosis (7), whereas the compensatory expansion of beta cell mass in response to increased insulin demand is mediated primarily by beta cell proliferation (97;103).

1.5.3 Beta Cell Mass in Animal Models of Fetal Programming

A common finding in numerous animal models of metabolic fetal programming is altered beta cell mass prior to the onset of glucose abnormalities (reviewed in (104-106)). However, the mechanisms through which beta cell loss occurs vary depending on the animal model. Maternal protein restriction results in smaller fetal islets, mainly due to a reduction in the proliferation rate combined with increased apoptosis (107-109). Maternal calorie restriction during the last week of gestation causes a reduction in beta cell mass at birth, which is attributed to altered islet neogenesis rather than altered proliferation or apoptosis (109;110). Ligation of the uterine artery on the last days of gestation (to mimic uteroplacental insufficiency) causes a reduction in beta cell proliferation at 14 days postnatally, but no change in beta cell apoptosis, which results in reduced beta cell mass beginning at 15 weeks of age (31;111). Regardless of mechanism, the loss of beta cell mass in all of these animal models is central to the impaired glucose tolerance observed in the adult offspring (reviewed in (104-106)). Fetal and neonatal nicotine exposure has been shown to cause adult-onset dysglycemia, but the possible contribution of impaired beta cell mass and/or function to this phenotype have not been examined in detail. Furthermore, the mechanisms through which exposure to nicotine during critical windows of pancreatic development may impact beta cell survival and function remain to be determined and are the focus of this thesis.

1.6 REGULATION OF BETA CELL SURVIVAL AND FUNCTION BY NICOTINE

Although there are numerous pathways through which beta cell survival and function may be regulated, this thesis will focus on selective pathways that were hypothesized to be likely targets of fetal and neonatal nicotine expsoure.

1.6.1 Pancreatic Nicotinic Acetylcholine Receptors

The nicotinic acetylcholine receptor (nAChR) belongs to a family of neurotransmitter-gated ion channels (112) that are homo- or heteropentamers comprised of various combinations of α- and β-subunits (α2 – α10 and β2 – β4) (113). The nAChRs are known to be particularly important for healthy brain development and their expression is tightly regulated during critical windows of neural development (80). Importantly, many of the adverse effects of prenatal nicotine exposure on neural development and long term neurobehavioral consequences have been attributed to the ability of nicotine to interact with and alter expression of the nAChR during critical windows of development (80). Although the nAChR is best characterized in the brain, there is also evidence for the presence of these neuronal receptors in various non-neuronal cell types (114;115), including a pancreatic beta cell line (116). Furthermore, Yoshikawa and colleagues have demonstrated that nicotine can directly interact with the nAChR in beta cells to inhibit both basal and glucose-stimulated insulin secretion (116). However, it is currently unknown whether the nAChR subunits are present in the developing pancreas and therefore, whether nicotine could be acting

directly on its receptor to adversely affect beta cell survival and function.

1.6.2 Oxidative Stress

Cellular oxidative status is a balance between oxidative stress and antioxidant capacity. A basal level of reactive oxygen species (ROS) is required for normal cell function, but if the level of ROS exceeds the antioxidant capacity of the cell, oxidative stress will ensue (117;118). Molecules that are classified as ROS include the superoxide anion (O2•-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (•OH) (119). If the cellular antioxidant defense system is overwhelmed, ROS can cause damage to cellular proteins, lipids and nucleic acids (118;120), ultimately leading to cell death (121).

Mitochondria are particularly susceptible to oxidative stress for a number of reasons. First, the iron-sulphur centers of the enzymes in the mitochondrial electron transport chain (ETC) are extremely sensitive to ROS inactivation (119). Secondly, mitochondrial DNA (mtDNA) is significantly more susceptible to ROS damage than nuclear DNA (nDNA) because it is not protected by a nuclear membrane or histone proteins, it contains only exons (coding regions of DNA) and its repair mechanisms are poor (119;122). However, mitochondria are not only susceptible to damage by ROS, but are also the major source of endogenous ROS production. When the enzyme complexes in the ETC are dysfunctional, electron leakage occurs, and ROS are formed by the donation of an electron to oxygen (119). Antioxidant enzymes such as manganese

superoxide dismutase (MnSOD) or copper-zinc (CuZn) SOD convert O2•- to H_2O_2, which can then be converted safely to water by glutathione peroxidase-1 (GPx-1) (119). If H_2O_2 is not detoxified by GPx-1, then it will be converted to the highly reactive •OH (119).

Maternal smoking is associated with increased levels of oxidative stress markers in mothers, newborns and infants (123;124). Furthermore there is considerable evidence *in vivo* and *in vitro* to suggest that exposure to nicotine alone results in increased oxidative stress in fetal, neonatal and adult tissues (125-127). In adult rats, nicotine exposure has been shown to increase oxidative stress in pancreatic tissue *in vitro* (127) and to produce oxidative tissue injuries *in vivo* (128;129). Because the pancreatic beta cell has low expression of antioxidant enzymes (130;131), it is particularly susceptible to oxidative stress-mediated tissue damage, including increased beta cell death (117;121;132-135). For these reasons, the effects of fetal and neonatal nicotine exposure on oxidative stress in the beta cells will be explored in this thesis.

1.6.3 Apoptosis Pathways

There are two major signaling pathways of programmed cell death, the mitochondrial pathway (intrinsic) and the death receptor pathway (extrinsic) (Figure 1.1). In the mitochondrial pathway, pro-apoptotic members of the Bcl-2 family (Bax, Bak or Bid) translocate to the mitochondrial outer membrane, and are involved in the formation of a mitochondrial permeability transition pore

(mtPTP) (119;121;136-140). Anti-apoptotic members of the Bcl2 family (Bcl-2 and Bcl-XL) sequester Bax, Bak and/or Bid, and therefore prevent translocation of these pro-apoptotic signaling molecules to the mtPTP. Opening of the mtPTP destroys the mitochondrial membrane potential, causing ion equilibration, swelling of the mitochondria and release of proteins from the intermembrane space into the cytosol (119). Cytochrome *c* release leads to the formation of an "apoptosome", consisting of Apaf-1 (apoptosis protease activating factor), caspase-9 and ATP, which in turn activates caspase-3 (121;137;140). Release of AIF (apoptosis-inducing factor) or EndoG (endonuclease G) leads to DNA fragmentation, while SMAC/DIABLO (second mitochondria-derived activator of caspases / direct IAP-associated binding protein with low pI) release causes inhibition of the IAP (inhibitor of apoptosis protein) family (121;137;139).

The death receptor pathway involves the binding of a death receptor (e.g., Fas) to a ligand (e.g., FasL), which results in activation of a caspase-signaling cascade to induce cell death (121;138;139;141). In particular, the Fas/FasL interaction leads to oligomerization of FasL, recruitment of FADD (Fas-associated death domain) protein and pro-caspase-8 to the cytoplasmic death domain of Fas, where a death-inducing signal complex (DISC) is formed (121;141). Formation of the DISC leads to activation caspase-8, which in turns causes cleavage of pro-caspase-3 to its active form (121;141). Alternatively, active caspase-8 can induce cleavage of Bid to tBid, which will then translocate to the mitochondrial outer membrane, resulting in release of mitochondrial proteins and

amplification of the Fas/FasL apoptosis signal (121;141).

Mitochondrial-mediated cell death plays a crucial role in the pathogenesis of type 2 diabetes, and specifically, in the survival of pancreatic beta cells (142). The mitochondrial-mediated apoptotic pathway has been shown to be involved in beta cell apoptosis caused by exposure to both chronic high glucose (143) and fatty acids (144). On the other hand, in healthy adult human islets, exposure to high concentrations of glucose induced beta cell apoptosis via the death receptor-mediated pathway (145). Therefore, both pathways may play a role in beta cell apoptosis; their relative involvement in nicotine-induced beta cell apoptosis following fetal and neonatal exposure remains to be determined.

1.6.4 Mitochondria

In beta cells, the mitochondria play a key role in signaling for apoptosis (as described above) and therefore regulating beta cell mass (119) (Figure 1.1 and 1.2). In addition, the mitochondria are critical for maintenance of beta cell function through the coupling of a glucose stimulus to insulin release (146-148) (Figure 1.3). Both human and animal studies have demonstrated impairment of mitochondrial function in islets of subjects with type 2 diabetes (149;150). Therefore, defects in mitochondrial function are hypothesized to be central to the pathogenesis of type 2 diabetes.

It is estimated that 98% of the energy for the beta cell is produced by

mitochondrial oxidative metabolism (148). The mitochondria are essential for both stages of glucose-stimulated insulin secretion, including glucose entry and metabolism, as well as insulin exocytosis from the beta cell (Figure 1.3). Glucose enters the beta cell via glucose transporter 2 (GLUT2) and is converted to pyruvate through glycolysis. The beta cells contain relatively low amounts of lactate dehydrogenase (151), meaning that nearly all of the pyruvate produced from glucose is routed to the mitochondria, rather than converted to lactate in the cytosol (146;148). NADH, a product of both glycolysis in the cytosol and the citric acid cycle within the mitochondrial matrix, is the major source of electrons for the electron transport chain (146;152). Electrons are transferred through the four protein complexes, and at complex IV, oxygen is reduced to water (146;152). At complexes I, III and IV, H+ ions are pumped from the mitochondrial matrix across the inner mitochondrial membrane, creating an electrochemical potential gradient (146;152). When the H+ ions are transported back to the mitochondrial matrix via ATP synthase, the energy stored within the electrochemical gradient is used to produce ATP from ADP. ATP production stimulates closure of ATP-sensitive K+ channels, depolarization of the beta cell membrane, opening of the voltage-gated Ca+ channels and finally a rise in intracellular Ca+ levels, which triggers insulin exocytosis (146).

Insulin exocytosis from the beta cell occurs in two phases. The first phase involves release of insulin-containing vesicles, which are stored and ready for exocytosis, in response to an acute rise in intracellular Ca+ levels (148;152). The

second phase involves sustained insulin release, which can only occur after ATP is produced by fuel metabolism in the mitochondria, allowing for the mobilization and priming of insulin granules from reserve pools to create new insulin vesicles (148;152). Therefore, if ATP production by the mitochondria is compromised, then insulin synthesis and secretion will also be adversely affected. Indeed islets from patients with type 2 diabetes show reduced insulin secretion in response to a glucose stimulus, as well as lower ATP levels and a lower ATP/ADP ratio (149). The role of mitochondria in regulating beta cell function remains to be determined following fetal and neonatal nicotine exposure.

1.7 PROTECTION OF BETA CELL SURVIVAL AND FUNCTION BY ANTIOXIDANTS

Loss of compensatory beta cell function is a crucial event in the progression from normal to impaired glucose tolerance (5-8;98). Furthermore, the inability of beta cells to secrete sufficient insulin to maintain healthy glucose homeostasis has been attributed to a reduction in beta cell mass (6;99). Since the mitochondria are central to both the beta cell apoptosis and loss of beta cell function in type 2 diabetes (119;146-148), improving mitochondrial function may be a viable option for protecting beta cell mass and function. Antioxidant interventions have been used successfully in various models of type 2 diabetes to improve markers of glucose homeostasis (153-161), and to protect beta cell mass and prevent beta cell apoptosis (154;157). However, the ability of antioxidants to protect beta cells during fetal and neonatal development has yet

not been examined.

Antioxidants are known to function optimally when a mixture is used instead of an individual compound because independently, antioxidants are often not only ineffective, but can potentially act as pro-oxidants, thus causing cellular damage (162). Furthermore, when several antioxidants are combined, they can act cooperatively as reduction/oxidation (redox) couples, thus increasing efficacy (162). For these reasons, an antioxidant diet containing a mixture of coenzyme Q10 (CoQ), alpha lipoic acid (ALA) and vitamin E (VitE) will be investigated in this animal model.

CoQ is a lipid-soluble benzoquinone compound that shuttles electrons to complex III in the respiratory chain and translocates protons across the inner mitochondrial membrane (163). In addition to its main role in energy production, CoQ protects the mitochondria through a number of mechanisms, including its ability to act as a free radical scavenger (163), inhibit mitochondrial depolarization independent of its free radical scavenging property (164), and function as a redox couple (162;165). When combined in an antioxidant cocktail, CoQ acted primarily to regenerate α-tocopherol (VitE) by direct reduction of the tocopheroxyl radical, while VitE functions as the direct radical scavenger (165). Vitamin E (α-tocopherol) is a lipid soluble chain breaking terminating antioxidant that acts primarily to scavenge lipid peroxyl radicals (162), and has also been shown to protect cells from mitochondrial damage (166). ALA functions as an essential cofactor for several mitochondrial enzyme complexes (including pyrvate

dehydrogenase), a free radical scavenger, and an important redox couple for CoQ and VitE (162). Treatment with ALA protects rodents from age-related defects in mitochondrial bioenergetics (167). Furthermore, all three of these antioxidants have independently been shown to prevent mitochondrial-mediated apoptosis in various cell types (164;166;168-171). The ability of these antioxidants to prevent nicotine-induced beta cell apoptosis and mitochondrial damage will be examined in this animal model.

1.8 SPECIFIC AIMS

1.8.1 Specific Aim 1

The first goal of this PhD thesis was to determine the critical windows of fetal and neonatal nicotine exposure for pancreatic beta cell development and adult-onset dysglycemia. First, I hypothesized that maternal nicotine exposure would cause beta cell apoptosis and loss of beta cell mass in the developing fetus and neonate. Furthermore, since rodent pancreatic development occurs during two important phases, (expansion during fetal development and remodeling during neonatal development), I hypothesized that nicotine exposure would be required during ***both*** pregnancy and lactation to cause permanent beta cell damage and metabolic reprogramming in the offspring.

1.8.2 Specific Aim 2

The second aim of this thesis was to determine the mechanisms through which fetal and neonatal exposure to nicotine results in: a) loss of beta cell mass during development, and b) impaired glucose homeostasis during adulthood. I hypothesized that nicotine acts directly on the developing pancreas via the nicotinic acetylcholine receptor to induce beta cell apoptosis and loss of beta cell mass. Mitochondria are particularly susceptible to damage by reactive oxygen species and are capable of signalling for cellular apoptosis following oxidative stress. Therefore, I proposed that nicotine exposure would induce oxidative stress in the developing beta cells, and consequently trigger the mitochondrial-mediated apoptotic signalling pathway. Furthermore, I proposed that alterations to the mitochondria during perinatal development would initiate a feed-forward chain of progressive postnatal mitochondrial damage, ultimately leading to impaired beta cell function. Therefore, in this animal model I hypothesized that nicotine-induced mitochondrial defects would contribute to loss of both beta cell mass (via apotosis) during development and beta cell function during adulthood.

1.8.3 Specific Aim 3

The third objective of my thesis was to determine whether an antioxidant intervention during pregnancy and lactation could prevent the nicotine-induced beta cell defects and dysglycemia in this animal model. I hypothesized that exposure to antioxidants during pregnancy and lactation would protect the beta

cell mitochondria from the damaging pro-oxidant effects of nicotine, thus preventing the early loss of beta cell mass. Furthermore, I proposed that protection of the mitochondria during critical windows of pancreatic development would prevent the progression of postnatal mitochondrial defects which contribute to impaired beta cell function and dysglycemia during adulthood.

1.9 REFERENCES (REFER TO CHAPTER 7)

The references for the general introduction and discussion have been compiled into one bibliography at the end of Chapter 7.

1.10 INTRODUCTION FIGURES

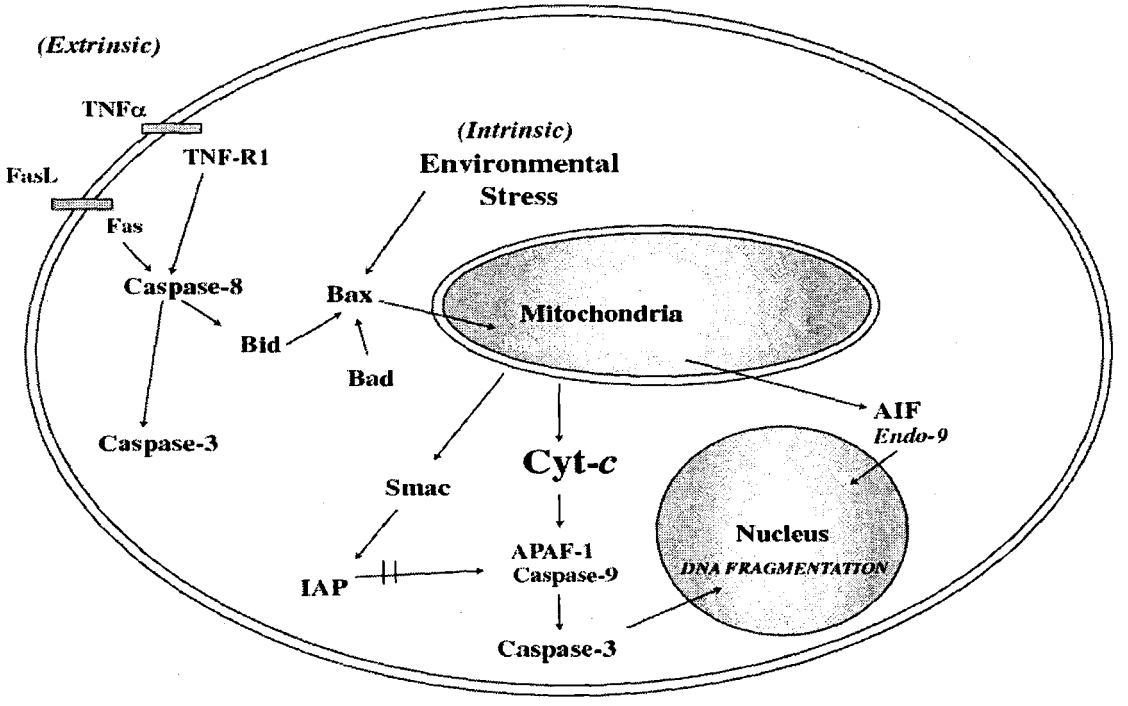


Figure 1.1: Extrinsic and intrinsic pathways of apoptosis (121).
The schematic diagram outlines key signaling molecules involved in the extrinsic (receptor-dependent) and intrinsic (mitochondrial) apoptosis pathways. In the extrinsic pathway, a death-inducing ligand, such as tumor necrosis factor α (TNFα) or Fas ligand (FasL), binds to its respective receptor (TNF-R1 or Fas, respectively), and triggers the formation of a death-inducing signal complex (DISC). DISC formation leads to activation of caspase-8, which can either lead to activation of caspase-3 or to the activation of Bid. Bid assists in the activation and translocation of Bax to the mitochondria, thus triggering the intrinsic pathway. Intrinsic apoptosis can also be triggered by environmental stress that either directly damages the mitochondria or activates the Bax. Bax translocates to the mitochondrial outer membrane, where it oligomerizes and allows release of proteins such as cytochrome c (Cyt-*c*), Smac or AIF from the mitochondria into the cytosol. Release of Cyt-*c* triggers the formation of an apoptosome complex with Apaf-1 and caspase-9, which signals for activation of caspase-3.

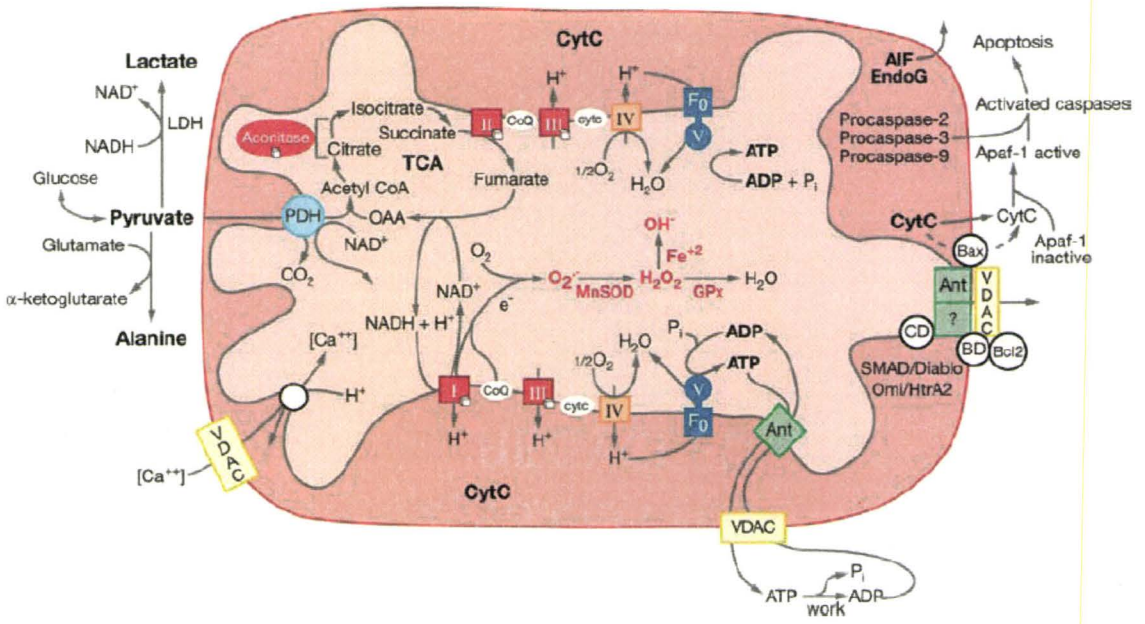


Figure 1.2: Oxidative stress, apoptosis and oxidative phosphorylation pathways in the mitochondria (119).

The schematic diagram integrates the multiple pathways that are all linked to the mitochondria. Antioxidant enzymes MnSOD and GPx detoxify reactive oxygen species which are formed by the donation of an electron to molecular oxygen by the complexes from the electron transport chain. Mitochondrial-mediated apoptosis is regulated by members of the Bcl2 family, which bind to the mitochondrial permeability transition pore (mtPTP, consisting of VDAC, Ant and CD) and trigger the release of proteins, including cytochrome *c* (CytC) and SMAD/Diablo into the cell cytosol.

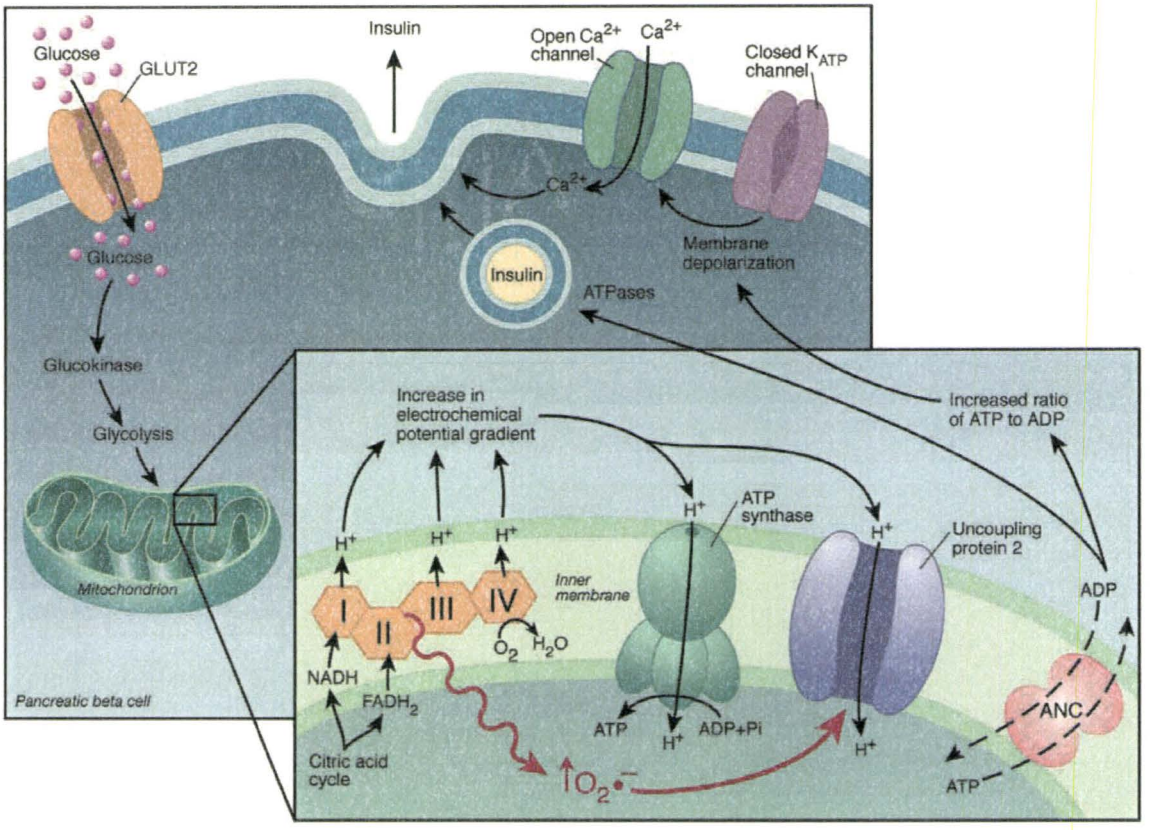


Figure 1.3: The role of the mitochondria in the beta cell (147).
The schematic diagram the outlines the central role of the mitochondria in regulating glucose stimulated insulin release from the beta cell. Glucose enters the beta cell via glucose transporter 2 (GLUT2) and is phosphorylated by glucokinase, so that it can enter the glycolysis pathway in the cytosol. Pyruvate, produced during glycolysis, enters the mitochondria and undergoes the citric acid cycle to produce NADH and FADH2, which provide electrons that enter the electron transport chain at complexes I and II. The transport of H+ ions across the inner membrane increases the electrochemical potential gradient and allows for ATP to be produced by ATP synthase. This increase in the ATP/ADP ratio triggers the closing of KATP channels and opening of Ca+ channels, which allows for insulin exocytosis from the beta cell. The presence of uncoupling protein 2 (UCP2) in the mitochondrial inner membrane causes leakage of H+ ions into the matrix and leads to inefficient production of ATP by ATP synthase. Superoxide generated by the electron transport chain stimulates proton leak activity of UCP2 protein, thus decreasing glucose-stimulated insulin secretion.

CHAPTER 2

FETAL AND NEONATAL NICOTINE EXPOSURE AND POSTNATAL GLUCOSE HOMEOSTASIS: IDENTIFYING CRITICAL WINDOWS OF EXPOSURE

BRUIN JE, KELLENBERGER LD, GERSTEIN HC, MORRISON KM, HOLLOWAY AC. FETAL AND NEONATAL NICOTINE EXPOSURE AND POSTNATAL GLUCOSE HOMEOSTASIS: IDENTIFYING CRITICAL WINDOWS OF EXPOSURE. *JOURNAL OF ENDOCRINOLOGY*. 2007. 194(1):171-8.

2.1 INTRODUCTION

Approximately 15-20% of all women smoke while pregnant (1;2), despite intentions to refrain from smoking during that period (3). Cigarette smoking during pregnancy remains one of the most important modifiable risk factors for adverse fetal, obstetrical and developmental outcomes (1;4). 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 (2;5-11). 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 (12). 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 birthweight, and postnatal impaired glucose homeostasis, hyperinsulinemia, increased body weight and dyslipidemia (13-17). The beta cell loss, impaired glucose tolerance and hyperinsulinemia observed in the nicotine-exposed offspring from this animal model (17) closely represents symptoms associated with type 2 diabetes in humans (18;19). However, the heterogeneous nature of smoking behaviour and nicotine replacement therapy use in the pregnant and breastfeeding population (20-22) 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 (23;24). However the effect of smoking cessation on metabolic disturbances later in life 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 the critical windows of fetal and neonatal exposure to maternal nicotine on the development of the pancreatic beta cell and glucose intolerance in the offspring.

2.2 MATERIALS AND METHODS

2.2.1 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 200-250g female Wistar rats (Harlan, Indianapolis, IN) were maintained under controlled lighting (12:12 L:D) and temperature (22°C) with *ad libitum* access to food and water. Dams were randomly assigned (N= 5 per group) to receive saline (vehicle) or nicotine bitartrate (1mg/kg/d, Sigma-Aldrich, St. Louis MO) via subcutaneous injection daily A) for 2 weeks prior to mating only; B) for 2 weeks prior to mating and 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 1 (PND1) 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 postnatal day 21 (PND21), male offspring were selected randomly and caged as sibling pairs.

2.2.2 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 (0900h), 30 and 120 minutes after rats were given 2g/kg glucose

(Sigma-Aldrich, St. Louis MO) in water by gavage as previously described (25). 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), and insulin levels were measured by an ultra sensitive rat insulin ELISA (Crystal Chem Inc., Downers Grove, IL).

2.2.3 Beta Cell Mass

To assess whether nicotine exposure during pregnancy and lactation alters prenatal and postnatal pancreas development, pancreas tissue was collected from a subset of pups at birth (PND1), 4 and 26 weeks of age. Animals at PND1 were euthanized by decapitation and at 4 and 26 weeks animals were euthanized 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) at 4° C overnight, washed in water and embedded in paraffin. Immunohistochemical detection of insulin was performed on 5μm serial sections, separated by an average of 30μm, of PND1, week 4 and week 26 pancreatic tissues from saline-exposed (5 sections per animal; 5 animals randomly selected to include animals from each saline exposure group) and nicotine exposed (5 sections per animal; 5 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 10mmol/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), 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) with diaminobenzadine as the chromogen. Tissue sections were counterstained with Harris's hematoxylin, destained with acid alcohol, dehydrated and mounted with Permount (Fisher Scientific, Fair Lawn, NJ). 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 Version 5.1 software (Media Cybernetics, Inc., Silver Spring MD) for automated cell counting and the calculation of beta cell area and total pancreas area. The beta cell area was calculated as a ratio of the beta cell area (immunopositive staining only) to the total pancreas area (immunopositive staining plus pancreas counterstaining). Beta cell mass was calculated as the product of the beta cell area and the corresponding total pancreas weight in milligrams.

2.2.4 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 labelling (TUNEL) assay according to manufacturer's instructions (Roche Applied Science, Laval, Quebec) 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 10mmol/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), overnight at 4°C followed by anti-rabbit Alexa Fluor 594 secondary antibody (1:400 dilution, Molecular Probes, Inc., Eugene OR) for 2 hours 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 minutes at room temperature and then incubated with the FITC-conjugated TUNEL enzyme for 60 minutes to detect DNA fragmentation. Nuclei were counterstained with DAPI (Sigma Aldrich, St. Louis, MO) and tissue sections were imaged with an Olympus BX-61 microscope and analyzed with Image Pro Plus Version 5.1 software (Media Cybernetics, Inc., Silver Spring MD). For analysis, 3 islets per section (3 sections per animal, 5 animals per group) were quantified for apoptosis and reported as the percentage of TUNEL+ beta

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, St. Louis, MO) overnight at 4oC. 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 (26). Immunostaining was identified using the Vectastain kit (Vector Laboratories, Burlinghame, CA) with diaminobenzadine as the chromogen. Tissue sections were counterstained with Harris's hematoxylin, destained with acid alcohol, dehydrated and mounted with Permount (Fisher Scientific, Fair Lawn, NJ). For analysis, 3 islets per section (3 sections per animal, 5 animals per group) were quantified. Islet cell proliferation was assessed as the percentage of total islet cells that were PCNA+.

2.2.5 Statistical Analysis

All statistical analyses were performed using SigmaStat (v.2.03, SPSS, Chicago, IL) and one-way analysis of variance (ANOVA) followed by *post-hoc* multiple comparisons when significance was indicated by ANOVA (α=0.05). When significance was indicated by ANOVA, OGTT results for each treatment group were compared to the saline controls (Bonferroni's t-test, α=0.05). There was no difference in the glucose or insulin response to the OGTT at baseline, 30 or 120 minutes between the 4 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 beta cell mass, apoptosis or proliferation between the 4 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 beta cell mass, proliferation and apoptosis among each treatment group were compared to each other using the Student-Neuman-Keuls test. 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. Area under the curve for the total glucose response during the glucose tolerance test was assessed using the trapezoidal rule.

2.3 RESULTS

2.3.1 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 ± 0.24 g food/100 g body weight vs nicotine: 8.9 ± 0.30 g food/100 g body weight, $p=0.30$) or during pregnancy (Table 2.1). 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 2.1).

2.3.2 Glucose Homeostasis

At 26 weeks of age nicotine exposure had no effect on fasting serum glucose ($p>0.05$) or insulin concentrations (Kruskal-Wallis one-way ANOVA on ranks $p>0.05$) in any treatment group (Table 2.2). Following an oral glucose challenge, animals in Group C (fetal and neonatal nicotine exposure) had a higher total glucose response (area under the curve; AUC) to the glucose load relative to the saline controls ($p<0.01$), an effect that was not observed in any other group (Figure 2.1). Furthermore, the peak glucose concentration at 30 minutes was higher in this group (saline 9.1 ± 0.29 mmol·l-1; Group C 10.9 ± 1.15 mmol·l-1; $p<0.05$) and the ability to clear the glucose load, determined by serum glucose concentrations at 120 minutes following the glucose challenge, was impaired (saline 8.2 ± 0.33 mmol·l-1; Group C 11.4 ± 1.21 mmol·l-1; $p<0.005$; Figure 1). The total insulin response (AUC) to the glucose challenge was also ($p<0.05$) elevated in the offspring in Group C relative to the control (saline-exposed) offspring (Table 2.2).

2.3.3 Beta 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 beta cell mass ($p<0.05$) relative to saline-exposed offspring (Figure 2.2A), an effect that was still

present at 4 weeks of age (Figure 2.2B). By 26 weeks, animals exposed to nicotine during pregnancy alone (Group B) had recovered their beta cell mass relative to saline controls (Figure 2.2C). The beta cell mass at 26 weeks of offspring exposed to nicotine during both fetal and neonatal development (Group C) continued to be lower than the beta cell mass of any other group (Figure 2.2C). 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 beta cell mass at any age examined (Figures 2.2A-C). At all three ages, changes in beta cell mass were a reflection of changes in the beta cell area, not a reduction in pancreas weight (Table 2.3).

2.3.4 Beta Cell Proliferation and Apoptosis

Although beta 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 beta 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 if 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 (Figure 2.3A). Animals with fetal and neonatal exposure to

nicotine (Group C) had an increased level of beta cell apoptosis relative to those with fetal exposure alone or to saline controls (Figure 2.3B).

2.4 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 (17). The dose of nicotine used in this study (1mg/kg/d nicotine bitartrate) results in maternal serum cotinine concentrations of 136 ng/ml (27), which is within the range of cotinine levels (80 to 163 ng/mL) reported in women who are considered "moderate smokers" (28). In addition, this dose of nicotine resulted in serum cotinine concentrations of 26 ng/ml in the nicotine-exposed offspring at birth (27), which is also within the range (5 to 30 ng/ml) observed in infants nursed by smoking mothers (29). Although 15-20% of pregnant women smoke (1;3) many women attempt to stop smoking during pregnancy and then relapse following parturition (20-22) 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 current 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 minutes), the nicotine from each daily injection is entirely cleared

before the next injection (30), 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 dysglycaemia at 26 weeks of age in adult 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 co-morbidities including cardiovascular disease, nephropathy, retinopathy and neuropathy (31;32)

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 (18;23;33). This defect in beta cell function is already observable in dysglycaemic individuals with impaired fasting glucose or impaired glucose tolerance, long before the onset of frank type 2 diabetes (33). Recent studies suggest that this insulin insufficiency may be due in part to a reduction in beta cell mass (18;34). Indeed, in humans pancreatic beta cell mass is reduced by 40-60% in patients with type 2 diabetes (23;35;36), and this reduction in beta cell mass precedes the diagnosis of diabetes (23). Similarly we have shown in an animal model that reduced beta cell mass preceded the loss of normal glycaemic control and that although insulin 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 (17).

We had previously suggested that nicotine-induced damage to the beta cell during fetal development may induce permanent changes in pancreatic structure and function evident as impaired glycaemia in adults (17). Similar findings have been reported for other *in utero* insults including glucocorticoid administration, uteroplacental insufficiency and fetal undernutrition (26;37-39). However, this work illustrates that *in utero* nicotine exposure alone transiently reduces beta cell mass without permanent metabolic defects. The full recovery of beta cell mass and function can be attributed to enhanced beta cell proliferation leading to increased expansion of beta cell mass between 1 and 6 months of age. Control animals experienced a 4.6 fold-increase in beta cell mass between 1 and 6 months of age, whereas animals with fetal exposure only (Group B) had lower absolute beta cell mass at 1 month of age but a 6.2 fold "catch-up" in beta cell mass such that by 26 weeks of age they had recovered to 98% of the saline control beta cell mass. There was no decrease in apoptosis found to explain this "catch-up" in beta 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 beta cell mass that was only 62% of controls at 26 weeks. Furthermore, the inhibited growth of beta cell mass in these offspring was due to increased beta cell apoptosis and not to decreased beta cell proliferation.

Other insults during fetal and neonatal developmental, such as maternal undernutrition, also cause a reduction in beta cell mass at birth, an effect which

was irreversible even though restoration of nutrition at the end of gestation resulted in normal beta cell proliferation during lactation (40). In contrast, the results from this study have demonstrated that increased beta cell replication following a reduction in beta cell mass at birth was able to fully restore beta cell mass and function. However, recovery only occurred 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 beta 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 beta cell mass were islet neogenesis and beta cell replication, while an adaptive reduction in beta cell mass was primarily due to increased beta cell apoptosis (41).

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 beta cell mass in early life. These results confirm previous findings that recovery of beta cell mass occurs during critical developmental windows, otherwise the beta 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 developmental differences between species. In rats, pancreatic development occurs both prenatally and postnatally, whereas in humans the majority of development is completed prenatally (42). However, regardless of differences in timing, the

essential principal that impairment of early pancreatic development will result in permanent changes remains the same. Therefore, data from the current 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.

2.5 REFERENCES

1. Andres,RL, Day,MC: Perinatal complications associated with maternal tobacco use. *Semin Neonatol* 5:231-241, 2000

2. Bergmann,KE, Bergmann,RL, Von,KR, Bohm,O, Richter,R, Dudenhausen,JW, Wahn,U: Early determinants of childhood overweight and adiposity in a birth cohort study: role of breast-feeding. *Int J Obes Relat Metab Disord* 27:162-172, 2003

3. Okuyemi,KS, Ahluwalia,JS, Harris,KJ: Pharmacotherapy of smoking cessation. *Arch Fam Med* 9:270-281, 2000

4. Hofhuis,W, de Jongste,JC, Merkus,PJ: Adverse health effects of prenatal and postnatal tobacco smoke exposure on children. *Arch Dis Child* 88:1086-1090, 2003

5. Blake,KV, Gurrin,LC, Evans,SF, Beilin,LJ, Landau,LI, Stanley,FJ, Newnham,JP: Maternal cigarette smoking during pregnancy, low birth weight and subsequent blood pressure in early childhood. *Early Hum Dev* 57:137-147, 2000

6. Montgomery,SM, Ekbom,A: Smoking during pregnancy and diabetes mellitus in a British longitudinal birth cohort. *BMJ* 324:26-27, 2002

7. Morley,R, Leeson,PC, Lister,G, Lucas,A: Maternal smoking and blood pressure in 7.5 to 8 year old offspring. *Arch Dis Child* 72:120-124, 1995

8. Toschke,AM, Koletzko,B, Slikker,W, Jr., Hermann,M, Von,KR: Childhood obesity is associated with maternal smoking in pregnancy. *Eur J Pediatr* 161:445-448, 2002

9. Von,KR, Toschke,AM, Koletzko,B, Slikker,W, Jr.: Maternal smoking during pregnancy and childhood obesity. *Am J Epidemiol* 156:954-961, 2002

10. Vik,T, Jacobsen,G, Vatten,L, Bakketeig,LS: Pre- and post-natal growth in children of women who smoked in pregnancy. *Early Hum Dev* 45:245-255, 1996

11. Wideroe,M, Vik,T, Jacobsen,G, Bakketeig,LS: Does maternal smoking during pregnancy cause childhood overweight? *Paediatr Perinat Epidemiol* 17:171-179, 2003

12. Lambers,DS, Clark,KE: The maternal and fetal physiologic effects of nicotine. *Semin Perinatol* 20:115-126, 1996

13. Williams,CM, Kanagasabai,T: Maternal adipose tissue response to nicotine administration in the pregnant rat: effects on fetal body fat and cellularity. *Br J Nutr* 51:7-13, 1984

14. Newman,MB, Shytle,RD, Sanberg,PR: Locomotor behavioral effects of prenatal and postnatal nicotine exposure in rat offspring. *Behav Pharmacol* 10:699-706, 1999

15. Pausova,Z, Paus,T, Sedova,L, Berube,J: Prenatal exposure to nicotine modifies kidney weight and blood pressure in genetically susceptible rats: a case of gene-environment interaction. *Kidney Int* 64:829-835, 2003

16. Gao,YJ, Holloway,AC, Zeng,ZH, Lim,GE, Petrik,JJ, Foster,WG, Lee,RM: Prenatal exposure to nicotine causes postnatal obesity and altered perivascular adipose tissue function. *Obes Res* 13:687-692, 2005

17. Holloway,AC, Lim,GE, Petrik,JJ, Foster,WG, Morrison,KM, Gerstein,HC: Fetal and neonatal exposure to nicotine in Wistar rats results in increased beta cell apoptosis at birth and postnatal endocrine and metabolic changes associated with type 2 diabetes. *Diabetologia* 48:2661-2666, 2005

18. Leahy,JL: Pathogenesis of type 2 diabetes mellitus. *Arch Med Res* 36:197-209, 2005

19. Pratley,RE, Weyer,C: The role of impaired early insulin secretion in the pathogenesis of Type II diabetes mellitus. *Diabetologia* 44:929-945, 2001

20. McBride,CM, Pirie,PL: Postpartum smoking relapse. *Addict Behav* 15:165-168, 1990

21. Thyrian,JR, Hannover,W, Roske,K, Rumpf,HJ, John,U, Hapke,U: Postpartum return to smoking: Identifying different groups to tailor interventions. *Addict Behav* 31:1785-1796, 2006

22. Castrucci,BC, Culhane,JF, Chung,EK, Bennett,I, McCollum,KF: Smoking in pregnancy: patient and provider risk reduction behavior. *J Public Health Manag Pract* 12:68-76, 2006

23. Lindley,AA, Becker,S, Gray,RH, Herman,AA: Effect of continuing or stopping smoking during pregnancy on infant birth weight, crown-heel length, head circumference, ponderal index, and brain:body weight ratio. *Am J Epidemiol* 152:219-225, 2000

24. Pickett,KE, Wakschlag,LS, Dai,L, Leventhal,BL: Fluctuations of maternal smoking during pregnancy. *Obstet Gynecol* 101:140-147, 2003

25. Cleasby,ME, Kelly,PA, Walker,BR, Seckl,JR: Programming of rat muscle and fat metabolism by in utero overexposure to glucocorticoids. *Endocrinology* 144:999-1007, 2003

26. Petrik,J, Reusens,B, Arany,E, Remacle,C, Coelho,C, Hoet,JJ, Hill,DJ: A low protein diet alters the balance of islet cell replication and apoptosis in the fetal and neonatal rat and is associated with a reduced pancreatic expression of insulin-like growth factor-II. *Endocrinology* 140:4861-4873, 1999

27. Holloway AC, Kellenberger LD, Petrik JJ: Fetal and Neonatal Exposure to Nicotine Disrupts Ovarian Function and Fertility in Adult Female Rats. *Endocrine* 30:213-216, 2006

28. Eskenazi,B, Bergmann,JJ: Passive and active maternal smoking during pregnancy, as measured by serum cotinine, and postnatal smoke exposure. I. Effects on physical growth at age 5 years. *Am J Epidemiol* 142:S10-S18, 1995

29. Luck,W, Nau,H: Nicotine and cotinine concentrations in serum and urine of infants exposed via passive smoking or milk from smoking mothers. *J Pediatr* 107:816-820, 1985

30. Matta,SG, Balfour,DJ, Benowitz,NL, Boyd,RT, Buccafusco,JJ, Caggiula,AR, Craig,CR, Collins,AC, Damaj,MI, Donny,EC, Gardiner,PS, Grady,SR, Heberlein,U, Leonard,SS, Levin,ED, Lukas,RJ, Markou,A, Marks,MJ, McCallum,SE, Parameswaran,N, Perkins,KA, Picciotto,MR, Quik,M, Rose,JE, Rothenfluh,A, Schafer,WR, Stolerman,IP, Tyndale,RF, Wehner,JM, Zirger,JM: Guidelines on nicotine dose selection for in vivo research. *Psychopharmacology (Berl)* 190:269-319, 2007

31. Clemens,A, Siegel,E, Gallwitz,B: Global risk management in type 2 diabetes: blood glucose, blood pressure, and lipids--update on the background of the current guidelines. *Exp Clin Endocrinol Diabetes* 112:493-503, 2004

32. Mlinar,B, Marc,J, Janez,A, Pfeifer,M: Molecular mechanisms of insulin resistance and associated diseases. *Clin Chim Acta* 375:20-35, 2007

33. Kahn,SE: The relative contributions of insulin resistance and beta-cell dysfunction to the pathophysiology of Type 2 diabetes. *Diabetologia* 46:3-19, 2003

34. Rhodes,CJ: Type 2 diabetes-a matter of beta-cell life and death?. *Science* 307:380-384, 2005

35. Sakuraba,H, Mizukami,H, Yagihashi,N, Wada,R, Hanyu,C, Yagihashi,S: Reduced beta-cell mass and expression of oxidative stress-related DNA damage in the islet of Japanese Type II diabetic patients. *Diabetologia* 45:85-96, 2002

36. Yoon,KH, Ko,SH, Cho,JH, Lee,JM, Ahn,YB, Song,KH, Yoo,SJ, Kang,MI, Cha,BY, Lee,KW, Son,HY, Kang,SK, Kim,HS, Lee,IK, Bonner-Weir,S: Selective beta-cell loss and alpha-cell expansion in patients with type 2 diabetes mellitus in Korea. *J Clin Endocrinol Metab* 88:2300-2308, 2003

37. Holness,MJ, Langdown,ML, Sugden,MC: Early-life programming of susceptibility to dysregulation of glucose metabolism and the development of Type 2 diabetes mellitus. *Biochem J* 349 Pt 3:657-665, 2000

38. Shen,CN, Seckl,JR, Slack,JM, Tosh,D: Glucocorticoids suppress beta-cell development and induce hepatic metaplasia in embryonic pancreas. *Biochem J* 375:41-50, 2003

39. Simmons,RA, Templeton,LJ, Gertz,SJ: Intrauterine growth retardation leads to the development of type 2 diabetes in the rat. *Diabetes* 50:2279-2286, 2001

40. Garofano,A, Czernichow,P, Breant,B: In utero undernutrition impairs rat beta-cell development. *Diabetologia* 40:1231-1234, 1997

41. Butler,AE, Janson,J, Soeller,WC, Butler,PC: Increased beta-cell apoptosis prevents adaptive increase in beta-cell mass in mouse model of type 2 diabetes: evidence for role of islet amyloid formation rather than direct action of amyloid. *Diabetes* 52:2304-2314, 2003

42. Hill,DJ, Duvillie,B: Pancreatic development and adult diabetes. *Pediatr Res* 48:269-274, 2000

2.6 TABLES AND FIGURES

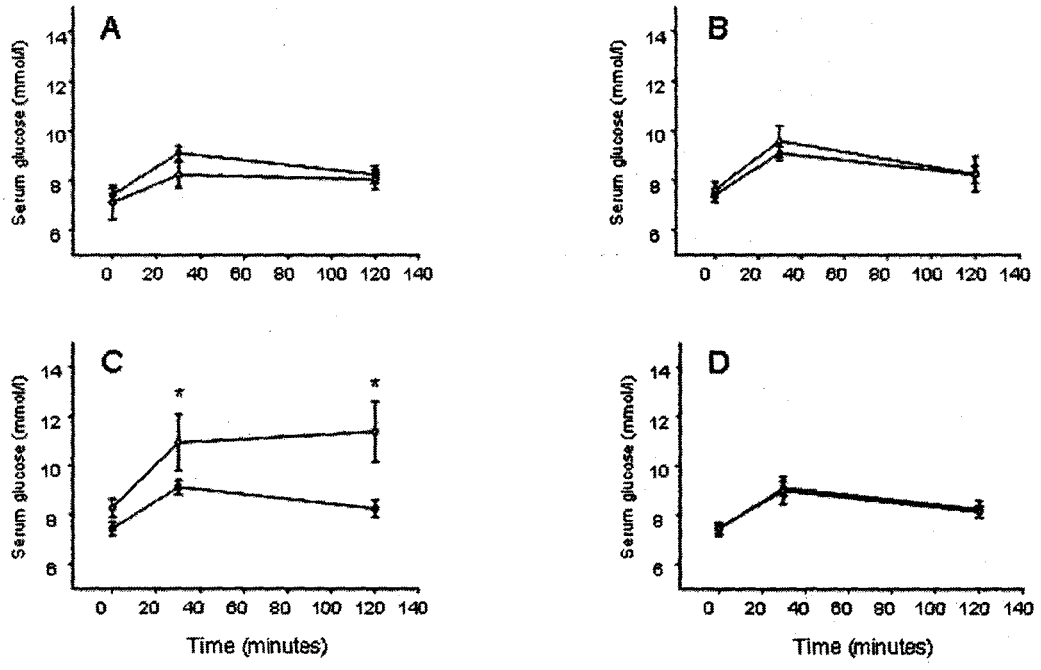


Figure 2.1: Oral glucose tolerance tests.
Serum glucose concentrations (mmol/l) following administration of an oral glucose load (2g/kg body weight) at 26 weeks of age for the offspring of female Wistar rats given saline (control: closed circles), or nicotine (1mg·kg-1·d-1, open circles) A) prior to 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 ± SEM. Values with an asterisk are significantly ($p<0.05$) different from saline controls.

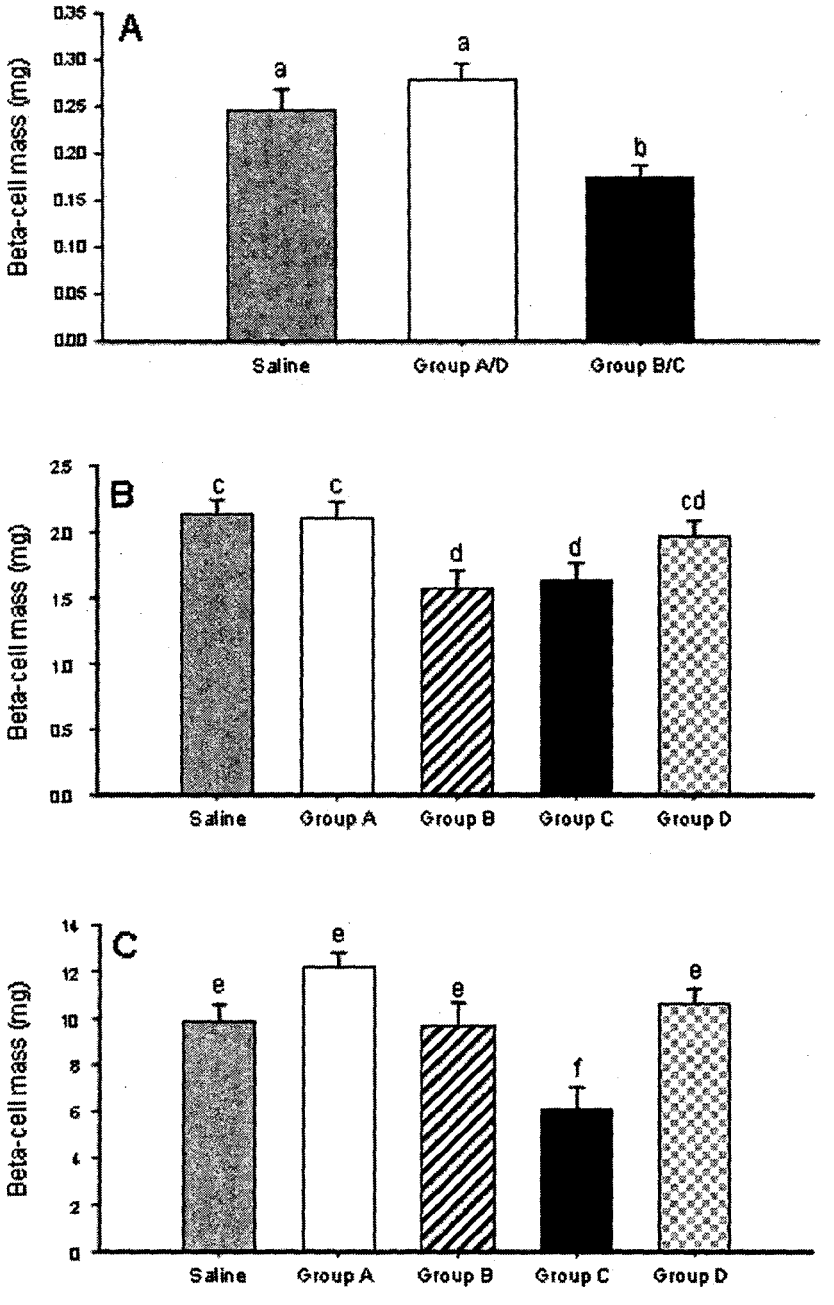


Figure 2.2: Beta cell mass.
Effect of fetal and neonatal exposure to nicotine bitartrate (1mg · kg-1 · d-1) on pancreatic beta cell mass (mg) of rats at: A) PND1; B) 4 weeks of age and C) 26 weeks of age. Beta cell mass was calculated using N=5 sections per animal, and N=5 animals per treatment group. Data are presented as mean ± SEM. Values with an asterisk are significantly ($p<0.05$) different from saline controls.

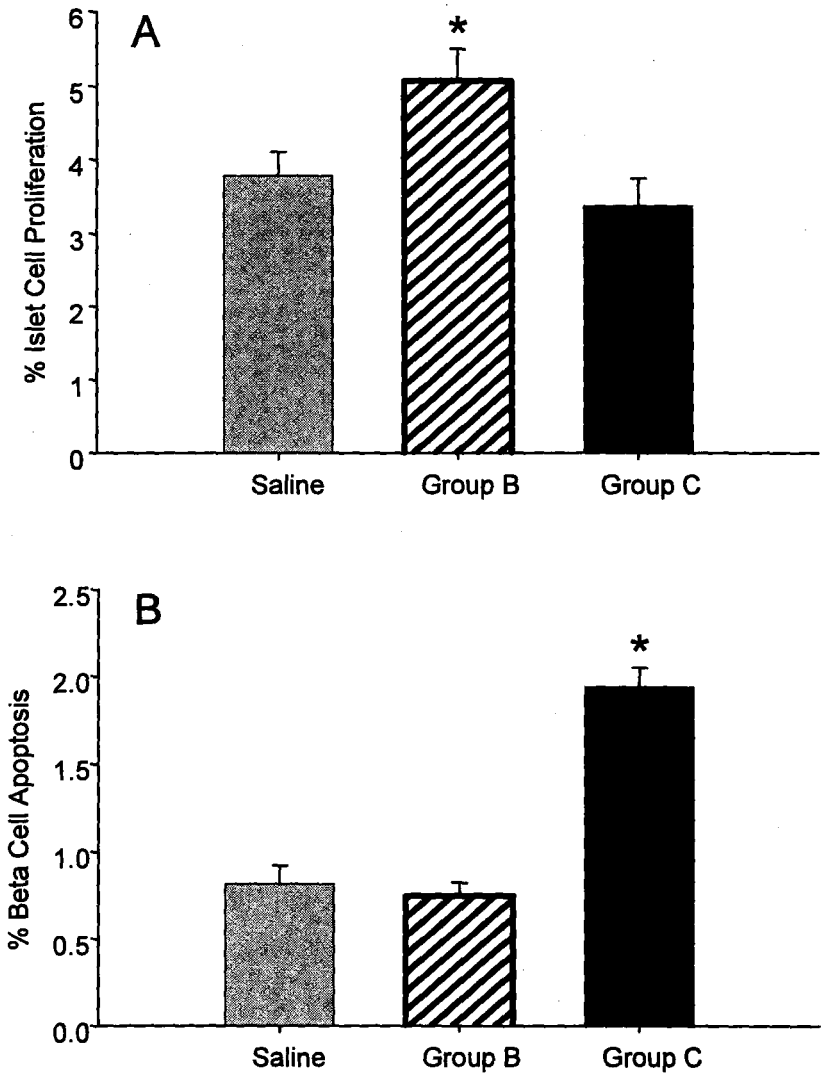


Figure 2.3: Islet cell proliferation and beta cell apoptosis.
Effect of fetal exposure only (Group B) and fetal and lactational exposure (Group C) to nicotine bitartrate (1mg · kg-1 · d-1) on: A) percent islet cell proliferation (PCNA+ islet cells) and B) percent beta cell apoptosis (TUNEL+ beta cells) 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 ± SEM. Values with an asterisk are significantly ($p<0.01$) different from saline controls.

Table 2.1: Maternal outcome and birth phenotype.

Treatment	Maternal food Consumption (g food/100g body weight)	Maternal weight gain during pregnancy (g)	Birthweight (g)	Litter size
Saline	7.9 ± 0.10	138.4 ± 7.30	6.0 ± 0.06	13.8 ± 0.97
Nicotine - A/D	7.9 ± 0.14	160.6 ± 12.66	5.9 ± 0.04	15.1 ± 0.88
Nicotine - B/C	8.1 ± 0.24	130.4 ± 10.05	6.2 ± 0.09	12.0 ± 1.07

Values are presented as mean ± SEM. 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 2.2: Effect of developmental exposure to nicotine in Wistar rats

Treatment	Fasting glucose (mmol/l)	Fasting insulin (ng/ml)	Insulin:Glucose	AUC Insulin
Saline	7.4 ± 0.27	1.9 ± 0.68	0.27 ± 0.063	221.8 ± 27.23
Nicotine - A	7.1 ± 0.68	2.0 ± 0.54	0.24 ± 0.050	300.2 ± 50.02
Nicotine - B	7.6 ± 0.30	1.4 ± 0.24	0.19 ± 0.030	250.2 ± 38.70
Nicotine - C	8.3 ± 0.36	1.8 ± 0.18	0.26 ± 0.014	342.2 ± 40.32*
Nicotine - D	7.4 ± 0.19	1.5 ± 0.31	0.21 ± 0.048	255.9 ± 31.88

Values are presented as mean ± SEM. Values with an asterisk are significantly different from the saline controls ($p<0.05$)

Table 2.3: Effect of developmental exposure to nicotine on beta cell mass in Wistar rats

Age	Treatment	Beta Cell Area (%)	Pancreas Mass (mg)	Beta Cell Mass (mg)
PND 1	Saline	2.0 ± 0.18	12.0 ± 0.90	0.24 ± 0.023
	Nicotine - A/D	2.0 ± 0.12	11.9 ± 0.11	0.28 ± 0.017
	Nicotine - 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 - A	0.6 ± 0.04	327 ± 22.0	2.09 ± 0.14
	Nicotine - B	0.4 ± 0.04*	347 ± 27.9	1.57 ± 0.14*
	Nicotine - C	0.5 ± 0.04*	343 ± 12.0	1.62 ± 0.14*
	Nicotine - 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 - A	1.3 ± 0.06	928 ± 44.0	12.17 ± 0.64
	Nicotine - B	0.90 ± 0.05	1072 ± 64.9	9.66 ± 1.02
	Nicotine - C	0.64 ± 0.03*	946 ± 118.0	6.09 ± 0.95*
	Nicotine - D	1.2 ± 0.07	964 ± 34.1	10.65 ± 0.61

Values are presented as mean ± SEM. 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 ($p<0.05$).

CHAPTER 3

INCREASED PANCREATIC BETA CELL APOPTOSIS FOLLOWING FETAL AND NEONATAL EXPOSURE TO NICOTINE IS MEDIATED VIA THE MITOCHONDRIA

BRUIN JE, GERSTEIN HC, MORRISON KM AND HOLLOWAY AC. INCREASED PANCREATIC BETA CELL APOPTOSIS FOLLOWING FETAL AND NEONATAL EXPOSURE TO NICOTINE IS MEDIATED VIA THE MITOCHONDRIA. *TOXICOLOGICAL SCIENCES*. 2008 JUN;103(2):362-70. EPUB 2008 JAN 17.

3.1 INTRODUCTION

Although cigarette smoking is associated with numerous adverse obstetrical and fetal outcomes (1-6), approximately 15-20% of all women smoke during pregnancy (1;7). Furthermore, recent epidemiologic studies have demonstrated a relationship between maternal smoking and the subsequent development of obesity, hypertension and type 2 diabetes in adult offspring (7-13). Our laboratory has previously shown in a rat model that maternal exposure to nicotine, the major addictive component of cigarettes, during pregnancy and lactation results in development of obesity and impaired glucose homeostasis in adult offspring (14;15). Furthermore, this fetal and neonatal nicotine exposure resulted in elevated pancreatic beta cell apoptosis and loss of beta cell mass at weaning, which persisted into adulthood (15). These results may partially explain the increased risk of type 2 diabetes in children born to women who smoked during pregnancy (11). However, the cellular pathway(s) which are involved in

nicotine-induced beta cell toxicity during fetal and neonatal development have not yet been identified.

There are two major signaling pathways of programmed cell death, the mitochondrial pathway (intrinsic) and the death receptor pathway (extrinsic) (Figure 3.1). In the mitochondrial pathway (Figure 3.1), pro-apoptotic members of the Bcl-2 family (Bax, Bak or Bid) translocate to the mitochondrial outer membrane, and are involved in the formation of a mitochondrial permeability transition pore (mtPTP) (16-22). Opening of the mtPTP destroys the mitochondrial membrane potential, causing ion equilibration, mitochondrial swelling and release of proteins, including cytochrome *c*, from the intermembrane space into the cytosol (16). Cytochrome *c* release leads to formation of an apoptosome, which in turn activates caspase-3 and ultimately induces cell apoptosis (16;19;22). Anti-apoptotic members of the Bcl2 family (Bcl-2 and Bcl-XL) sequester Bax, Bak and/or Bid in the cytosol, thus preventing translocation of these pro-apoptotic signaling molecules to the mtPTP and inhibiting apoptosis.

The death receptor pathway involves the binding of a death receptor (e.g., Fas) to a ligand (e.g., FasL), which results in activation of a caspase-signaling cascade to induce cell death (16;20;21;23). In particular, the Fas/FasL interaction leads to oligomerization of FasL, recruitment of FADD (Fas-associated death domain) protein and pro-caspase-8 to the cytoplasmic death domain of Fas, where a death-inducing signal complex (DISC) is formed (16;23). DISC

formation leads to the activation of caspase-8, which in turn causes cleavage of pro-caspase-3 to its active form (16;23). Alternatively, active caspase-8 can induce cleavage of Bid to tBid, which will then translocate to the mitochondrial outer membrane, resulting in release of mitochondrial proteins and amplification of the Fas/FasL apoptosis signal (16;23). The goal of the current study was to determine whether the beta cell apoptosis observed in this animal model following fetal and neonatal nicotine exposure is mediated via the mitochondrial and/or death receptor pathway.

3.2 MATERIALS AND METHODS

3.2.1 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 200-250g female Wistar rats (Harlan, Indianapolis, IN) were maintained under controlled lighting (12:12 L:D) and temperature (22°C) with *ad libitum* access to food and water. Dams were randomly assigned (n=10 per group) to receive saline (vehicle) or nicotine bitartrate (1mg/kg/d, Sigma-Aldrich, St. Louis MO) via subcutaneous injection daily for 2 weeks prior to mating until weaning. At postnatal day 1 (PND1) litters were culled to eight 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, male offspring (n=5

per treatment group) were selected randomly for the experiments described below. Immediately following sacrifice, pancreas tissue was excised and either frozen in liquid nitrogen for western blotting analysis, fixed in 10% neutral buffered formalin for immunohistochemistry, or fixed in 2% glutaraldehyde, 0.1M cacodylate buffer (pH 7.4) for electron microscopy experiments as described below.

3.2.2 Western Blotting

Protein expression was measured in either whole pancreas homogenates (n=5 per group) or mitochondria/cytosol fractions (n=5 per group) from pancreas of nicotine and saline-exposed offspring. Protein was extracted from whole frozen pancreas using RIPA lysis buffer (15 mM Tris–HCl, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, 167 mM NaCl, 0.5% (w/v) sodium deoxycholatic acid), with Complete Mini EDTA-free protease inhibitors (Roche Applied Science, Laval, PQ). To separate the mitochondrial and cytosolic fractions, the Compartmental Protein Extraction Kit (K3013010; Biochain Institute Inc., Hayward, CA) was used according to manufacturer's instructions. For Western blots of whole pancreas homogenates, 30 µg of protein was loaded; for Western blots of the mitochondrial/cytosolic fractions, 20µg of protein was loaded. Protein was subjected to SDS-PAGE and then electro-transferred to PVDF blotting membrane (BioRad Laboratories, Hercules, CA). Membranes were blocked overnight with 5 % (wt/vol) skim milk in TTBS at 4°C and then incubated for 1 h at room

temperature in primary antibody on a rocking platform. The membrane was cut horizontally; one half was incubated with the antibody for the protein of interest and the other half with a loading control antibody (α-tubulin or β-actin, depending on which molecular weight was compatible with the primary protein of interest). The following antibodies were used for this study (all rabbit polyclonal except Bcl2): Fas (1:1000; 50 kDa; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), FasL (1:1000; antibody reacts with both the membrane bound form [40 kDa] and the soluble form [26 kDa]; Santa Cruz), Caspase-8 (1:12,000; antibody reacts with both the inactive form [55kDa] and the active form [17 kDa]; AbCam, Cambridge, MA), Bax (1:1000; 23 kDa; Santa Cruz), Bcl2 (1:1000; mouse monoclonal; 26 kDa; Santa Cruz), Cytochrome *c* (1:1500; 11 kDa; Santa Cruz), Caspase-3 (1:2000; antibody reacts with both the inactive form [35 kDa] and the active form [17 kDa]; Santa Cruz), β-Actin (1:2000; 43 kDa; AbCam) and α-Tubulin (1:2000; 55 kDa; AbCam). After washing with TTBS (TBS, 0.5% (v/v) Tween 20), blots were incubated with peroxidase-conjugated secondary anti-rabbit (1:2000; Santa Cruz) or anti-mouse (1:2000; Amersham Biosciences, Piscataway, NJ) antibodies for 1 h at room temperature on a rocking platform. Blots were washed thoroughly in TTBS, followed by TBS after immunoblotting. Reactive protein was detected with ECL Plus chemiluminescence (Amersham Biosciences) and Bioflex X-ray film (Clonex Corporation, Markham, ON). Densitometric analysis of immunoblots was performed using ImageJ 1.37v software (National Institutes of Health, Bethesda, MD); all proteins were

quantified relative to the loading control. For antibodies that recognized two forms of the protein at different molecular weights (i.e., caspase-3, caspase-8 and FasL), both bands were quantified from the same sample relative to the loading control from that lane.

3.2.3 Immunohistochemistry

To determine the cellular localization of the active apoptotic pathways, immunohistochemical staining for active caspase-3 was performed in pancreas sections of nicotine and saline-exposed offspring. Active caspase-3 was selected because it is the final executioner caspase common to both the extrinsic and intrinsic pathways of programmed cell death. The pancreas from each animal (n=5 per group) was fixed by immersion in 10% (v/v) neutral buffered formalin (EM Science, Gibbstown, NJ) at 4° C overnight, washed in water and embedded in paraffin. Tissue sections (5μm) 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) at 37°C and blocking with 10% (v/v) normal goat serum and 1% (w/v) BSA at room temperature. Sections were then incubated with the primary antibody, a polyclonal rabbit anti-active caspase-3 antibody (1:10 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. Sections were washed in PBS, and immunostaining was identified using the Vectastain kit (Vector Laboratories, Burlinghame, CA) with diaminobenzadine as the chromogen. Tissue sections

were counterstained with Harris's hematoxylin, destained with acid alcohol, dehydrated and mounted with Permount (Fisher Scientific, Fair Lawn, NJ). Negative control sections were incubated with 1% (w/v) BSA in PBS in place of the primary antibody.

3.2.4 Electron Microscopy

Pancreas tissue from saline and nicotine-exposed offspring (n=5 per group) were cut into small pieces and immersed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 4˚C for 1-3 days. Samples were then washed with 0.2 M sodium cacodylate buffer (pH 7.4), fixed for 1 hour in 1% OsO4 in 0.1M sodium cacodylate buffer (pH 7.4) at room temperature, dehydrated in an ethanol series followed by 100% propylene oxide (PO). Infiltration was performed at room temperature by immersion of tissues in 50% epon-araldite / 50% PO for 30 min, followed by 75% epon-araldite / 25% PO for 30 min and 2 x 60 min in 100% epon-araldite. Tissues were embedded in 100% epon-araldite and polymerized overnight at 65˚C. All chemicals used for electron microscopy were purchased from Canemco Inc., Montreal, PQ, unless otherwise stated. Thick sections (approximately 1µm) were cut on an Ultracut E ultramicrotome (Leica Microsystems, Wetzlar, Germany), stained with toluidine blue and examined under a light microscope to ensure the presence of islets. Thin sections (approximately 70nm) were then cut from areas of the tissue containing islets, mounted on a Cu/Pd grid (200 mesh), and stained with

saturated uranyl acetate and lead citrate. Grids were examined with a JEOL 1200EX transmission electron microscope (JEOL Ltd., Tokyo, Japan) and representative photographs were taken. All photographs were analysed using Image Pro Plus Version 5.1 software (Media Cybernetics, Inc., Silver Spring, MD). Beta cells were identified within the pancreas sections by the presence of insulin granules. The number of mitochondria was calculated relative to the area of a beta cell in at least four different cells per animal. To assess mitochondrial swelling, the average mitochondrial area and optical intensity were determined by manually circling a minimum of 140 mitochondria within the beta cells of each animal. Higher optical intensity values (a measure of brightness) represent increased empty, white spaces and less tightly formed cristae within a mitochondrion, which is an indication of organelle swelling.

3.2.5 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 ($\alpha = 0.05$). Where data failed normality or equal variance test, data were reanalyzed using Mann-Whitney rank sum test.

3.3 RESULTS

3.3.1 Mitochondrial-Mediated Apoptosis Pathway

To examine the mitochondrial-mediated pathway of apoptosis, protein expression of total Bcl2, Bax, and caspase-3 were measured in whole pancreas homogenates from saline- and nicotine-exposed offspring. In addition, the mitochondrial and cytosolic fractions were isolated from pancreas samples for measurement of Bax translocation to the mitochondrial membrane and cytochrome *c* release from the mitochondria into the cytosol. Nicotine-exposed offspring had decreased Bcl-2 expression ($p<0.05$; Figure 3.2B), but no change in Bax expression ($p>0.05$; Figure 3.2A) relative to saline controls. This resulted in a 2.7-fold increase in the ratio of Bax (pro-apoptotic) to Bcl2 (anti-apoptotic) compared to control animals ($p<0.05$; Figure 3.2C). Although there was no change in total Bax, the ratio of Bax expression in the cytosolic fraction relative to the mitochondrial fraction was reduced ($p<0.05$) following nicotine exposure (Figure 3.3A) suggesting increased translocation of Bax from the cytosol to the mitochondria. Additionally, nicotine-exposed offspring had an increase ($p<0.05$) in the ratio of cytochrome *c* expression in the cytosolic fraction relative to the mitochondrial fraction compared to saline-exposed animals (Figure 3.3B), implying that nicotine exposure increases cytochrome *c* release from the mitochondria into the cytosol. Finally, caspase-3, the last protein activated in the apoptosis signaling cascade, was examined. There was no change in the inactive 35kDa form of caspase-3 (Figure 3.4A), but an increase in the 17kDa

active form of the protein (Figure 3.4B), which translated into a 3.3-fold increase ($p<0.05$) in the ratio of active to inactive caspase-3 protein in nicotine-exposed relative to saline-exposed offspring (Figure 3.4C).

3.3.2 Death Receptor-Mediated Apoptosis Pathway

To examine the death-receptor pathway of apoptosis, protein expression of the Fas receptor (Fas), Fas ligand (FasL) and caspase-8 were measured in whole pancreas homogenates from saline and nicotine-exposed offspring. Nicotine exposure caused a significant ($p<0.05$) upregulation of Fas and the soluble form of FasL (Figure 3.5A and B) relative to saline exposure, but no change in expression of membrane-bound FasL (Figure 3.5B). There were no significant differences in expression of either the inactive or active forms of caspase-8 (Figure 3.6A), or the ratio of inactive to active caspase-8 (Figure 3.6B).

3.3.3 Immunohistochemical Localization of Active Caspase-3

Immunohistochemical staining of pancreas sections from saline and nicotine-exposed offspring revealed that all active caspase-3 protein was localized within the islet cells; there was no immunopositive staining in the acinar tissue of these sections (Figure 3.7).

3.3.4 Electron Microscopy

Electron microscopy photographs of pancreatic beta cells from saline and nicotine-exposed offspring (Figure 3.8A and B, respectively) were examined to assess changes in mitochondrial number and morphology. The average optical intensity of mitochondria in nicotine-exposed beta cells was increased by approximately 11% compared to saline-exposed mitochondria ($p < 0.05$; Figure 3.8E). There was no difference in the number of mitochondria per beta cell area ($p = 0.332$; Figure 3.8C) or the average mitochondrion area within beta cells of saline and nicotine-exposed animals ($p = 0.160$; Figure 8D).

3.4 DISCUSSION

Humans with type 2 diabetes are characterized by the inability to produce a sufficient amount of insulin to compensate for peripheral insulin resistance (24-26). This insulin insufficiency is observed prior to the diagnosis of type 2 diabetes (25) and is attributed to a reduction in beta cell mass (26;27). Indeed, recent studies have demonstrated a 40-60% reduction in beta cell mass from human patients with type 2 diabetes prior to diagnosis compared to weight-matched controls (24;28;29). Similarly, in our animal model, fetal and neonatal exposure to nicotine results in permanent loss of beta cell mass, a defect that precedes the onset of glucose intolerance (15). The nicotine-induced reduction in beta cell mass in this rodent model has been attributed to elevated levels of beta cell apoptosis and an impaired capacity for islet cell proliferation (15).

However, the specific apoptotic pathways responsible for this nicotine toxicity were unknown. Results from the current study suggest that exposure to nicotine during fetal and neonatal development triggers apoptotic signaling in pancreatic beta cells via the mitochondrial-mediated pathway.

For this study, markers of both major apoptotic signaling cascades, the death receptor and mitochondrial-mediated pathways, were examined. Although nicotine exposure resulted in some alterations to the death receptor-mediated pathway (increased Fas and soluble FasL), apoptosis appears to be mediated primarily through the mitochondrial pathway. Bcl2, which normally sequesters Bax in the cytosol (16), was significantly reduced in the pancreas following nicotine exposure, thus explaining the observed increase in translocation of Bax to the mitochondria. The presence of Bax on the mitochondrial outer membrane would trigger mtPTP opening, mitochondrial swelling and release of proteins such as cytochrome *c* into the cell cytosol (16). Indeed, mitochondrial swelling was confirmed by the increased mitochondrial optical density in beta cells of nicotine-exposed offspring observed with electron microscopy. Furthermore, this swelling resulted in increased release of cytochrome *c* from the mitochondria and triggered activation of caspase-3 in nicotine-exposed offspring. The apoptotic cysteine protease, caspase-3, is normally expressed as a 32 kDa precursor, but following apoptosome formation by cytochrome *c*, the protein is cleaved, first into p20 and p12 fragments, and then the p20 subunit is further proteolyzed to form the mature 17 kDa fragment (30). The active form of caspase-3 is classified as

an "executioner" caspase, and is responsible for the majority of cellular apoptotic events (21). For this reason, active caspase-3 was selected as a representative protein for localization of apoptotic signaling within the pancreas. Immunopositive staining of the active "executioner" caspase was restricted to pancreatic islets, indicating that the observed activation of the mitochondrial-mediated apoptotic pathway was specific to endocrine cells. Furthermore, the nicotine-induced mitochondrial swelling observed by electron microscopy was only quantified in insulin-secreting beta cells. Therefore, it is likely that the changes to the mitochondrial-mediated apoptosis markers observed by Western blotting in the whole pancreas homogenates are localized to the pancreatic islets.

Activation of caspase-3 can also be triggered by binding of Fas to its ligand FasL, via the death receptor pathway. This interaction triggers either caspase-8-mediated cleavage of pro-caspase-3 or DISC-mediated translocation of tBid to the mitochondria, leading to release of mitochondrial proteins (16;23). Results from the current study showed an upregulation of Fas protein, as well as the soluble form of FasL in the pancreas, but no change in activation of caspase-8. Without caspase-8 activation, downstream signaling events in the death receptor cascade such as cleavage of pro-caspase-3 to its active form, and subsequent apoptosis do not occur. Furthermore, induction of soluble FasL may not have a significant impact on apoptotic signaling, since soluble FasL has reduced biological activity compared to the membrane-bound form of the ligand (31). These data suggest that the death receptor pathway is not activated in the

pancreas following developmental nicotine exposure. Instead, this study indicates that nicotine-induced beta cell toxicity in this animal model is mediated by the mitochondria. Similarly, beta cell apoptosis in response to high glucose levels has also been shown to be mediated via the mitochondrial apoptosis pathway (32). Indeed, glucose stimulation resulted in alterations to the mitochondrial apoptotic pathway in beta cells that are similar to those seen in this study: namely an increased Bax/Bcl2 ratio, Bax translocation, cytochrome *c* release and caspase-3 activation (32).

Taken together, this study overwhelmingly points to the mitochondria as the principal mediator of beta cell apoptosis resulting from fetal and neonatal exposure to nicotine. However, mitochondria are involved not only in regulation of apoptosis in beta cells, but are also central to maintenance of beta cell function (33-35). Since we have demonstrated that mitochondria are affected by nicotine exposure, results from this study may have further consequences for beta cell function. Transgenic mice with beta cell-specific mitochondrial defects have decreased glucose-stimulated insulin release (i.e. impaired beta cell function) (36;37), suggesting that mitochondrial dysfunction can lead to development of type 2 diabetes. Indeed, both human and animal studies have observed impaired mitochondrial function in pancreatic islets of subjects with type 2 diabetes (38;39). Therefore, the effect of fetal and neonatal nicotine exposure on mitochondria may have implications beyond determining the early fate of beta cells and may in fact be the underlying cause of dysglycaemia in nicotine-

exposed adult offspring. These results suggest a mechanism by which fetal and neonatal exposure to nicotine, delivered through maternal smoking or nicotine replacement therapy, may result in postnatal glucometabolic abnormalities and suggests that the long-term postnatal health consequences of nicotine exposure warrants further investigation.

3.5 REFERENCES

1. Andres,RL, Day,MC: Perinatal complications associated with maternal tobacco use. *Semin Neonatol* 5:231-241, 2000

2. Cnattingius,S, Lambe,M: Trends in smoking and overweight during pregnancy: prevalence, risks of pregnancy complications, and adverse pregnancy outcomes. *Semin Perinatol* 26:286-295, 2002

3. Faiz,AS, Ananth,CV: Etiology and risk factors for placenta previa: an overview and meta-analysis of observational studies. *J Matern Fetal Neonatal Med* 13:175-190, 2003

4. Nordentoft,M, Lou,HC, Hansen,D, Nim,J, Pryds,O, Rubin,P, Hemmingsen,R: Intrauterine growth retardation and premature delivery: the influence of maternal smoking and psychosocial factors. *Am J Public Health* 86:347-354, 1996

5. Robinson,JS, Moore,VM, Owens,JA, McMillen,IC: Origins of fetal growth restriction. *Eur J Obstet Gynecol Reprod Biol* 92:13-19, 2000

6. Shiverick,KT, Salafia,C: Cigarette smoking and pregnancy I: ovarian, uterine and placental effects. *Placenta* 20:265-272, 1999

7. Bergmann,KE, Bergmann,RL, Von,KR, Bohm,O, Richter,R, Dudenhausen,JW, Wahn,U: Early determinants of childhood overweight and adiposity in a birth cohort study: role of breast-feeding. *Int J Obes Relat Metab Disord* 27:162-172, 2003

8. Wideroe,M, Vik,T, Jacobsen,G, Bakketeig,LS: Does maternal smoking during pregnancy cause childhood overweight? *Paediatr Perinat Epidemiol* 17:171-179, 2003

9. Toschke,AM, Koletzko,B, Slikker,W, Jr., Hermann,M, Von,KR: Childhood obesity is associated with maternal smoking in pregnancy. *Eur J Pediatr* 161:445-448, 2002

10. Power,C, Jefferis,BJ: Fetal environment and subsequent obesity: a study of maternal smoking. *Int J Epidemiol* 31:413-419, 2002

11. Montgomery,SM, Ekbom,A: Smoking during pregnancy and diabetes mellitus in a British longitudinal birth cohort. *BMJ* 324:26-27, 2002

12. Von,KR, Toschke,AM, Koletzko,B, Slikker,W, Jr.: Maternal smoking during pregnancy and childhood obesity. *Am J Epidemiol* 156:954-961, 2002

13. Vik,T, Jacobsen,G, Vatten,L, Bakketeig,LS: Pre- and post-natal growth in children of women who smoked in pregnancy. *Early Hum Dev* 45:245-255, 1996

14. Holloway,AC, Lim,GE, Petrik,JJ, Foster,WG, Morrison,KM, Gerstein,HC: Fetal and neonatal exposure to nicotine in Wistar rats results in increased beta cell apoptosis at birth and postnatal endocrine and metabolic changes associated with type 2 diabetes. *Diabetologia* 48:2661-2666, 2005

15. Bruin,JE, Kellenberger,LD, Gerstein,HC, Morrison,KM, Holloway,AC: Fetal and neonatal nicotine exposure and postnatal glucose homeostasis: Identifying critical windows of exposure. *J Endocrinol* 194:171-178, 2007

16. Wallace,DC: A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. *Annu Rev Genet* 39:359-407, 2005

17. Ryter,SW, Kim,HP, Hoetzel,A, Park,JW, Nakahira,K, Wang,X, Choi,AM: Mechanisms of cell death in oxidative stress. *Antioxid Redox Signal* 9:49-89, 2007

18. Green,DR, Kroemer,G: The pathophysiology of mitochondrial cell death. *Science* 305:626-629, 2004

19. Gulbins,E, Dreschers,S, Bock,J: Role of mitochondria in apoptosis. *Exp Physiol* 88:85-90, 2003

20. Gupta,S: Molecular signaling in death receptor and mitochondrial pathways of apoptosis (Review). *Int J Oncol* 22:15-20, 2003

21. Zimmermann,KC, Bonzon,C, Green,DR: The machinery of programmed cell death. *Pharmacol Ther* 92:57-70, 2001

22. Jiang,X, Wang,X: Cytochrome C-mediated apoptosis. *Annu Rev Biochem* 73:87-106, 2004

23. Timmer,T, de Vries,EG, de,JS: Fas receptor-mediated apoptosis: a clinical application? *J Pathol* 196:125-134, 2002

24. Butler,AE, Janson,J, Bonner-Weir,S, Ritzel,R, Rizza,RA, Butler,PC: Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. *Diabetes* 52:102-110, 2003

25. Kahn,SE: The relative contributions of insulin resistance and beta-cell dysfunction to the pathophysiology of Type 2 diabetes. *Diabetologia* 46:3-19, 2003

26. Leahy,JL: Pathogenesis of type 2 diabetes mellitus. *Arch Med Res* 36:197-209, 2005

27. Rhodes,CJ: Type 2 diabetes-a matter of beta-cell life and death? *Science* 307:380-384, 2005

28. Sakuraba,H, Mizukami,H, Yagihashi,N, Wada,R, Hanyu,C, Yagihashi,S: Reduced beta-cell mass and expression of oxidative stress-related DNA damage in the islet of Japanese Type II diabetic patients. *Diabetologia* 45:85-96, 2002

29. Yoon,KH, Ko,SH, Cho,JH, Lee,JM, Ahn,YB, Song,KH, Yoo,SJ, Kang,MI, Cha,BY, Lee,KW, Son,HY, Kang,SK, Kim,HS, Lee,IK, Bonner-Weir,S: Selective beta-cell loss and alpha-cell expansion in patients with type 2 diabetes mellitus in Korea. *J Clin Endocrinol Metab* 88:2300-2308, 2003

30. Han,Z, Hendrickson,EA, Bremner,TA, Wyche,JH: A sequential two-step mechanism for the production of the mature p17:p12 form of caspase-3 in vitro. *J Biol Chem* 272:13432-13436, 1997

31. Date,T, Mochizuki,S, Belanger,AJ, Yamakawa,M, Luo,Z, Vincent,KA, Cheng,SH, Gregory,RJ, Jiang,C: Differential effects of membrane and soluble Fas ligand on cardiomyocytes: role in ischemia/reperfusion injury. *J Mol Cell Cardiol* 35:811-821, 2003

32. Kim,WH, Lee,JW, Suh,YH, Hong,SH, Choi,JS, Lim,JH, Song,JH, Gao,B, Jung,MH: Exposure to chronic high glucose induces beta-cell apoptosis through decreased interaction of glucokinase with mitochondria: downregulation of glucokinase in pancreatic beta-cells. *Diabetes* 54:2602-2611, 2005

33. Del Guerra,S, Lupi,R, Marselli,L, Masini,M, Bugliani,M, Sbrana,S, Torri,S, Pollera,M, Boggi,U, Mosca,F, Del Prato,S, Marchetti,P: Functional and molecular defects of pancreatic islets in human type 2 diabetes. *Diabetes* 54:727-735, 2005

34. Lowell,BB, Shulman,GI: Mitochondrial dysfunction and type 2 diabetes. *Science* 307:384-387, 2005

35. MacDonald,PE, Joseph,JW, Rorsman,P: Glucose-sensing mechanisms in pancreatic beta-cells. *Philos Trans R Soc Lond B Biol Sci* 360:2211-2225, 2005

36. Silva,JP, Kohler,M, Graff,C, Oldfors,A, Magnuson,MA, Berggren,PO, Larsson,NG: Impaired insulin secretion and beta-cell loss in tissue-specific knockout mice with mitochondrial diabetes. *Nat Genet* 26:336-340, 2000

37. Silva,JP, Larsson,NG: Manipulation of mitochondrial DNA gene expression in the mouse. *Biochim Biophys Acta* 1555:106-110, 2002

38. Anello,M, Lupi,R, Spampinato,D, Piro,S, Masini,M, Boggi,U, Del,PS, Rabuazzo,AM, Purrello,F, Marchetti,P: Functional and morphological alterations of mitochondria in pancreatic beta cells from type 2 diabetic patients. *Diabetologia* 48:282-289, 2005

39. Simmons,RA, Suponitsky-Kroyter,I, Selak,MA: Progressive accumulation of mitochondrial DNA mutations and decline in mitochondrial function lead to beta-cell failure. *J Biol Chem* 280:28785-28791, 2005

3.6 FIGURES

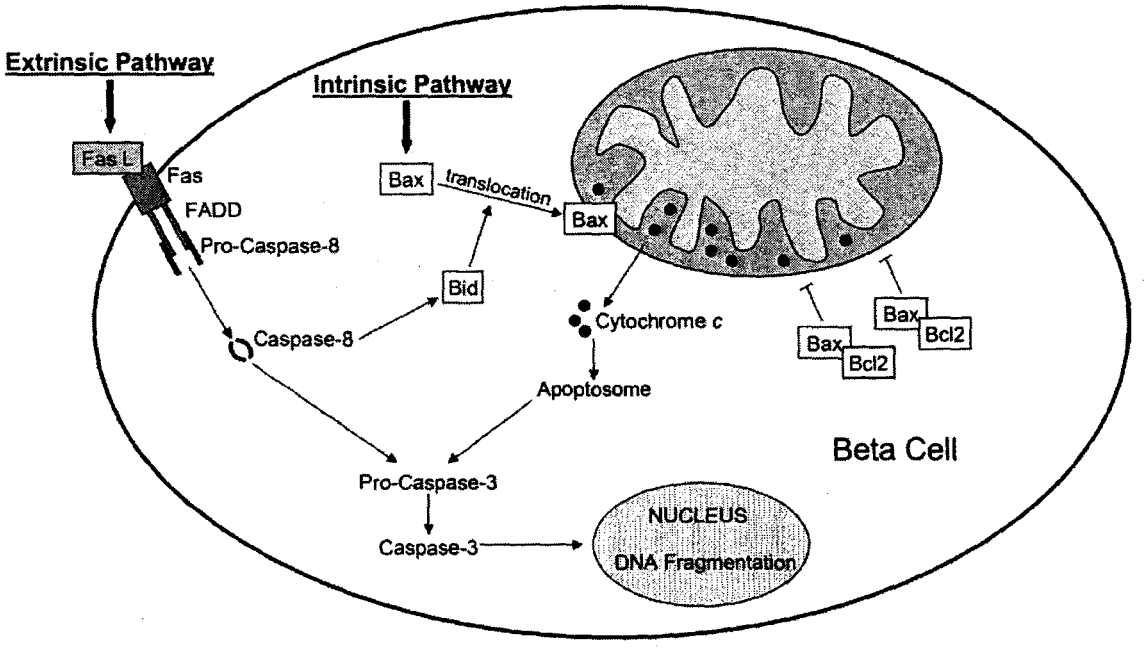


Figure 3.1: Extrinsic and intrinsic pathways of apoptosis.
The schematic diagram outlines key signaling molecules involved in the extrinsic (death receptor) and intrinsic (mitochondrial) apoptosis pathways. In the extrinsic pathway, a death-inducing ligand, such as Fas ligand (FasL), binds to its receptor (Fas), and initiates recruitment of FADD (Fas-associated death domain) and pro-caspase-8, which combine to form a death-inducing signal complex (DISC). DISC formation leads to activation of caspase-8, and can cause either activation of caspase-3 or truncation of Bid. Bid assists in the activation and translocation of Bax to the mitochondria, thus triggering the intrinsic pathway. Intrinsic apoptosis can also be triggered by environmental stress that either directly damages the mitochondria or activates Bax. Bax translocates to the mitochondrial outer membrane, where it oligomerizes and causes release of proteins such as cytochrome *c* from the mitochondria into the cell cytosol. Release of cytochrome *c* triggers the formation of an apoptosome complex with Apaf-1 and caspase-9, which then signals for activation of caspase-3 and subsequently, cellular apoptosis.

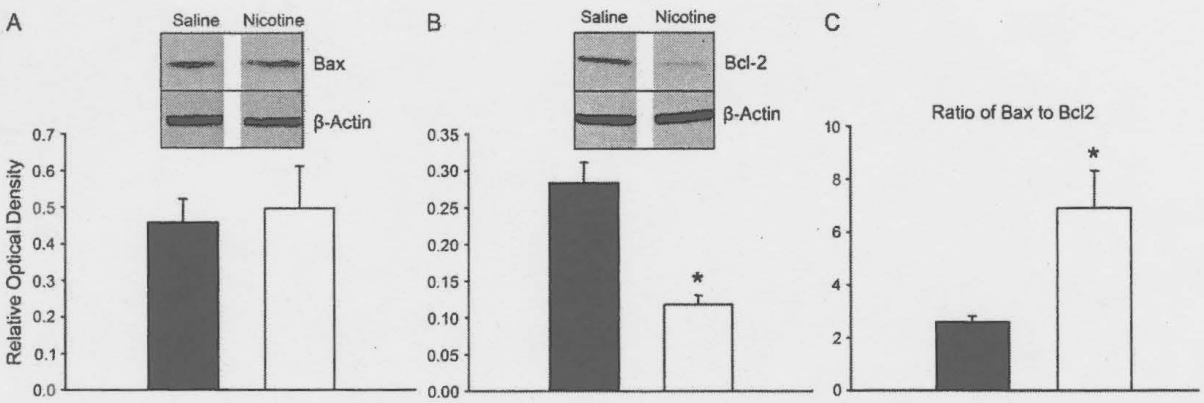


Figure 3.2: Mitochondrial-mediated apoptosis; bax and bcl2.
Protein expression for: A) Bax, B) Bcl2 and C) the Bax/Bcl2 ratio in saline (black bars) and nicotine (white bars) -exposed whole pancreas homogenates (n=4 per group) at 3 weeks of age. A representative western blot for each protein is provided. All protein expression was quantified relative to a β-actin loading control. Data are presented as the mean ± SEM. Values with an asterisk are significantly different from the saline control ($p<0.05$).

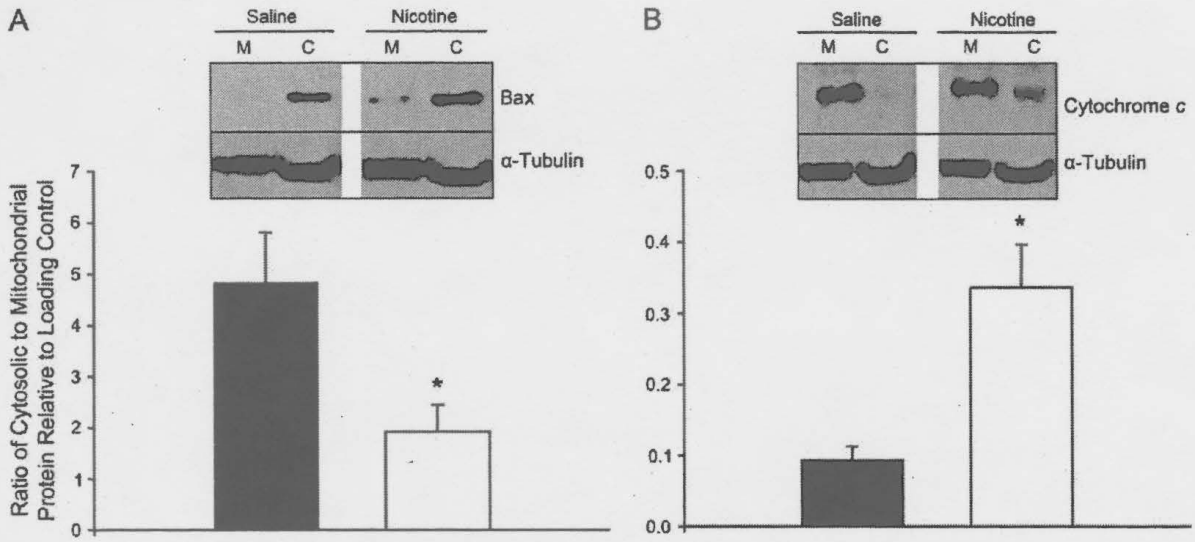


Figure 3.3: Mitochondrial-mediated apoptosis; bax translocation and cytochrome *c* release.

Protein expression in the cytosol (C) relative to the mitochondrial fraction (M) for: A) bax, and B) cytochrome *c* in saline (black bars) and nicotine (white bars) -exposed whole pancreas, fractionated homogenates (n=4 per group) at 3 weeks of age. A representative western blot for each protein is provided. All protein expression was quantified relative to a β-actin loading control. Data are presented as the mean ± SEM. Values with an asterisk are significantly different from the saline control ($p<0.05$).

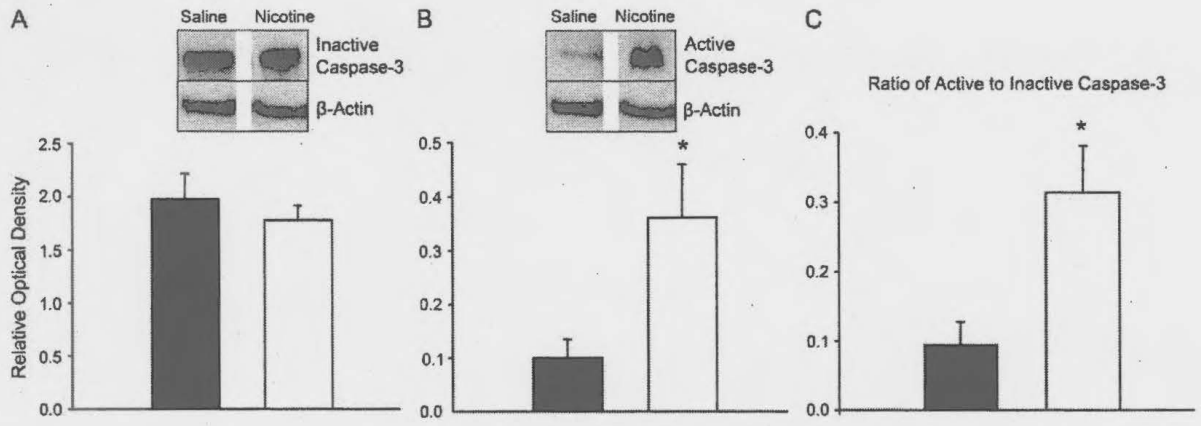


Figure 3.4: Mitochondrial-mediated apoptosis; caspase-3.
Protein expression for: A) inactive caspase-3, B) active caspase-3, and C) the ratio of active to inactive caspase-3 in saline (black bars) and nicotine (white bars) -exposed pancreas homogenates (n=5 per group) at 3 weeks of age. A representative western blot for each protein is provided. All protein expression was quantified relative to a β-actin loading control. Data are presented as mean ± SEM. Values with an asterisk are significantly different from the saline control ($p<0.05$).

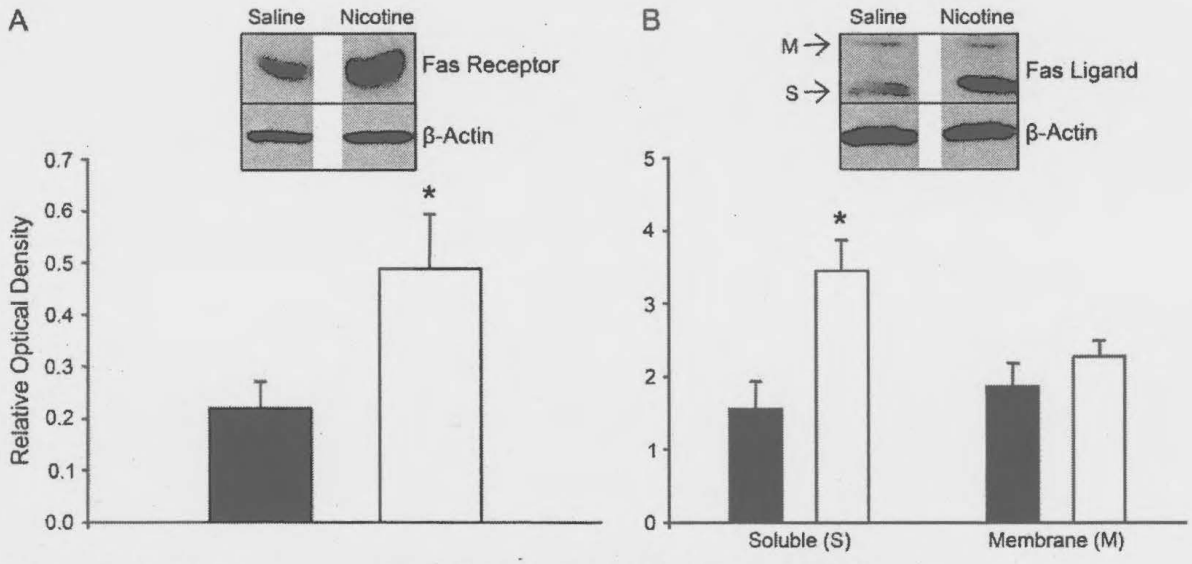


Figure 3.5: Death receptor-mediated apoptosis; fas and fasL.
Protein expression for: A) Fas, and B) FasL (membrane and soluble forms) from saline (black bars) and nicotine (white bars)-exposed pancreas (n=5 per group) at 3 weeks of age. A representative western blot for each protein is provided. All protein expression was quantified relative to a β-actin loading control. Data are presented as mean ± SEM. Values with an asterisk are significantly different from the saline control ($p<0.05$).

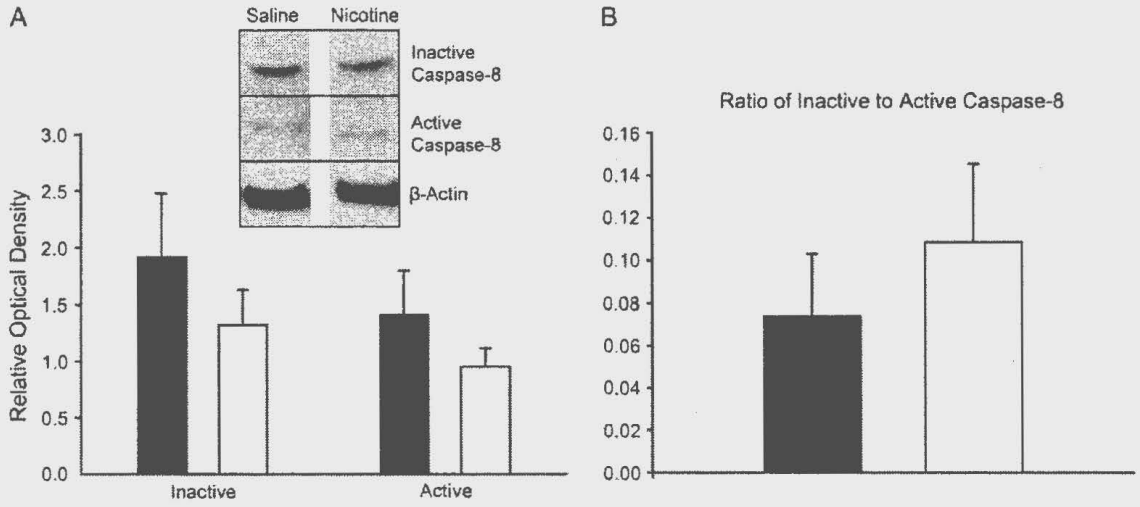


Figure 3.6: Death receptor-mediated apoptosis; caspase-8.
Protein expression for: A) inactive and active caspase-8, and B) the ratio of inactive to active caspase-8 from saline (black bars) and nicotine (white bars)-exposed pancreas (n=5 per group) at 3 weeks of age. A representative western blot for each protein is provided. All protein expression was quantified relative to a β-actin loading control. Data are presented as mean ± SEM. There were no significant differences in expression of any form of the caspase-8.

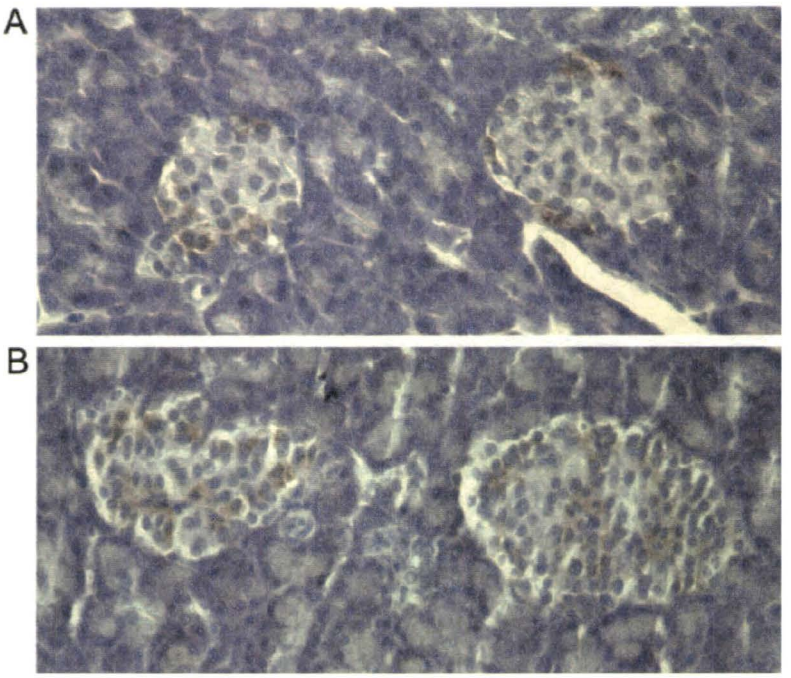


Figure 3.7: Active caspase-3 immunohistochemistry.
Representative photographs of pancreas sections containing two islet cell clusters from each of a A) saline, and B) nicotine-exposed offspring at 4 weeks of age. The brown staining represents active caspase-3 protein; all positive staining was localized within the islets.

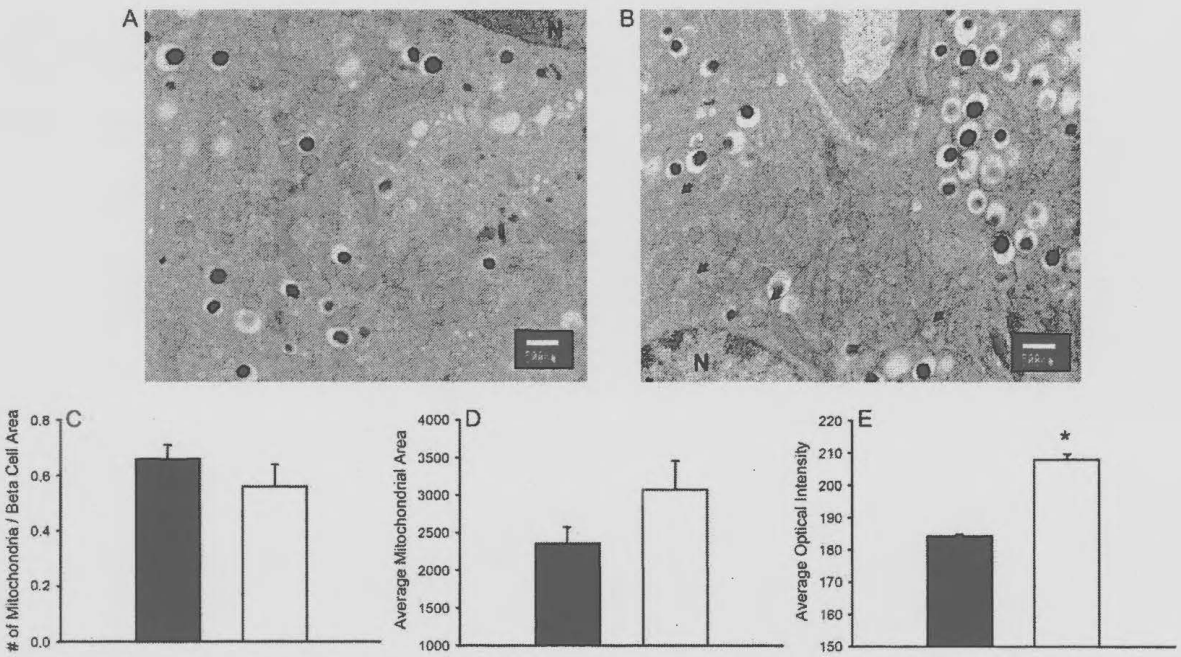


Figure 3.8: Electron microscopy.
Representative electron microscopy photographs of: A) saline (black bars) and B) nicotine (white bars) exposed pancreas (n=5 per group) at 3 weeks of age. Black arrows point to examples of mitochondrial swelling; N = nucleus. Mitochondria were quantified within the pancreatic beta cells and presented as: C) the number of mitochondria per beta cell area, D) the average mitochondrial area, and E) the average mitochondrial optical intensity. Data are presented as mean ± SEM. Values with an asterisk are significantly different from the saline control ($p<0.05$).

CHAPTER 4

MATERNAL EXPOSURE TO NICOTINE INCREASES OXIDATIVE STRESS IN THE PANCREAS OF THE OFFSPRING.

BRUIN JE, PETRE MA, LEHMAN MA, RAHA S, GERSTEIN HC, MORRISON KM AND HOLLOWAY AC. MATERNAL EXPOSURE TO NICOTINE INCREASES OXIDATIVE STRESS IN THE PANCREAS OF THE OFFSPRING. *FREE RADICAL BIOLOGY AND MEDICINE*. 2008 JUN 1;44(11):1919-25. EPUB 2008 MAR 4.

4.1 INTRODUCTION

Cigarette smoking during pregnancy is associated with a number of adverse obstetrical outcomes including placenta previa, premature rupture of the membranes, preterm birth and low birthweight (1-6). Moreover, recent epidemiologic studies have shown a strong relationship between maternal smoking and subsequent obesity, hypertension and type 2 diabetes in the offspring (7;8). We have previously shown in a rat model that maternal exposure to nicotine alone during pregnancy and lactation results in permanent loss of beta cell mass and function in the offspring (9;10). These results may partially explain the increased risk of type 2 diabetes in children born to women who smoked during pregnancy (11), but the mechanism(s) underlying this beta cell loss have not yet been identified.

It has been demonstrated that maternal smoking is associated with

increased levels of oxidative stress markers in mothers, newborns and infants (12;13). Furthermore, there is considerable evidence *in vivo* and *in vitro* to suggest that exposure to nicotine results in increased oxidative stress in fetal, neonatal and adult tissues (14-17). Indeed, in adult rats, nicotine exposure has been shown to increase oxidative stress in pancreatic tissue *in vitro* (16) and to produce oxidative tissue injuries *in vivo* (18;19). Because the pancreatic beta cell has low expression of antioxidant enzymes (20;21), it is particularly susceptible to oxidative stress-mediated tissue damage including increased beta cell death (22-27).

The current study uses an animal model of fetal and neonatal exposure to nicotine, which has previously been shown to cause increased beta cell apoptosis at birth and weaning (9;10;28). It was hypothesized that maternally-administered nicotine would activate the fetal and neonatal pancreatic nicotinic acetylcholine receptors (nAChR), to cause an imbalance in the pro-oxidative/anti-oxidative status of the beta cell (oxidative stress), thus signaling for beta cell apoptosis. The nAChR belongs to a family of neurotransmitter-gated ion channels (29) that are homo- or heteropentamers comprised of various combinations of α- and β-subunits (α2 – α10 and β2 – β4) (30). The nAChR is best characterized in the brain; however, these neuronal receptors also exist in various non-neuronal cell types (31;32), including adult pancreatic beta cells (33). It has been suggested that nicotine may affect adult pancreatic function by direct interaction with the pancreatic nAChR (33). However, expression of nAChR

subunits has not been previously examined in the developing fetal or neonatal pancreas.

Therefore, the goals of this study were: 1) to determine the pattern of nAChR subunit expression in the developing pancreas, 2) to examine whether fetal and neonatal nicotine exposure alters pro- and / or anti-oxidant markers in the pancreas, and 3) to assess whether the pancreatic and / or systemic oxidative balance has been disrupted.

4.2 MATERIALS AND METHODS

4.2.1 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 200-250g female Wistar rats (Harlan, Indianapolis, IN) were maintained under controlled lighting (12:12 L:D) and temperature (22°C) with *ad libitum* access to food and water. Dams were randomly assigned (n=10 per group) to receive saline (vehicle) or nicotine bitartrate (1mg/kg/d, Sigma-Aldrich, St. Louis MO) via subcutaneous injection daily for 2 weeks prior to mating until weaning. At postnatal day 1 (PND1) litters were culled to eight 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 postnatal day 21 (PND21), male offspring (n=10 per group) were selected randomly for the

experiments described below.

4.2.2 Reverse Transcription (RT) and Real-Time PCR

Pancreatic tissue from pups born to saline- and nicotine-exposed mothers was removed at PND1 and PND21 and immediately placed in RNA later (Sigma-Aldrich Inc., St. Louis, MO, USA) for analysis by either semi-quantitative RT-PCR or quantitative real-time PCR. The mRNA expression of nAChR subunits (α2, α3, α4, α5, α6, α7, β2, β3, and β4) was determined at birth (PND1) and weaning (PND21) by RT-PCR to assess the pattern of the receptor subunit expression during fetal and neonatal development. The mRNA expression of the anti-oxidant enzymes heme-oxygenase 1 (HO-1), glutathione peroxidase (GPx), manganese superoxide dismutase (MnSOD) and copper/zinc superoxide dismutase (CuZnSOD) was determined at PND21 by real-time PCR to compare the antioxidant response following nicotine or saline exposure. RNA was extracted using a Qiagen RNA Extraction Kit (RNeasy Mini Kit, Mississauga, ON, Canada) and treated with TURBO DNA-*free*TM DNase-1 (Ambion, Austin, TX) according to manufacturer's instructions; RNA was stored at -80°C prior to use.

RNA samples (n=6 per group) were reverse transcribed to cDNA in a 20 µL reaction mixture containing 1 µg of extracted RNA, 1 µL of random primers, 1µL of dNTPs (10 mM), 4 µL of 5x First-Strand Buffer, 1 µL of 0.1 M DTT, 1 µL of RNase OUT, 1 µL of Superscript III RT and DNase/RNase-free water (Invitrogen,

Carrisbad, CA, USA), according to the standard protocol supplied with each product. The reaction was carried out in an iCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA) using the following program: 5 minutes at 25°C, 60 minutes at 50°C, and 15 minutes at 70°C. The cDNA was stored at -20°C until use.

Reverse transcription (RT)-PCR reactions to determine the pattern of nAChR subunit expression were prepared in 50 µL reactions with: 40.1 µL of RNase/DNase free water, 5.0 µL of 10x Mg-free PCR Buffer, 1.5 µL of $MgCl_2$ (50 mM), 1.0 µL of dNTPs (10 mM), 0.4 µL of Taq DNA Polymerase (Invitrogen, Carrisbad, CA, USA), 1.0 µL template cDNA, and 1.0 µL of the primer mix (10 µM, MOBIX, McMaster University, Hamilton ON, Canada). Primer sequences for nAChR subunits are provided in Table 4.1. The cDNA was amplified in the iCycler Thermal Cycler using the following program: 2 minutes at 94°C, followed by 35 cycles of 30 seconds denaturing at 94°C, 30 seconds annealing at 55°C, 1 min elongation at 72°C, and then storage at 4°C. PCR products were separated on a 2% agarose gel and visualized using ethidium bromide (EMD, Gibbstown, NJ, USA). PCR products were imaged with the UVP Bioimaging Systems Epi Chemi II Darkroom and Labworks software (UVP Inc., Upland CA).

Real-time PCR to measure antioxidant enzyme expression in saline- and nicotine- exposed pancreas was performed using SYBR® Green chemistry (n=5 per treatment group). 25µL reactions were prepared with 10.5 µL of RNase/DNase free water, 12.5 µL of iQ SYBR Green Supermix (Biorad Laboratories, CA), 1.0µL of template cDNA, 1.0 µL of the primer mix (25 µM for

Beta Actin; 10 μM for MnSOD, CuZnSOD and GPx-1; 2 μM for HO-1; MOBIX, McMaster University, Hamilton ON, Canada). Primer sequences for antioxidant enzymes are provided in Table 4.1. The cDNA was amplified in an iCycler Thermal Cycler coupled with an iCycler IQ Multicolor real-time PCR Detection System using iCycler IQ software v3.1.7050 (Biorad Laboratories, CA). Real-time PCR was run for 1 cycle (50°C for 2 min, 95°C for 10 min) followed immediately by 40 cycles (95°C for 15 s, 60°C for 60 s), and fluorescence was measured after each of the repetitive cycles. Emission data were quantified using the threshold cycle (Ct) value. A melting point dissociation curve generated by the instrument was used to confirm that only a single product was present with each set of primers. Gene expression of each antioxidant enzyme was quantified as the average Ct value normalized to the beta actin Ct value for the same sample.

4.2.3 Western Blotting

Protein expression was measured in whole pancreas homogenates from nicotine and saline-exposed offspring at weaning (PND21). Protein was extracted from the pancreas (n=4 per group) using RIPA lysis buffer (15 mM Tris–HCl, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, 167 mM NaCl, 0.5% (w/v) sodium deoxycholatic acid), with Complete Mini EDTA-free protease inhibitors (Roche Applied Science, Laval QC, Canada). 30 μg of protein was subjected to SDS-PAGE using a 10% separating gel and then electro-transferred to PVDF

blotting membrane (BioRad Laboratories, CA). Membranes were blocked overnight with 5% (w/v) skim milk in TBST (TBS, 0.5% (v/v) Tween 20) at 4°C and then incubated for 1 h at room temperature in primary antibody on a rocking platform. The membrane was cut horizontally at approximately 40 kDa and the upper molecular weight portion was incubated in rabbit polyclonal anti-beta actin for all blots (47 kDa running weight; 1:2000 dilution, AbCam, MA). The lower molecular weight portion was incubated with primary antibodies for MnSOD (rabbit polyclonal, 1:5000, 25kDa, Santa Cruz Biotechnology, CA), CuZnSOD (rabbit polyclonal, 1:1000, 23 kDa, Santa Cruz Biotechnology, CA), GPx-1 (rabbit polyclonal, 1:5000, 22kDa, AbCam, MA) and HO-1 (mouse monoclonal, 1:4000, 35kDa, Stressgen Biotechnologies, BC, Canada). After washing with TBST, blots were incubated with peroxidase-conjugated secondary anti-rabbit (1:2000; SantaCruz, CA) or anti-mouse (1:2000; Amersham Biosciences, NJ) antibodies for 1 h at room temperature on a rocking platform. Blots were washed thoroughly in TBST followed by TBS after immunoblotting. Reactive protein was detected with ECL Plus chemiluminescence (Amersham Biosciences, NJ) and Bioflex X-ray film (Clonex Corporation, ON). Densitometric analysis of immunoblots was performed using ImageJ 1.37v software (National Institutes of Health, Bethesda, MD); all proteins were quantified relative to beta actin.

4.2.4 Reactive Oxidative Species Production by Isolated Islets

Islet isolation from rats was performed as previously described (34).

Briefly, the pancreas was immediately excised following sacrifice and placed in 6 mL of Hank's balanced salt solution (HBSS) (HyClone, Logan UT) containing 4 mg/mL collagenase type IA (Sigma-Aldrich, St. Louis, MO), 100 IU/mL penicillin G and 0.25 µg/mL streptomycin (Gibco, Grand Island, NY). The pancreas was minced finely and the resulting suspension was incubated at 37°C for 40 min. The reaction was then quenched with 20 mL HBSS supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT), 100 IU/mL penicillin G and 0.25 µg/mL streptomycin. Islets were manually picked from the suspension using a small glass pipette and a dissecting microscope. The islets were incubated at 37°C, 5% CO2 / 95% normal atmosphere in 5 mL RPMI 1640 (Life Technologies, Burlington, ON) supplemented with 10% FBS, 100 IU penicillin G and 0.25 µg/mL streptomycin for 48 hours.

ROS production by isolated islets at PND21 following saline and nicotine exposure was measured using 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Molecular Probes Inc., Eugene, OR) fluorescence as previously described (35). Briefly, 100 islets from saline- and nicotine-exposed offspring (n=3 per group) were washed twice with PBS. Following centrifugation, the supernatant was removed and the pelleted islets were then resuspended in 100 µL of PBS containing 100µM H_2DCFDA and incubated for 3 h at 37°C. Because of the relatively low number of cells in this assay, a long incubation period allows for the diffusion of the oxidized dye from inside the cell back out into the culture medium (35). This approach has been previously validated to determine ROS

production by isolated islet cells in rats (35). In addition, since H_2DCFDA must be made fresh immediately prior to use, islets isolated on different days were incubated in different batches of reagent. To account for day-to-day variability within the experiment, a 43 µM hydrogen peroxide reaction was prepared with each batch of H_2DCFDA to calibrate the performance of the dye. The hydrogen peroxide was added to 100µM H_2DCFDA and incubated in parallel with the islet reactions. Following the incubation period, the islets were vigorously disrupted to release intracellular H_2DCFDA. Both the islet suspensions and the hydrogen peroxide control were centrifuged, and the supernatants were transferred to black 96-well plates (BD Falcon, Mississauga, ON). Fluorescence of the 2',7'-dichlorofluorescein product was determined using a SpectaMax Gemini XS (Molecular Devices Corp., Sunnyvale CA) microplate spectrofluorometer at excitation and emission wavelengths of 505 nm and 540 nm, respectively. All measurements of islet ROS production were normalized to the 0.043 mM hydrogen peroxide control.

4.2.5 Protein Carbonyl Detection

To assess oxidative damage to pancreatic proteins, the presence of protein carbonyl groups was quantified using the OxyBlot™ Protein Oxidation Detection Kit (Chemicon International, Temecula CA). Formation of protein carbonyl groups was measured at PND21 in both whole pancreas homogenates (n=5 per group) and in the mitochondrial fraction of the pancreas (n=4 per group)

from saline- and nicotine-exposed offspring. Whole pancreas homogenates were prepared and quantified as described above. To separate the mitochondrial fraction, the Compartmental Protein Extraction Kit (K3013010; Biochain Institute Inc., Hayward, CA) was used according to manufacturer's instructions. Protein samples were then prepared with the OxyblotTM Kit, according to manufacturer's instructions. Briefly, equal amounts of protein (16 µg for whole pancreas, and 10 µg for mitochondrial fractions) were derivatized with either 2,4-dinitrophenylahydrazine (DNPH) or a derivatization-control solution. Gel electrophoresis, transfer to a PVDF membrane, immunoblotting conditions and detection of reactive protein were the same as above. Blots were incubated for 1 h in rabbit-DNP antibody (1:150), followed by 1 h in secondary goat anti-rabbit IgG (HRP-conjugated; 1:300). No reactive protein was detected in the derivatization-control immunoblots. Densitometric analysis of immunoblots was performed using ImageJ 1.37v software (National Institutes of Health, Bethesda, MD).

4.2.6 8-iso Prostaglandin F2α

Blood samples (n=10 per group) for the analysis of 8-iso-prostaglandin F2α (8-isoPG), a marker of lipid peroxidation, were collected immediately after sacrifice from saline and nicotine-exposed animals at weaning (PND21). Blood samples were allowed to clot at 4°C, were then centrifuged and the serum was stored at -80°C until assayed. 8-isoPG concentrations were determined using

the CorrelateTM EIA Direct 8-iso-prostaglandin F2α Enzyme Immunoassay Kit (Assay Designs, MI) according to manufacturer's instructions.

4.2.7 Statistical Analysis

All statistical analyses were performed using SigmaStat (v.2.03, SPSS, Chicago, IL). Data from nicotine-exposed offspring were compared to the control group using Student's *t*-test (α=0.05).

4.3 RESULTS

4.3.1 nAChR Subunit mRNA Expression

At PND1 and PND21 all of the nAChR subunits except α5 were present in the pancreas of both saline- and nicotine-exposed offspring (Figure 4.1).

4.3.2 Antioxidant Enzyme Expression

Fetal and neonatal exposure to nicotine did not alter mRNA expression of HO-1, GPx-1, MnSOD or CuZnSOD in the pancreas at PND21 (Table 4.2). Nicotine exposure significantly increased the protein expression of both GPx-1 and MnSOD in the pancreas at PND21 (Figures 4.2B and C respectively; $p<0.05$), but did not alter the protein expression of HO-1 or CuZnSOD (Figures 4.2A and D respectively).

4.3.3 Reactive Oxygen Species Production by Isolated Islets

ROS production by isolated pancreatic islets was significantly elevated in nicotine-exposed offspring relative to saline controls at PND21 (Figure 4.3; $p<0.05$).

4.3.4 Protein Carbonyl Formation

To assess whether the balance of ROS production and antioxidant enzyme expression has been disrupted by nicotine exposure, oxidative damage to protein was measured in the pancreas at PND21. Total protein carbonyl levels were unchanged in the whole pancreas (Figure 4.4), but a significant increase in oxidative damage to a 25 kDa protein was observed (Figure 4.4; $p<0.05$). Furthermore, total protein carbonyl levels were approximately 3 times higher in the mitochondrial fraction from nicotine-exposed relative to saline-exposed pancreas (Figure 4.4; $p<0.001$).

4.3.5 8-iso Prostaglandin F2α

Mean serum levels of 8-isoPG in nicotine-exposed animals were 3 times higher than in control offspring, but due to variability in the nicotine-exposed offspring, this difference did not reach significance (saline 256 ± 48.6 pg/ml, nicotine 767 ± 375.4 pg/ml; $p=0.17$).

4.4 DISCUSSION

Recent epidemiological studies have shown that the offspring of women who smoke during pregnancy have an increased risk of developing obesity, hypertension and type 2 diabetes (7;8;11;36-40), although the mechanism(s) underlying these associations are unknown. We have previously demonstrated in a rat model that maternal nicotine exposure during pregnancy and lactation causes beta cell apoptosis in fetal and neonatal offspring, followed by development of impaired glucose metabolism in early adult life (9;10;28). We hypothesized that nicotine acts directly on the nicotinic acetylcholine receptor in the developing pancreas to induce oxidative stress, and ultimately, beta cell apoptosis. Indeed, results from this study demonstrate that subunits of the nAChR are present during early postnatal life, and that in the pancreas, perinatal nicotine exposure results in both increased oxidative stress and increased beta cell apoptosis.

In this study, the α2 - α4, α6 - α7 and β2 - β4 subunits of nAChR were all present in the pancreas at birth and weaning. Nicotine-exposed offspring have a mean serum cotinine (the major metabolite of nicotine) concentration of 26.2 ± 1.78 ng/ml at birth (41), which provides evidence that maternally-administered nicotine reaches the pups. The presence of the nAChR subunits in the fetal and neonatal pancreas suggests that this maternally-derived nicotine may act via non-neuronal nicotinic acetylcholine receptors to cause the observed increase in beta cell death in the offspring (9;10;28). However, further binding studies and

co-administration of an nAChR antagonist would be required to determine conclusively that nicotine is exerting its effects by direct activation of the nAChR.

Nicotine exposure has been shown to increase oxidative stress in pancreatic tissue *in vitro* (16) and it is well established that pancreatic beta cells are particularly susceptible to oxidative stress-mediated tissue damage due to their low level of antioxidant enzyme expression (20;21). Therefore, we hypothesized that the observed increase in beta cell apoptosis in neonatal nicotine-exposed offspring (9;10;28) might be due, in part to nicotine-induced oxidative stress in the developing pancreas. Indeed, nicotine-exposed offspring had elevated expression of antioxidant proteins, MnSOD and GPx-1, in the pancreas and a concomitant rise in islet ROS production. Cellular oxidative status is a balance between oxidative stress and antioxidant capacity. Therefore if the level of ROS exceeds the antioxidant capacity of the cell, oxidative stress will ensue (25;42). Data from this study suggest that fetal and neonatal nicotine exposure triggers both the pro-oxidant and anti-oxidant response in the pancreas.

The consequences of oxidative stress include damage to mitochondria, cellular proteins, lipids and nucleic acids (42;43), which can lead to cell death through a variety of mechanisms (22). Indeed, nicotine-exposed animals had evidence of oxidative protein damage, as there was increased protein carbonyl formation in the nicotine-exposed offspring relative to saline controls. Interestingly, individual proteins in both the whole pancreas and isolated mitochondrial fraction appeared to be particularly susceptible to oxidative

damage. This trend has also been observed by other groups who report increased protein carbonyl modification to susceptible proteins (44-46). In a future study, we plan to identify specific proteins that are targeted by oxidative stress in this animal model using 2-dimensional gel electrophoresis and mass spectrometry. Furthermore, our data suggest that the oxidative stress is not limited to the pancreas, as there was a trend towards higher levels of 8-isoPG (a marker of whole body oxidative stress) in the nicotine-exposed offspring at weaning. Taken together, these results suggest that developmental nicotine exposure induces an antioxidant response that is associated with loss of redox balance in the pancreas.

Results from this study also indicate that the increased oxidative stress in the pancreas may differentially affect the mitochondria. A striking increase in protein carbonyl levels was observed in the isolated mitochondrial fraction from the pancreas following nicotine exposure whereas there was no significant difference in protein carbonyl formation in the whole pancreas homogenate. Furthermore, the increase in MnSOD protein expression, but not CuZnSOD protein expression, also indicates that the oxidative stress may be localized within the mitochondria. The superoxide dismutase antioxidant response is essential for protecting the cell from oxidative stress; SOD enzymes catalyze the conversion of the highly reactive superoxide anion to hydrogen peroxide, which can then be safely converted to water and molecular oxygen by catalase and glutathione peroxidase (47). Mitochondrial DNA is more vulnerable to ROS

damage than nuclear DNA (48), which may explain why elevated levels of ROS in the islets induced the mitochondrial SOD response (MnSOD) and not the cytosolic response (CuZnSOD). In addition, the mitochondria are not only targeted by ROS, but are also the major source of ROS production in the cell. The iron-sulfur centers of the electron transport chain (ETC) enzyme complexes within the mitochondrial inner membrane are extremely sensitive to ROS inactivation (43;49). When the function of electron carrier complexes is impaired, electrons build up at the initial stages of the ETC, leading to further production of ROS (43;43). Oxidative damage within the mitochondria can lead to mitochondrial swelling and ultimately trigger programmed cell death (22;49;50). The exact role of the mitochondria in the observed beta cell apoptosis in this animal model has been examined in a separate study (51).

Results from this study have shown that maternal nicotine, delivered to the fetus and neonate either through cigarette smoking or nicotine replacement therapy, during pregnancy and lactation, may act directly on the nAChR in the developing pancreas to induce oxidative stress and subsequent beta cell loss in the pancreas. Furthermore, this oxidative stress may target the mitochondria, suggesting a potential mechanism through which fetal and neonatal nicotine exposure leads to beta cell apoptosis. An early reduction in beta cell mass is associated with an increased risk of developing type 2 diabetes later in life (52;53), which may explain, in part, the increased risk of type 2 diabetes in children born to women who smoked during pregnancy. This study also provides

further support to the recent concerns about the safety of nicotine replacement therapy during pregnancy and lactation (54).

4.5 REFERENCES

1. Andres,RL, Day,MC: Perinatal complications associated with maternal tobacco use. *Semin Neonatol* 5:231-241, 2000

2. Cnattingius,S, Lambe,M: Trends in smoking and overweight during pregnancy: prevalence, risks of pregnancy complications, and adverse pregnancy outcomes. *Semin Perinatol* 26:286-295, 2002

3. Faiz,AS, Ananth,CV: Etiology and risk factors for placenta previa: an overview and meta-analysis of observational studies. *J Matern Fetal Neonatal Med* 13:175-190, 2003

4. Nordentoft,M, Lou,HC, Hansen,D, Nim,J, Pryds,O, Rubin,P, Hemmingsen,R: Intrauterine growth retardation and premature delivery: the influence of maternal smoking and psychosocial factors. *Am J Public Health* 86:347-354, 1996

5. Robinson,JS, Moore,VM, Owens,JA, McMillen,IC: Origins of fetal growth restriction. *Eur J Obstet Gynecol Reprod Biol* 92:13-19, 2000

6. Shiverick,KT, Salafia,C: Cigarette smoking and pregnancy I: ovarian, uterine and placental effects. *Placenta* 20:265-272, 1999

7. Bergmann,KE, Bergmann,RL, Von,KR, Bohm,O, Richter,R, Dudenhausen,JW, Wahn,U: Early determinants of childhood overweight and adiposity in a birth cohort study: role of breast-feeding. *Int J Obes Relat Metab Disord* 27:162-172, 2003

8. Wideroe,M, Vik,T, Jacobsen,G, Bakketeig,LS: Does maternal smoking during pregnancy cause childhood overweight? *Paediatr Perinat Epidemiol* 17:171-179, 2003

9. Holloway,AC, Lim,GE, Petrik,JJ, Foster,WG, Morrison,KM, Gerstein,HC: Fetal and neonatal exposure to nicotine in Wistar rats results in increased beta cell apoptosis at birth and postnatal endocrine and metabolic changes associated with type 2 diabetes. *Diabetologia* 48:2661-2666,

2005

10. Bruin J.E., Kellenberger L.D., Gerstein H.C., Morrison K.M., Holloway A.C.: Fetal and neonatal nicotine exposure and postnatal glucose homeostasis: identifying critical windows of exposure. *Journal of Endocrinology* 194:1-9, 2007

11. Montgomery,SM, Ekbom,A: Smoking during pregnancy and diabetes mellitus in a British longitudinal birth cohort. *BMJ* 324:26-27, 2002

12. Noakes,PS, Thomas,R, Lane,C, Mori,TA, Barden,AE, Devadason,SG, Prescott,SL: Maternal smoking is associated with increased infant oxidative stress at 3 months of age. *Thorax* Epub - ahead of print.: 2007

13. Ermis,B, Ors,R, Yildirim,A, Tastekin,A, Kardas,F, Akcay,F: Influence of smoking on maternal and neonatal serum malondialdehyde, superoxide dismutase, and glutathione peroxidase levels. *Ann Clin Lab Sci* 34:405-409, 2004

14. Zhao,Z, Reece,EA: Nicotine-induced embryonic malformations mediated by apoptosis from increasing intracellular calcium and oxidative stress. *Birth Defects Res B Dev Reprod Toxicol* 74:383-391, 2005

15. Crowley-Weber,CL, Dvorakova,K, Crowley,C, Bernstein,H, Bernstein,C, Garewal,H, Payne,CM: Nicotine increases oxidative stress, activates NF-kappaB and GRP78, induces apoptosis and sensitizes cells to genotoxic/xenobiotic stresses by a multiple stress inducer, deoxycholate: relevance to colon carcinogenesis. *Chem Biol Interact* 145:53-66, 2003

16. Wetscher,GJ, Bagchi,M, Bagchi,D, Perdikis,G, Hinder,PR, Glaser,K, Hinder,RA: Free radical production in nicotine treated pancreatic tissue. *Free Radic Biol Med* 18:877-882, 1995

17. Ozokutan,BH, Ozkan,KU, Sari,I, Inanc,F, Guldur,ME, Kilinc,M: Effects of maternal nicotine exposure during lactation on breast-fed rat pups. *Biol Neonate* 88:113-117, 2005

18. Husain,K, Scott,BR, Reddy,SK, Somani,SM: Chronic ethanol and nicotine interaction on rat tissue antioxidant defense system. *Alcohol* 25:89-97, 2001

19. Yildiz,D: Nicotine, its metabolism and an overview of its biological effects. *Toxicon* 43:619-632, 2004

20. Lenzen,S, Drinkgern,J, Tiedge,M: Low antioxidant enzyme gene

expression in pancreatic islets compared with various other mouse tissues. *Free Radic Biol Med* 20:463-466, 1996

21. Tiedge,M, Lortz,S, Drinkgern,J, Lenzen,S: Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells. *Diabetes* 46:1733-1742, 1997

22. Ryter,SW, Kim,HP, Hoetzel,A, Park,JW, Nakahira,K, Wang,X, Choi,AM: Mechanisms of cell death in oxidative stress. *Antioxid Redox Signal* 9:49-89, 2007

23. Donath,MY, Ehses,JA, Maedler,K, Schumann,DM, Ellingsgaard,H, Eppler,E, Reinecke,M: Mechanisms of beta-cell death in type 2 diabetes. *Diabetes* 54 Suppl 2:S108-S113, 2005

24. Kajimoto,Y, Kaneto,H: Role of oxidative stress in pancreatic beta-cell dysfunction. *Ann N Y Acad Sci* 1011:168-176, 2004

25. Kaneto,H, Kawamori,D, Matsuoka,TA, Kajimoto,Y, Yamasaki,Y: Oxidative stress and pancreatic beta-cell dysfunction. *Am J Ther* 12:529-533, 2005

26. Robertson,RP: Oxidative stress and impaired insulin secretion in type 2 diabetes. *Curr Opin Pharmacol* 6:615-619, 2006

27. Robertson,RP, Harmon,JS: Diabetes, glucose toxicity, and oxidative stress: A case of double jeopardy for the pancreatic islet beta cell. *Free Radic Biol Med* 41:177-184, 2006

28. Holloway,AC, Petrik,JJ, Bruin,JE, Gerstein,HC: Rosiglitazone prevents diabetes by increasing beta-cell mass in an animal model of type 2 diabetes characterized by reduced beta-cell mass at birth. *Diabetes Obes Metab* 2007

29. Karlin,A, Akabas,MH: Toward a structural basis for the function of nicotinic acetylcholine receptors and their cousins. *Neuron* 15:1231-1244, 1995

30. Dajas-Bailador,F, Wonnacott,S: Nicotinic acetylcholine receptors and the regulation of neuronal signalling. *Trends Pharmacol Sci* 25:317-324, 2004

31. Maus,AD, Pereira,EF, Karachunski,PI, Horton,RM, Navaneetham,D, Macklin,K, Cortes,WS, Albuquerque,EX, Conti-Fine,BM: Human and rodent bronchial epithelial cells express functional nicotinic acetylcholine

receptors. *Mol Pharmacol* 54:779-788, 1998

32. Conti-Fine,BM, Navaneetham,D, Lei,S, Maus,AD: Neuronal nicotinic receptors in non-neuronal cells: new mediators of tobacco toxicity? *Eur J Pharmacol* 393:279-294, 2000

33. Yoshikawa,H, Hellstrom-Lindahl,E, Grill,V: Evidence for functional nicotinic receptors on pancreatic beta cells. *Metabolism* 54:247-254, 2005

34. Ge,Q, Dong,YM, Hu,ZT, Wu,ZX, Xu,T: Characteristics of Ca2+-exocytosis coupling in isolated mouse pancreatic beta cells. *Acta Pharmacol Sin* 27:933-938, 2006

35. Simmons,RA, Suponitsky-Kroyter,I, Selak,MA: Progressive accumulation of mitochondrial DNA mutations and decline in mitochondrial function lead to beta-cell failure. *J Biol Chem* 280:28785-28791, 2005

36. Blake,KV, Gurrin,LC, Evans,SF, Beilin,LJ, Landau,LI, Stanley,FJ, Newnham,JP: Maternal cigarette smoking during pregnancy, low birth weight and subsequent blood pressure in early childhood. *Early Hum Dev* 57:137-147, 2000

37. Morley,R, Leeson,PC, Lister,G, Lucas,A: Maternal smoking and blood pressure in 7.5 to 8 year old offspring. *Arch Dis Child* 72:120-124, 1995

38. Toschke,AM, Koletzko,B, Slikker,W, Jr., Hermann,M, Von,KR: Childhood obesity is associated with maternal smoking in pregnancy. *Eur J Pediatr* 161:445-448, 2002

39. Vik,T, Jacobsen,G, Vatten,L, Bakketeig,LS: Pre- and post-natal growth in children of women who smoked in pregnancy. *Early Hum Dev* 45:245-255, 1996

40. Von,KR, Toschke,AM, Koletzko,B, Slikker,W, Jr.: Maternal smoking during pregnancy and childhood obesity. *Am J Epidemiol* 156:954-961, 2002

41. Holloway AC, Kellenberger LD, Petrik JJ: Fetal and Neonatal Exposure to Nicotine Disrupts Ovarian Function and Fertility in Adult Female Rats. *Endocrine* 30:213-216, 2006

42. Luo,ZC, Fraser,WD, Julien,P, Deal,CL, Audibert,F, Smith,GN, Xiong,X, Walker,M: Tracing the origins of "fetal origins" of adult diseases:

programming by oxidative stress? *Med Hypotheses* 66:38-44, 2006

43. Simmons,RA: Developmental origins of diabetes: the role of oxidative stress. *Free Radic Biol Med* 40:917-922, 2006

44. Castegna,A, Aksenov,M, Aksenova,M, Thongboonkerd,V, Klein,JB, Pierce,WM, Booze,R, Markesbery,WR, Butterfield,DA: Proteomic identification of oxidatively modified proteins in Alzheimer's disease brain. Part I: creatine kinase BB, glutamine synthase, and ubiquitin carboxy-terminal hydrolase L-1. *Free Radic Biol Med* 33:562-571, 2002

45. Reinheckel,T, Korn,S, Mohring,S, Augustin,W, Halangk,W, Schild,L: Adaptation of protein carbonyl detection to the requirements of proteome analysis demonstrated for hypoxia/reoxygenation in isolated rat liver mitochondria. *Arch Biochem Biophys* 376:59-65, 2000

46. Dalle-Donne,I, Rossi,R, Giustarini,D, Milzani,A, Colombo,R: Protein carbonyl groups as biomarkers of oxidative stress. *Clin Chim Acta* 329:23-38, 2003

47. Tomohiro,T, Kumai,T, Sato,T, Takeba,Y, Kobayashi,S, Kimura,K: Hypertension aggravates glomerular dysfunction with oxidative stress in a rat model of diabetic nephropathy. *Life Sci* 80:1364-1372, 2007

48. Droge,W: Free radicals in the physiological control of cell function. *Physiol Rev* 82:47-95, 2002

49. Wallace,DC: A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. *Annu Rev Genet* 39:359-407, 2005

50. Gulbins,E, Dreschers,S, Bock,J: Role of mitochondria in apoptosis. *Exp Physiol* 88:85-90, 2003

51. Bruin J.E., Gerstein H.C., Morrison K.M., Holloway A.C.: Increased pancreatic beta cell apoptosis following fetal and neonatal exposure to nicotine is mediated via the mitochondria. *Toxicological Sciences* (In Press): 2008

52. Leahy,JL: Pathogenesis of type 2 diabetes mellitus. *Arch Med Res* 36:197-209, 2005

53. Rhodes,CJ: Type 2 diabetes-a matter of beta-cell life and death? *Science* 307:380-384, 2005

54. Ginzel,KH, Maritz,GS, Marks,DF, Neuberger,M, Pauly,JR, Polito,JR, Schulte-Hermann,R, Slotkin,TA: Critical review: nicotine for the fetus, the infant and the adolescent? *J Health Psychol* 12:215-224, 2007

4.6 TABLES AND FIGURES

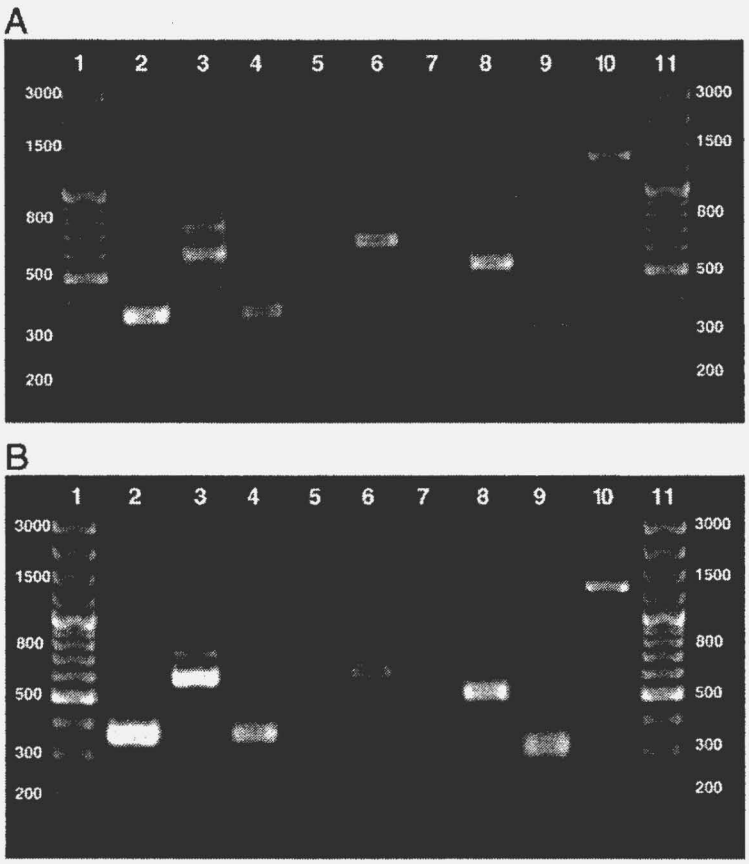


Figure 4.1: mRNA expression for nAChR subunits.
Representative PCR products are shown on an agarose gel from saline control pancreas at: A) PND1 and B) PND21. Lane: 1) 100bp DNA ladder; 2) α2; 3) α3; 4) α4; 5) α5; 6) α6; 7) α7; 8) β2; 9) β3; 10) β4; 11) 100bp DNA ladder.

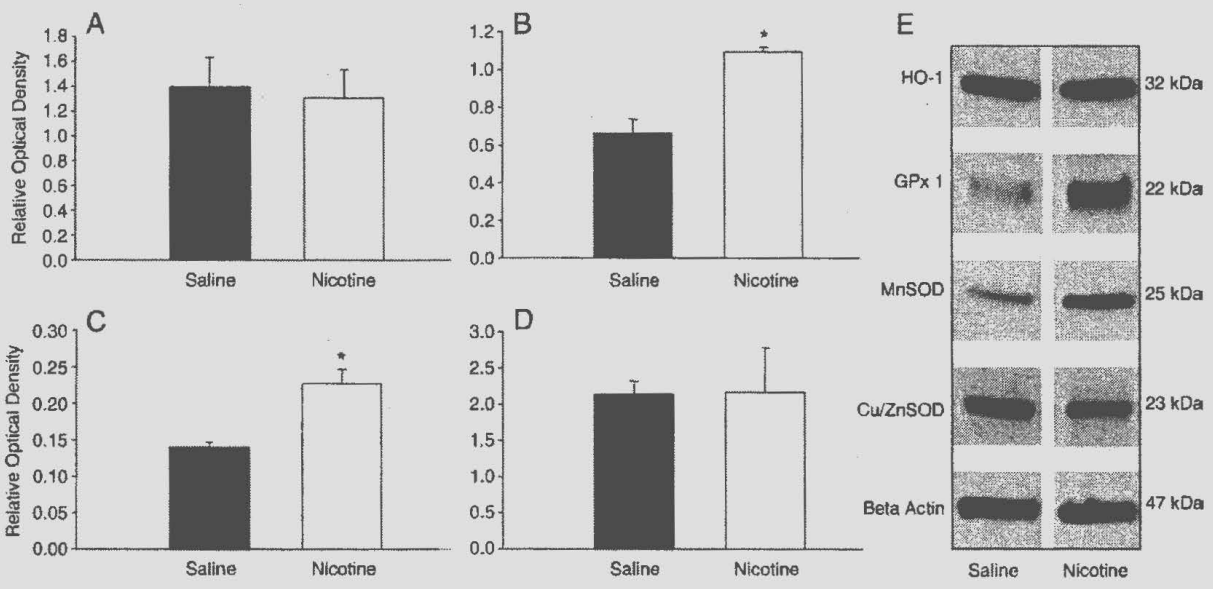


Figure 4.2: Quantification of protein expression for antioxidant enzymes.
A) HO-1, B) GPx-1, C) MnSOD and D) CuZnSOD in saline (black bar) and nicotine (white bar) -exposed pancreas at 3 weeks of age (n=4 per group). A representative Western blot for each protein is presented in panel E. All protein expression was quantified relative to a β-actin loading control. Data are presented as the mean ± SEM. Values with an asterisk are significantly different from the saline control ($p<0.05$).

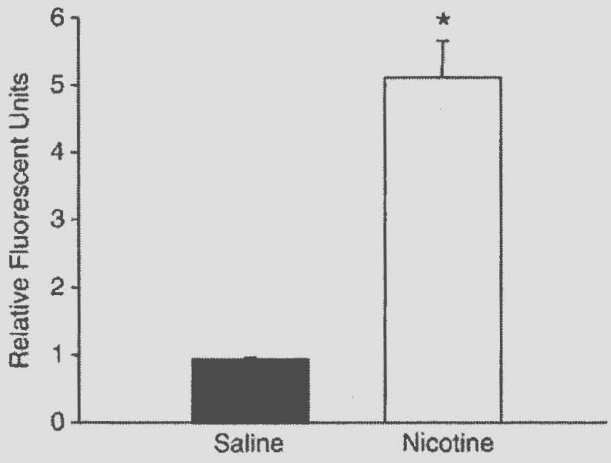


Figure 4.3: ROS production in isolated islets at postnatal day 21.
Islets were isolated from saline (black bar) and nicotine (white bar)-exposed offspring (n=3 per group) at weaning (postnatal day 21). Data are expressed as relative fluorescent units (normalized to a hydrogen peroxide control in H_2DCFDA) ± SEM. Values with an asterisk are significantly different ($p<0.05$).

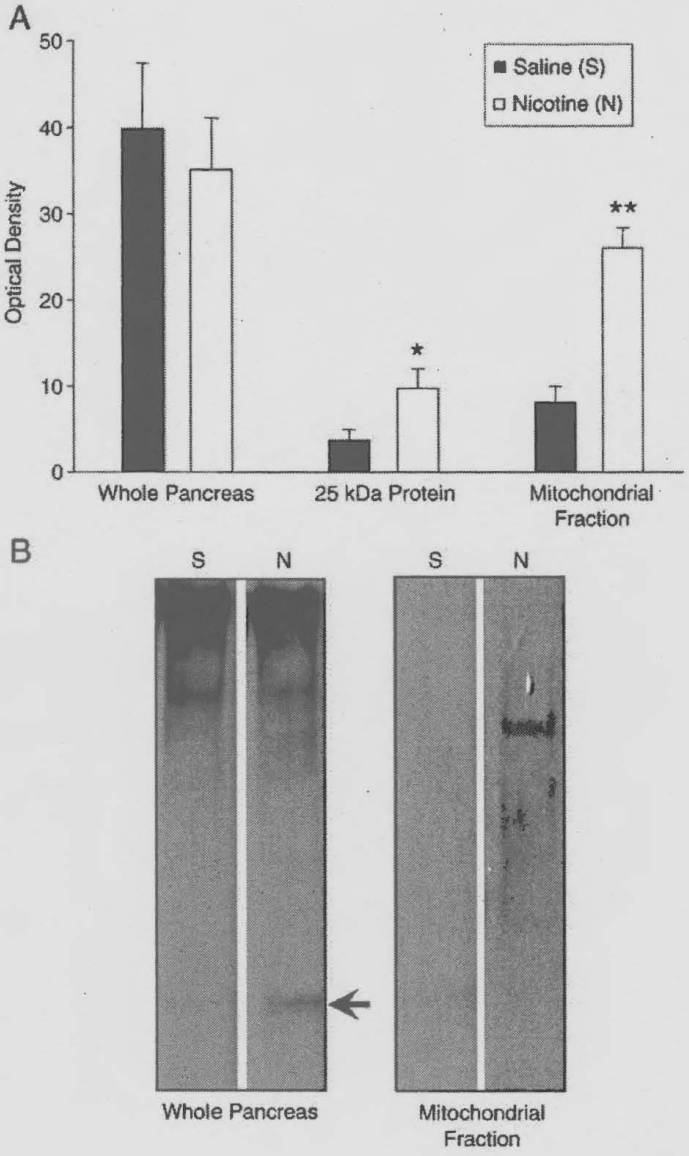


Figure 4.4: Pancreatic protein carbonyl formation at postnatal day 21.
A) Quantification of protein carbonyl groups in whole tissue homogenates (n=5 per group) and mitochondrial fractions (n=4 per group) from the pancreas of saline (black bar) and nicotine (white bar) – exposed offspring at 3 weeks of age. B) A representative Western blot of protein carbonyl groups is shown for the whole pancreas and mitochondrial fraction from saline (S) and nicotine (N) – exposed offspring. The black arrow indicates a 25 kDa protein that is significantly altered by fetal and neonatal nicotine exposure in the whole pancreas homogenates. Data are presented as the mean ± SEM. Values with an asterisk are significantly different from the saline control ($p<0.05$); a double asterisk indicates a p value < 0.001.

Table 4.1: Primer sequences for reverse transcription and real-time PCR.

Gene	Forward Primer	Reverse Primer
GPx-1	5' - CGA CAT CGA ACC CGA TAT AGA	5' - CCA TCA CCA AGC CCA GAT AC
MnSOD	5' - CCT TTC CCT GAC AAG GTA CAC	5' - CAA ATG CTG CAC AGG AAT ACA
CuZnSOD	5' - TGG GTT CCA TGT CCA TCA ATA	5' - CTG GAC CGC CAT GTT TCT TA
HO-1	5' - ACA CCA GCC ACA CAG CAC TA	5' - CCA GCA GCT CAG GAT GAG TA
nAChR α2	5' - CTC CTG CAG CAT CGA TGT GAC CTT CTT	5' - GAG ATG CAC AGC GTG ATC TTC TCT CCA C
nAChR α3	5' - GGA GAA GTG ACT TGG ATC C	5' - CAA GTG GGC ATG GTG TGT G
nAChR α4	5' - GCC ATC TAT AAG AGC TCC TGC AGC ATC	5' - CTT CTC GCC AAA CTC TGA AGG CAG ATA G
nAChR α5	5' - CGA ACG TCT GGT TGA AGC	5' - CAC CAT AAT GGA ATA GGG
nAChR α6	5' - TCT TAA GTA CGA TGG GGT GAT AAC	5' - AAC ATG GTC TTC ACC CAC TTG
nAChR α7	5' - TTG CCA GTA TCT CCC TCC AG	5' - CTT CTC ATT CCT TTT GCC AG
nAChR β2	5' - GCT GAC GGC ATG TAC GAA G	5' - GGA GGT GGG AGG CAC AAT C
nAChR β3	5' - CTC ATT ATC CAC CTC CGT TT	5' - CTG TAT CAC TCT CCT TTC CAT CC
nAChR β4	5' - GGT TGC CTG ACA TCG TGT TG	5' - GCC AAT GAG CGG TAT GTC
Beta Actin	5' - GCT GTG CTA TGT TGC CCT AGA C	5' - ACC GCT CAT TGC CGA TAG T

Table 4.2: mRNA expression of antioxidant proteins in the pancreas at 3 weeks of age, as determined by quantitative real-time PCR.

Subunit	Saline	Nicotine	p value
HO-1	1.43 ± 0.018	1.42 ± 0.016	0.628
GPx	1.17 ± 0.016	1.19 ± 0.022	0.486
MnSOD	1.36 ± 0.089	1.35 ± 0.020	0.640
CuZnSOD	1.23 ± 0.012	1.21 ± 0.021	0.377

All gene expression was quantified relative to a β-actin loading control. Data are presented as the mean ± SEM.

CHAPTER 5

FETAL AND NEONATAL NICOTINE EXPOSURE IN WISTAR RATS CAUSES PROGRESSIVE PANCREATIC MITOCHONDRIAL DAMAGE AND BETA CELL DYSFUNCTION.

BRUIN JE, PETRE MA, RAHA S, MORRISON KM, GERSTEIN HC, HOLLOWAY AC. FETAL AND NEONATAL NICOTINE EXPOSURE IN WISTAR RATS CAUSES A PROGRESSION OF PANCREATIC MITOCHONDRIAL ALTERATIONS AND LEADS TO BETA CELL DYSFUNCTION. 2008. *PLoS ONE* 3(10): E3371. DOI:10.1371/JOURNAL.PONE.0003371.

5.1 INTRODUCTION

Cigarette smoking is associated with numerous adverse obstetrical and fetal outcomes (1-6), yet 15-20% of women reportedly smoke during pregnancy (1;7). Furthermore, mounting epidemiologic evidence indicates that maternal smoking is associated with an increased risk of obesity, hypertension and type 2 diabetes in the offspring (7-13), although the mechanisms underlying this relationship are unknown. Our laboratory has previously demonstrated in a rat model that maternal exposure to nicotine, the major addictive component of cigarettes, during pregnancy and lactation results in postnatal obesity and impaired glucose homeostasis in adult offspring (14;15). Because nicotine replacement therapy (NRT) is recommended for pregnant women who cannot quit smoking by other means (16), these results may have significant public health implications. In our animal model, postnatal dysglycemia following fetal

and neonatal nicotine exposure was associated with a loss of beta cell mass, beginning at birth and persisting into adulthood (14). This reduction in beta cell mass following developmental nicotine exposure may partially explain the increased risk of type 2 diabetes in the offspring of women who smoked during pregnancy (8).

Individuals with type 2 diabetes are unable to produce sufficient insulin to maintain normal glucose homeostasis (17). This has been attributed, in part, to reduced beta cell mass and impaired beta cell function (17;18). In beta cells, the mitochondria are involved in triggering apoptosis, thereby contributing to the regulation of beta cell mass (19;20). We have previously shown that fetal and neonatal exposure to nicotine results in beta cell loss due to increased oxidative stress (21) and beta cell apoptosis (14;15). Furthermore, we have demonstrated that this nicotine-induced oxidative stress differentially targeted the mitochondria in the pancreas (21), resulting in mitochondrial-mediated beta cell apoptosis (22). However, in addition to regulating beta cell mass (via apoptosis), the mitochondria are also critical for maintenance of beta cell function through the coupling of a glucose stimulus to insulin release (23-25). Both human and animal studies have demonstrated mitochondrial dysfunction in islets of subjects with type 2 diabetes (26;27). Therefore, we hypothesize that the dysglycemia observed in this animal model following fetal and neonatal nicotine exposure is likely mediated by pancreatic mitochondrial defects. This study will examine the effect of fetal and neonatal exposure to nicotine on postnatal mitochondrial

structure and function, as well as subsequent beta cell function.

5.2 MATERIALS AND METHODS

5.2.1 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 200-250g female Wistar rats (Harlan, Indianapolis, IN, USA) were maintained under controlled lighting (12:12 L:D) and temperature (22°C) with *ad libitum* access to food and water. Dams were randomly assigned (n=30 per group) to receive saline (vehicle) or nicotine bitartrate (1mg/kg/d, Sigma-Aldrich, St. Louis, MO, USA) via subcutaneous injection daily for 2 weeks prior to mating until weaning (postnatal day 21). We have previously demonstrated that this dose of nicotine (1mg/kg/d) results in cotinine concentrations in maternal serum that are similar to "moderate" female smokers and in nicotine-exposed offspring serum at birth that are comparable to infants nursed by smoking mothers (28). At postnatal day 1, litters were culled to eight 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.

5.2.2 Oral Glucose Tolerance

Glucose homeostasis was investigated in nicotine-exposed and saline control rats at 4, 15 and 26 weeks of age (n=15 per group) using sequential oral glucose tolerance tests (OGTT) as previously described (14;15). Briefly, after an overnight fast insulin and glucose were measured in saphenous vein samples, collected by repeated puncture, at baseline, 30 and 120 minutes after rats were given 2g· kg-1 glucose (Sigma-Aldrich, St. Louis, MO, USA) in water by gavage. 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). Data are presented as the average area under the curve (AUC) ± SEM for saline- and nicotine-exposed offspring at each age.

5.2.3 Electron Microscopy

Pancreas tissue from offspring at 3 weeks (n=4 per group), 15 weeks (saline: n=3 and nicotine: n=4), and 26 weeks (n=3 per group) were collected and processed for electron microscopy as previously described (22). All chemicals used for electron microscopy were purchased from Canemco Inc., Montreal, QC, Canada unless otherwise stated. Thick sections (approximately 1μm) were cut on an Ultracut E ultramicrotome (Leica Microsystems, Wetzlar,

Germany), stained with toluidine blue and examined under a light microscope to ensure the presence of islets. Thin sections (approximately 70nm) were then cut from areas of the tissue containing islets, mounted on a Cu/Pd grid (200 mesh), and stained with saturated uranyl acetate and lead citrate. Grids were examined with a JEOL 1200EX transmission electron microscope (JEOL Ltd., Tokyo, Japan) and representative photographs were taken at either 5000x or 12000x magnification. All photographs were analysed by a single investigator blinded to the treatment groups using Image Pro Plus Version 5.1 software (Media Cybernetics, Inc., Silver Spring, MD, USA).

Beta cells were identified within the pancreas sections by the presence of insulin granules. Insulin granules were classified as filled (dense-core), immature (light gray granule) or empty (no insulin). The number of insulin granules and mitochondria were calculated relative to the area of a beta cell. Individual mitochondrial morphology was assessed by quantifying: a) the average mitochondrion area; b) the proportion of mitochondria with blebbing and/or merging with other mitochondria (refer to Figure 5.1E for examples); c) the proportion of mitochondria in each of five defined stages of progressive deterioration. The definitions for each mitochondrial stage were created using a modification of a previously described scale for assessing mitochondrial morphologies (29). Stage 1 mitochondria were classified as structurally healthy, with dense, intact cristae. Stage 2 mitochondria had visible swelling, but maintained distinctive intact cristae structure. Stage 3 mitochondria had more

severe swelling and minimal evidence of intact cristae. Stage 4 mitochondria displayed severe swelling, minimal cristae structure and formation of vacuoles. Stage 5 mitochondria were extremely large and swollen, with essentially complete loss of defined structure within the mitochondrial membrane. An example of mitochondria at each defined morphological stage is provided in Figure 5.2F.

5.2.4 Mitochondrial Enzyme Activity

Pancreas tissue was excised from offspring at 3 weeks (saline: n=7, nicotine: n=5), 15 weeks (n=5 per group) and 26 weeks (n=5 per group), frozen on dry ice and stored at -80°C until analysis. Tissue samples were homogenized in homogenization buffer (5mM HEPES pH 7.4, 100mM KCl, 70mM sucrose, 220 mM mannitol, 1mM EGTA) with Complete Mini EDTA-free protease inhibitors (Roche Applied Science, Laval, QC, Canada) using Tenbroeck tissue grinders. Homogenates were spun for 10 min at 600xg, the supernatant removed, flash frozen in liquid nitrogen and stored at -80°C until use. Citrate synthase activity (an indicator of total mitochondrial mass) was measured using the thiol reagent 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB, Sigma Chemical Co., St. Louis, MO, USA). Complex IV (cytochrome *c* oxidase) activity was assessed by measuring the rate of cytochrome *c* (from equine heart; Sigma Chemical Co., St. Louis, MO, USA) oxidation. Both activity assays were performed using UV-spectrophotometry (Varian Inc., Palo Alto, CA, USA) as previously described

(30). Data are expressed as the mean enzyme activity relative to the wet weight of tissue.

5.2.5 Islet Isolation

Islet isolation was performed as previously described (21) at 26 weeks of age. Briefly, the pancreas was immediately excised following sacrifice, minced finely and placed in 6 mL of Hank's balanced salt solution (HBSS) (HyClone, Logan, UT, USA) containing 4 mg/mL collagenase type IA (Sigma-Aldrich, St. Louis, MO, USA), 100 IU/mL penicillin G and 0.25 µg/mL streptomycin (Gibco, Grand Island, NY, USA). Following an incubation period of 40 min at 37°C, the reaction was quenched with 20 mL HBSS supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA), 100 IU/mL penicillin G and 0.25 µg/mL streptomycin. Islets were manually picked from the suspension using a small glass pipette and a dissecting microscope. The islets were incubated at 37°C, 5% CO2 / 95% normal atmosphere in 5 mL RPMI 1640 with 3.0 mM glucose (Life Technologies, Burlington, ON, Canada) supplemented with 10% FBS, 100 IU penicillin G and 0.25 µg/mL streptomycin for 24 hours.

5.2.6 Reactive Oxygen Species Production by isolated Islets

Reactive oxygen species (ROS) production by isolated islets following saline and nicotine exposure was measured using 2',7'-

dichlorodihydrofluorescein diacetate (H_2DCFDA) (Molecular Probes Inc., Eugene, OR, USA) fluorescence as previously described (21). Since islet ROS production at weaning has been previously confirmed in this animal model (21), oxidative stress was only assessed at the endpoint of the current study (26 weeks). Briefly, 80 islets from saline- and nicotine-exposed offspring (n=6 per group) were washed with PBS. Following centrifugation, the supernatant was removed and the pelleted islets were then resuspended in 100 µL of PBS containing 100µM H_2DCFDA and incubated for 3 h at 37°C. Because of the relatively low number of cells in this assay, a long incubation period allows for the diffusion of the oxidized dye from inside the cell back out into the culture medium (27). This approach has been previously validated to determine ROS production by isolated islet cells in rats (21;27). In addition, since H_2DCFDA must be made fresh immediately prior to use, islets isolated on different days were incubated in different batches of reagent. To account for day-to-day variability within the experiment, a 43 µM hydrogen peroxide reaction was prepared with each batch of H_2DCFDA to calibrate the performance of the dye. The hydrogen peroxide was added to 100µM H_2DCFDA and incubated in parallel with the islet reactions. Following the incubation period, the islets were vigorously disrupted to release intracellular H_2DCFDA. Both the islet suspensions and the hydrogen peroxide control were centrifuged, and the supernatants were transferred to black 96-well plates (BD Falcon, Mississauga, ON, Canada). Fluorescence of the 2',7'-dichlorofluorescein product was determined using a SpectaMax Gemini XS (Molecular Devices

Corp., Sunnyvale, CA, USA) microplate spectrofluorometer at excitation and emission wavelengths of 505 nm and 540 nm, respectively. All measurements of islet ROS production were normalized to the 43 μM hydrogen peroxide control and expressed as a percentage of the average saline control.

5.2.7 Oxyblot Detection of Protein Carbonyls in Isolated Islets

To assess oxidative damage by reactive oxygen species to islet proteins, the presence of protein carbonyl groups was quantified using the OxyBlotTM Protein Oxidation Detection Kit (Chemicon International, Temecula, CA, USA). Formation of protein carbonyl groups was measured at 26 weeks in isolated pancreatic islets (saline: n=6, nicotine: n=5). 100 islets were hand-picked into eppendorf tubes and centrifuged for 3 minutes at 300 rcf. The supernatant was removed and islets were resuspended in 100 μL of homogenization buffer with protease inhibitors (as described above) and frozen at -80°C until use. Upon thawing, cells were lysed using a sonication probe. Protein samples (5μL) were then prepared with the OxyblotTM Kit, according to manufacturer's instructions. Derivatized protein was subjected to SDS-PAGE using a 12 % separating gel and then electro-transferred to PVDF blotting membrane (BioRad Laboratories, Hercules, CA, USA). Membranes were blocked for 2 h at room temperature with 5 % (w/v) skim milk in TBST (TBS, 0.5% (v/v) Tween 20), incubated overnight at 4°C in rabbit-DNP antibody (1:150), and finally 1 h at room temperature in secondary goat anti-rabbit IgG (HRP-conjugated; 1:300). Blots were washed

thoroughly in TBST followed by TBS after immunoblotting. Reactive protein was detected with ECL Plus chemiluminescence (Amersham Biosciences, Piscataway, NJ, USA) and Bioflex X-ray film (Clonex Corporation, Markham, ON, Canada). Densitometric analysis of immunoblots was performed using ImageJ 1.37v software (National Institutes of Health, Bethesda, MD, USA); all proteins were quantified relative to a Ponceau S (Sigma Aldrich, St. Louis, MO, USA) loading control.

5.2.8 Glucose-Stimulated Insulin Secretion in Isolated Islets

Glucose stimulated insulin secretion (GSIS) was examined as a marker of beta cell function at 26 weeks of age. Briefly, 20 islets from both saline- and nicotine-exposed offspring (n=6 per group) were incubated in 100 µL of Krebs Ringer Bicarbonate buffer, pH 7.4 (135mM NaCl, 3.6mM KCl, 5mM NaHCO3, 0.5mM $NaH_2PO_4 \cdot 2H_2O$, 0.5mM $MgCl_2 \cdot 6H_2O$, 1.5mM $CaCl_2 \cdot 2H_2O$, 10mM Hepes, 0.1% BSA) with either 3.0 mM glucose (basal) or 16.7 mM glucose (stimulated) for 2 hours at 37°C. All reactions were performed in duplicate. Following the incubation, islets were centrifuged, the media removed and stored at -80°C until use. The pelleted islets were resuspended in 25 µL of cell homogenization buffer (as described above), sonicated to release insulin and frozen at -80°C until use. Insulin levels were measured in both the media and the pellet by an ultra sensitive rat insulin ELISA (Crystal Chem Inc., Downers Grove, IL, USA). Results were expressed as the concentration of insulin in the media relative to

the concentration remaining in the pellet, to normalize for variability in the size of islets. Glucose-stimulated insulin release was determined by comparing the amount of insulin released from the pellet into the media at 16.7 mM glucose relative to 3 mM glucose.

5.2.9 Statistical Analysis

All statistical analyses were performed using SigmaStat (v.3.1, SPSS, Chicago, IL, USA). The results are expressed as mean ± SEM. Data were checked for normality and equal variance and were tested using unpaired Student's *t*-tests ($\alpha = 0.05$) at each age. Where data failed normality or equal variance test, data were reanalyzed using Mann-Whitney rank sum test.

5.3 RESULTS

5.3.1 Oral Glucose Tolerance Tests

At 4 weeks of age there was no effect of nicotine exposure ($p>0.05$) on the total glucose response (area under the curve; AUC) to the oral glucose load. By 15 weeks of age the nicotine-exposed animals had a higher total glucose response (AUC) relative to the saline controls ($p<0.05$), an effect which was also evident at 26 weeks of age (Figure 5.3).

5.3.2 Mitochondrial Structure

There was no difference in the number of mitochondria per beta cell area at any age (Figure 5.1A), but the mean individual mitochondrion area was significantly higher ($p<0.05$) following nicotine exposure at 15 and 26 weeks of age (Figure 5.1B) compared to saline controls. Furthermore, the proportion of mitochondria with either blebbing or merging with neighboring mitochondria dramatically increased in the nicotine-, but not saline-exposed animals with age (Figure 5.1C).

Structural abnormalities were also evident in mitochondria of nicotine-exposed offspring starting at weaning (postnatal day 21). At all ages, more than 75% of the mitochondria from saline-exposed offspring were classified as stage 1 (structurally intact; Figure 5.2A). In contrast, nicotine-exposed offspring had a significant decrease in the proportion of healthy, stage 1 mitochondria beginning at 3 weeks of age, followed by a continual decline with age, such that by 26 weeks only 31% of nicotine-exposed mitochondria were classified as stage 1 (Figure 5.2A). Coinciding with the loss of healthy stage 1 mitochondria was a significant 4.7-fold increase in the proportion of stage 2 mitochondria (visible swelling) at 3 weeks in the nicotine-exposed offspring (Figure 5.2B). Furthermore, by 15 weeks of age nearly 20% of the mitochondria in beta cells of nicotine-exposed offspring were classified as either stages 3, 4 or 5 and by 26 weeks this proportion had increased to 57% (Figure 5.2C-E). In contrast, at all ages examined less than 1% of the mitochondria from the saline control group

were at stages 3, 4 and 5 combined (Figure 5.2C-E). It was not possible to perform statistics on the data presented in Figures 5.2C, D or E due to lack of variability in the saline treatment group replicates (nearly all 0%).

5.3.3 Mitochondrial Enzyme Activity

By 26 weeks, complex IV activity (a marker of mitochondrial function) was significantly reduced in the nicotine-exposed animals compared to saline controls ($p<0.05$; Figure 5.4A). There was no difference in citrate synthase activity (an indicator of mitochondrial mass) in the pancreas at any age examined ($p>0.05$; Figure 5.4B).

5.3.4 Insulin Granule Characteristics

By 26 weeks of age, the nicotine-exposed animals had 32% fewer insulin granules in total ($p<0.05$; Figure 5.5A) and an 86% reduction in the number of immature granules per beta cell area ($p<0.05$; Figure 5.5B). The number of filled insulin granules per beta cell area was lower at all ages examined following nicotine exposure, but did not reach statistical significance ($p>0.05$; Figure 5.5C).

5.3.5 Oxidative Stress

Islet ROS production in adult animals at 26 weeks of age was increased by approximately 20% following fetal and neonatal exposure to nicotine relative to

saline ($p<0.05$; Figure 5.6). Furthermore, there was a 35% increase in the formation of protein carbonyl groups in the islets of nicotine-exposed offspring compared to saline controls ($p<0.05$; Figure 5.6).

5.3.6 Glucose-Stimulated Insulin Secretion

Fetal and neonatal exposure to nicotine resulted in impaired GSIS from pancreatic islets isolated from 26 week old animals (Figure 5.7).

5.4 DISCUSSION

Results from this study clearly demonstrate that fetal and neonatal nicotine exposure alters both mitochondrial structure and function postnatally. Mitochondrial structural abnormalities are observable prior to the onset of glucose intolerance and progressively worsen with age even though nicotine exposure is discontinued at weaning. Furthermore, as nicotine-exposed animals age, the observed mitochondrial defects appear to impact both mitochondrial function and beta cell function. These data raise concerns about the long term health consequences to the offspring following cigarette smoking or nicotine replacement therapy use during pregnancy and lactation.

The first observable mitochondrial alteration following developmental nicotine exposure was abnormal mitochondrial ultrastructure in the neonates at weaning (3 weeks of age). These early structural alterations in the nicotine-

exposed offspring coincided with increases in both pancreatic oxidative stress (21) and mitochondrial-mediated beta cell apoptosis (22). However, these changes in mitochondrial structure precede any observable alterations in glucose homeostasis. As the nicotine-exposed animals age, the proportion of beta cell mitochondria with severe structural abnormalities of the inner membrane (stages 3-5) and outer membrane (indicated by blebbing and / or merging) increased dramatically, despite discontinuation of nicotine exposure at weaning. These profound structural defects were not associated with any changes to the number of mitochondria within the beta cells, but were accompanied by a modest decline in mitochondrial enzyme activity, degranulation of beta cells, decreased beta cell function and impaired glucose tolerance (IGT). Therefore, we suggest that nicotine-induced mitochondrial damage has a significant role in the development of glucose intolerance in this animal model.

It is estimated that 98% of the energy for the beta cell is produced by mitochondrial oxidative metabolism (25). Mitochondria are essential for both stages of glucose-stimulated insulin secretion from beta cells, including glucose entry and metabolism, as well as insulin exocytosis (25;31). In this study, fetal and neonatal nicotine exposure resulted in reduced complex IV enzyme activity at 26 weeks of age, an effect that was not associated with loss of mitochondrial number or mass. Since the respiratory chain enzymes are located within the inner membrane of the mitochondria, impairment of complex IV activity was expected given the observed deterioration of the inner membrane structural

integrity in this animal model following perinatal nicotine exposure. Conversely, we did not detect a change in citrate synthase activity. However, citrate synthase is located within the mitochondrial matrix and therefore does not depend on mitochondrial membrane integrity. Furthermore, since citrate synthase is an indicator of mitochondrial mass, a decline in activity would only be expected if nicotine exposure resulted in a reduction in the number of mitochondria.

We propose that exposure of the beta cell mitochondria to reactive oxygen species (ROS) likely contributed to the loss of respiratory enzyme function and mitochondrial structural integrity in this animal model. ROS have been shown to inactivate the iron-sulfur centers of the electron transport chain complexes, thus causing defects in mitochondrial energy production (32). In addition, when the function of one of the electron carrier complexes is impaired electrons are not shuttled properly through the electron transport chain (ETC) and are increasingly lost to molecular oxygen, resulting in increased ROS formation (19). We hypothesize that in our animal model, this cycle was initiated during fetal and neonatal exposure to nicotine, a compound shown to have pro-oxidant properties *in vitro* and *in vivo* (33-35). We have previously demonstrated that nicotine exposure during fetal and neonatal development leads to increased islet ROS production and oxidative damage at weaning (21). We propose that this nicotine-induced increase in ROS likely triggered early, but undetectable damage to the ETC enzymes, thus initiating a feed-forward chain of progressive mitochondrial damage and additional ROS production. Furthermore, once dysglycemia has

been established (by 15 weeks of age in the current study), chronic high glucose levels likely also contributed to the observed deterioration of pancreatic mitochondrial structure and function, as well as the loss of beta cell function. Chronic exposure to high glucose has previously been shown to induce mitochondrial-mediated beta cell apoptosis (36), as well as mitochondrial superoxide production and beta cell dysfunction in isolated islets (37). As predicted, during adulthood nicotine-exposed offspring had elevated islet ROS production that was associated with increased formation of protein carbonyl groups in isolated islets, an indication that the redox balance has been disrupted in these cells. Therefore, in this animal model, perinatal nicotine exposure increases islet ROS production both at the end of lactation (i.e. during the nicotine exposure) (21) prior to the observable changes in ETC enzyme activity, and at 26 weeks of age when impaired complex IV activity and the most pronounced mitochondrial structural abnormalities were observed. We predict that the early mitochondrial structural alterations are likely initiated by nicotine-induced ROS, whereas the dramatic worsening of these defects between 15 and 26 weeks may be a consequence of chronic exposure to high glucose combined with ROS production by previously damaged mitochondrial ETC enzymes.

Although only a subtle reduction in respiratory enzyme activity was detected in the whole pancreas at 26 weeks following nicotine exposure, this may represent a more profound change in the beta cells, which comprise approximately 1% of the adult rodent pancreas (14). Beta cells are known to be

particularly susceptible to ROS damage since they have relatively low expression of antioxidant enzymes (38;39). We observed dramatic mitochondrial structural abnormalities by electron microscopy in the beta cells of nicotine-exposed offspring as early as 3 weeks of age. On the contrary, loss of respiratory enzyme activity measured in the whole pancreas was not detectable until 26 weeks. Therefore, it is possible that whole tissue measurements were simply not sensitive enough to detect differences between saline and nicotine exposure at the level of the beta cell in the younger animals. We propose that as the damage to mitochondrial protein accumulates with age, these changes become detectable at the whole tissue level.

Based on the numerous mitochondrial defects observed in this study (both structural and functional), it was expected that the nicotine-exposed offspring in this animal model would have altered beta cell function. As anticipated, developmental nicotine exposure resulted in altered insulin granule morphology and impaired GSIS compared to saline controls at 26 weeks of age. Electron microscopy (EM) analysis revealed a reduction in the total number of insulin granules within beta cells of nicotine-exposed offspring. Furthermore, there was a pronounced reduction (18-fold) in the number of pale, immature secretory granules by 26 weeks, suggesting that proinsulin biosynthesis may be impaired in this animal model and thus contribute to the loss of beta cell function. Indeed, proinsulin gene transcription has been previously shown to be crucial for maintaining proinsulin biosynthesis, retaining islet insulin stores, and ultimately

regulating glucose homeostasis (40). However, this finding of fewer immature insulin granules conflicts with EM studies in other animal models of dysglycemia, which have reported increased numbers of immature granules (41;42). These differences may be related to the profound alterations in mitochondrial structure observed in our model relative to previous studies. For example, in other models where the number of immature secretory granules was increased, no changes in mitochondrial structure were reported (41;42). In contrast, a significant proportion of the mitochondria in nicotine-exposed offspring were visibly swollen and vacuolated (stage 3-5) by 15 (20%) and 26 (56%) weeks of age. Another major difference between our animal model and the Zucker *fa/fa* rat model is that in Zucker *fa/fa* rats beta cells with a high proportion of immature granules had *increased* sensitivity to glucose, suggesting that the beta cells in this animal model are hyperactive (42). In contrast, the nicotine-exposed rats in this animal model of dysglycemia exhibited a diminished ability to secrete insulin in response to a glucose stimulus. Similarly, transgenic mice with beta cell-specific mitochondrial defects have decreased GSIS (i.e. impaired beta cell function) (43;44). Therefore, the impaired GSIS observed in this study may be attributed to the inability of damaged mitochondria to: a) regulate proinsulin biosynthesis, and b) couple a glucose stimulus to insulin synthesis and exocytosis.

In conclusion, data from this study indicate that fetal and neonatal nicotine exposure adversely affects postnatal mitochondrial structure and function, which in turn leads to impaired beta cell function and dysglycemia in adult offspring.

These data suggest a mechanism to explain, in part, the increased risk of type 2 diabetes in children born to women who smoked during pregnancy. This study also provides further support to the recent concerns about the safety of nicotine replacement therapy during pregnancy and lactation (45).

5.5 REFERENCES

1. Andres,RL, Day,MC: Perinatal complications associated with maternal tobacco use. *Semin Neonatol* 5:231-241, 2000

2. Cnattingius,S, Lambe,M: Trends in smoking and overweight during pregnancy: prevalence, risks of pregnancy complications, and adverse pregnancy outcomes. *Semin Perinatol* 26:286-295, 2002

3. Faiz,AS, Ananth,CV: Etiology and risk factors for placenta previa: an overview and meta-analysis of observational studies. *J Matern Fetal Neonatal Med* 13:175-190, 2003

4. Nordentoft,M, Lou,HC, Hansen,D, Nim,J, Pryds,O, Rubin,P, Hemmingsen,R: Intrauterine growth retardation and premature delivery: the influence of maternal smoking and psychosocial factors. *Am J Public Health* 86:347-354, 1996

5. Robinson,JS, Moore,VM, Owens,JA, McMillen,IC: Origins of fetal growth restriction. *Eur J Obstet Gynecol Reprod Biol* 92:13-19, 2000

6. Shiverick,KT, Salafia,C: Cigarette smoking and pregnancy I: ovarian, uterine and placental effects. *Placenta* 20:265-272, 1999

7. Bergmann,KE, Bergmann,RL, Von,KR, Bohm,O, Richter,R, Dudenhausen,JW, Wahn,U: Early determinants of childhood overweight and adiposity in a birth cohort study: role of breast-feeding. *Int J Obes Relat Metab Disord* 27:162-172, 2003

8. Montgomery,SM, Ekbom,A: Smoking during pregnancy and diabetes mellitus in a British longitudinal birth cohort. *BMJ* 324:26-27, 2002

9. Power,C, Jefferis,BJ: Fetal environment and subsequent obesity: a study of maternal smoking. *Int J Epidemiol* 31:413-419, 2002

10. Toschke,AM, Koletzko,B, Slikker,W, Jr., Hermann,M, Von,KR: Childhood obesity is associated with maternal smoking in pregnancy. *Eur J Pediatr* 161:445-448, 2002

11. Vik,T, Jacobsen,G, Vatten,L, Bakketeig,LS: Pre- and post-natal growth in children of women who smoked in pregnancy. *Early Hum Dev* 45:245-255, 1996

12. Von,KR, Toschke,AM, Koletzko,B, Slikker,W, Jr.: Maternal smoking during pregnancy and childhood obesity. *Am J Epidemiol* 156:954-961, 2002

13. Wideroe,M, Vik,T, Jacobsen,G, Bakketeig,LS: Does maternal smoking during pregnancy cause childhood overweight? *Paediatr Perinat Epidemiol* 17:171-179, 2003

14. Bruin,JE, Kellenberger,LD, Gerstein,HC, Morrison,KM, Holloway,AC: Fetal and neonatal nicotine exposure and postnatal glucose homeostasis: identifying critical windows of exposure. *J Endocrinol* 194:171-178, 2007

15. Holloway,AC, Lim,GE, Petrik,JJ, Foster,WG, Morrison,KM, Gerstein,HC: Fetal and neonatal exposure to nicotine in Wistar rats results in increased beta cell apoptosis at birth and postnatal endocrine and metabolic changes associated with type 2 diabetes. *Diabetologia* 48:2661-2666, 2005

16. Ontario Medical Association. Rethinking stop-smoking medications: myths and facts. 1999.

17. Butler,AE, Janson,J, Bonner-Weir,S, Ritzel,R, Rizza,RA, Butler,PC: Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. *Diabetes* 52:102-110, 2003

18. Marchetti,P, Del,PS, Lupi,R, Del,GS: The pancreatic beta-cell in human Type 2 diabetes. *Nutr Metab Cardiovasc Dis* 16 Suppl 1:S3-S6, 2006

19. Wallace,DC: A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. *Annu Rev Genet* 39:359-407, 2005

20. Green,DR, Kroemer,G: The pathophysiology of mitochondrial cell death. *Science* 305:626-629, 2004

21. Bruin,JE, Petre,MA, Lehman,MA, Raha,S, Gerstein,HC, Morrison,KM, Holloway,AC: Maternal nicotine exposure increases oxidative stress in the offspring. *Free Radic Biol Med* 44:1919-1925, 2008

22. Bruin,JE, Gerstein,HC, Morrison,KM, Holloway,AC: Increased pancreatic beta cell apoptosis following fetal and neonatal exposure to nicotine is mediated via the mitochondria. *Toxicol Sci* 103:362-370, 2008

23. Maechler,P, de Andrade,PB: Mitochondrial damages and the regulation of insulin secretion. *Biochem Soc Trans* 34:824-827, 2006

24. Lowell,BB, Shulman,GI: Mitochondrial dysfunction and type 2 diabetes. *Science* 307:384-387, 2005

25. MacDonald,PE, Joseph,JW, Rorsman,P: Glucose-sensing mechanisms in pancreatic beta-cells. *Philos Trans R Soc Lond B Biol Sci* 360:2211-2225, 2005

26. Anello,M, Lupi,R, Spampinato,D, Piro,S, Masini,M, Boggi,U, Del,PS, Rabuazzo,AM, Purrello,F, Marchetti,P: Functional and morphological alterations of mitochondria in pancreatic beta cells from type 2 diabetic patients. *Diabetologia* 48:282-289, 2005

27. Simmons,RA, Suponitsky-Kroyter,I, Selak,MA: Progressive accumulation of mitochondrial DNA mutations and decline in mitochondrial function lead to beta-cell failure. *J Biol Chem* 280:28785-28791, 2005

28. Holloway,AC, Kellenberger,LD, Petrik,JJ: Fetal and neonatal exposure to nicotine disrupts ovarian function and fertility in adult female rats. *Endocrine* 30:213-216, 2006

29. Sun,MG, Williams,J, Munoz-Pinedo,C, Perkins,GA, Brown,JM, Ellisman,MH, Green,DR, Frey,TG: Correlated three-dimensional light and electron microscopy reveals transformation of mitochondria during apoptosis. *Nat Cell Biol* 9:1057-1065, 2007

30. Parise,G, Phillips,SM, Kaczor,JJ, Tarnopolsky,MA: Antioxidant enzyme activity is up-regulated after unilateral resistance exercise training in older adults. *Free Radic Biol Med* 39:289-295, 2005

31. Maechler,P, Carobbio,S, Rubi,B: In beta-cells, mitochondria integrate and generate metabolic signals controlling insulin secretion. *Int J Biochem Cell Biol* 38:696-709, 2006

32. Simmons,RA: Developmental origins of diabetes: the role of oxidative stress. *Free Radic Biol Med* 40:917-922, 2006

33. Wetscher,GJ, Bagchi,M, Bagchi,D, Perdikis,G, Hinder,PR, Glaser,K, Hinder,RA: Free radical production in nicotine treated pancreatic tissue. *Free Radic Biol Med* 18:877-882, 1995

34. Ozokutan,BH, Ozkan,KU, Sari,I, Inanc,F, Guldur,ME, Kilinc,M: Effects of maternal nicotine exposure during lactation on breast-fed rat pups. *Biol Neonate* 88:113-117, 2005

35. Husain,K, Scott,BR, Reddy,SK, Somani,SM: Chronic ethanol and nicotine interaction on rat tissue antioxidant defense system. *Alcohol* 25:89-97, 2001

36. Kim,WH, Lee,JW, Suh,YH, Hong,SH, Choi,JS, Lim,JH, Song,JH, Gao,B, Jung,MH: Exposure to chronic high glucose induces beta-cell apoptosis through decreased interaction of glucokinase with mitochondria: downregulation of glucokinase in pancreatic beta-cells. *Diabetes* 54:2602-2611, 2005

37. Tang,C, Han,P, Oprescu,AI, Lee,SC, Gyulkhandanyan,AV, Chan,GN, Wheeler,MB, Giacca,A: Evidence for a role of superoxide generation in glucose-induced beta-cell dysfunction in vivo. *Diabetes* 56:2722-2731, 2007

38. Lenzen,S, Drinkgern,J, Tiedge,M: Low antioxidant enzyme gene expression in pancreatic islets compared with various other mouse tissues. *Free Radic Biol Med* 20:463-466, 1996

39. Tiedge,M, Lortz,S, Drinkgern,J, Lenzen,S: Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells. *Diabetes* 46:1733-1742, 1997

40. Leibowitz,G, Uckaya,G, Oprescu,AI, Cerasi,E, Gross,DJ, Kaiser,N: Glucose-regulated proinsulin gene expression is required for adequate insulin production during chronic glucose exposure. *Endocrinology* 143:3214-3220, 2002

41. Momose,K, Nunomiya,S, Nakata,M, Yada,T, Kikuchi,M, Yashiro,T: Immunohistochemical and electron-microscopic observation of beta-cells in pancreatic islets of spontaneously diabetic Goto-Kakizaki rats. *Med Mol Morphol* 39:146-153, 2006

42. Chan,CB, Wright,GM, Wadowska,DW, MacPhail,RM, Ireland,WP, Sulston,KW: Ultrastructural and secretory heterogeneity of fa/fa (Zucker) rat islets. *Mol Cell Endocrinol* 136:119-129, 1998

43. Silva,JP, Kohler,M, Graff,C, Oldfors,A, Magnuson,MA, Berggren,PO, Larsson,NG: Impaired insulin secretion and beta-cell loss in tissue-specific knockout mice with mitochondrial diabetes. *Nat Genet* 26:336-340, 2000

44. Silva,JP, Larsson,NG: Manipulation of mitochondrial DNA gene expression in the mouse. *Biochim Biophys Acta* 1555:106-110, 2002

45. Ginzel,KH, Maritz,GS, Marks,DF, Neuberger,M, Pauly,JR, Polito,JR, Schulte-Hermann,R, Slotkin,TA: Critical review: nicotine for the fetus, the infant and the adolescent? *J Health Psychol* 12:215-224, 2007

5.6 FIGURES

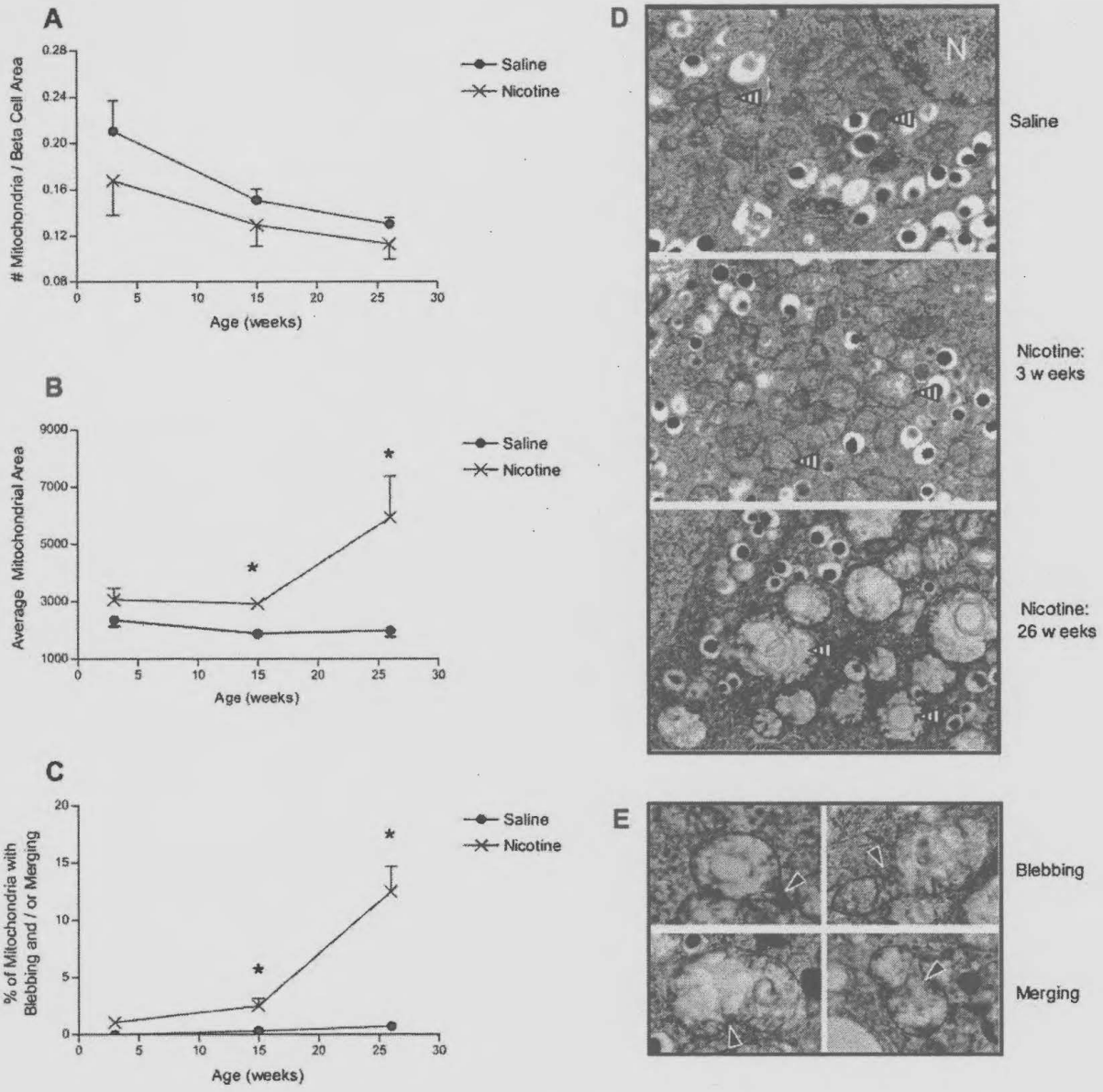
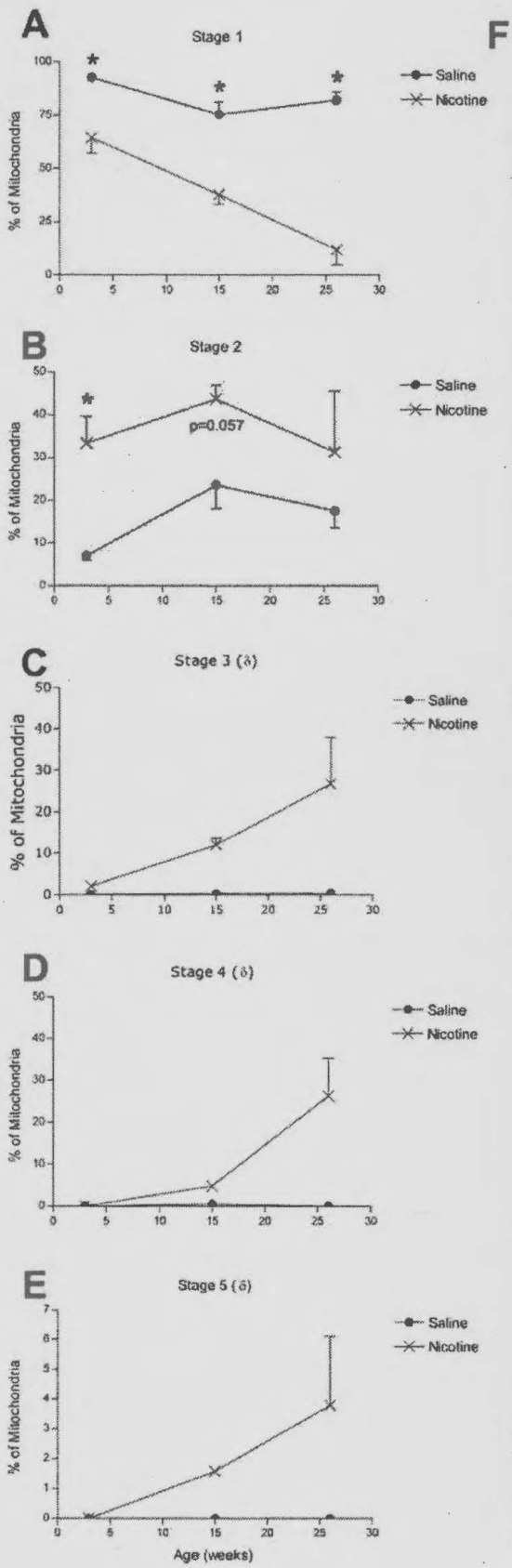


Figure 5.1: Mitochondrial morphology during postnatal development.
A) The number of mitochondria per beta cell area; B) individual mitochondrion area; and C) percentage of mitochondria with blebbing and / or merging with a neighboring mitochondria from offspring at 3, 15 and 26 weeks of age following exposure to either saline or nicotine during fetal and neonatal development. Representative electron microscopy photographs are provided to illustrate: D) typical mitochondrial structure (indicated by striped arrows) in the beta cells of saline and nicotine-exposed offspring during postnatal development, and E) examples of mitochondrial blebbing and merging (indicated by solid black arrows); N = nucleus. All data are presented as the mean ± SEM. Values with an asterisk are significantly different from the saline control ($p<0.05$).



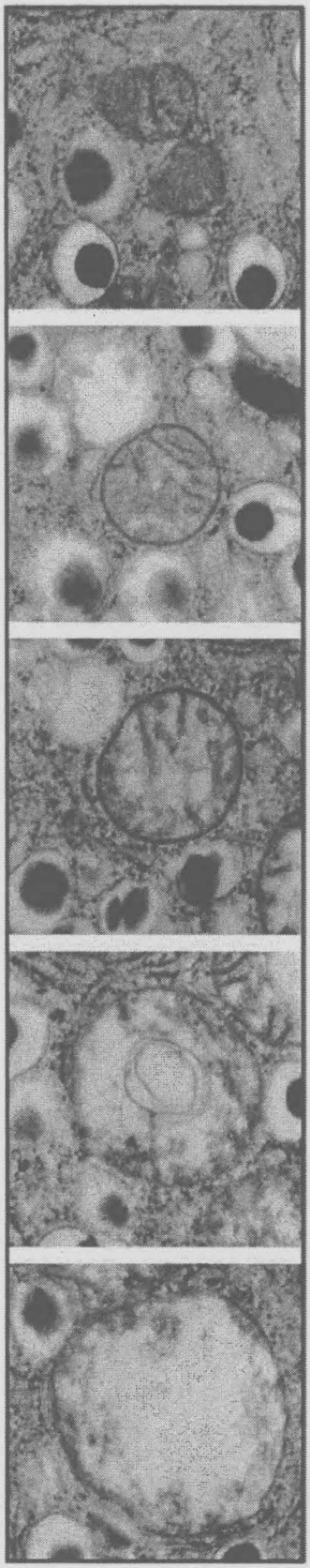


Figure 5.2: Stages of mitochondrial health during postnatal development. The percentage of mitochondria within beta cells at: A) stage 1, B) stage 2, C) stage 3, D) stage 4, and E) stage 5. F) Electron microscopy images with examples of mitochondria at each of the five morphological stages. δ indicates that statistical analysis was not performed on these data due to lack of variability in the saline treatment group replicates. All data are presented as mean ± SEM.

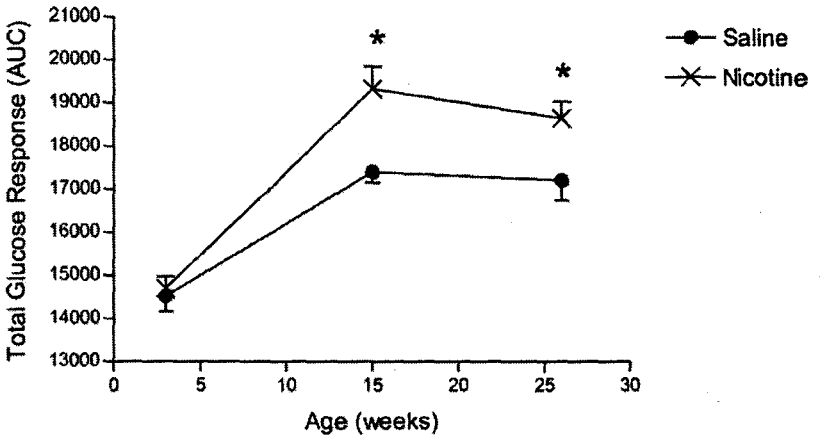


Figure 5.3: Oral glucose tolerance tests during postnatal development.
Area under the curve (AUC) for the total glucose response to an oral glucose challenge at 4, 15 and 26 weeks of age in saline (closed circles) and nicotine-exposed (open circles) animals. All data are presented as mean ± SEM. Values with an asterisk are significantly ($p<0.05$) different from saline controls.

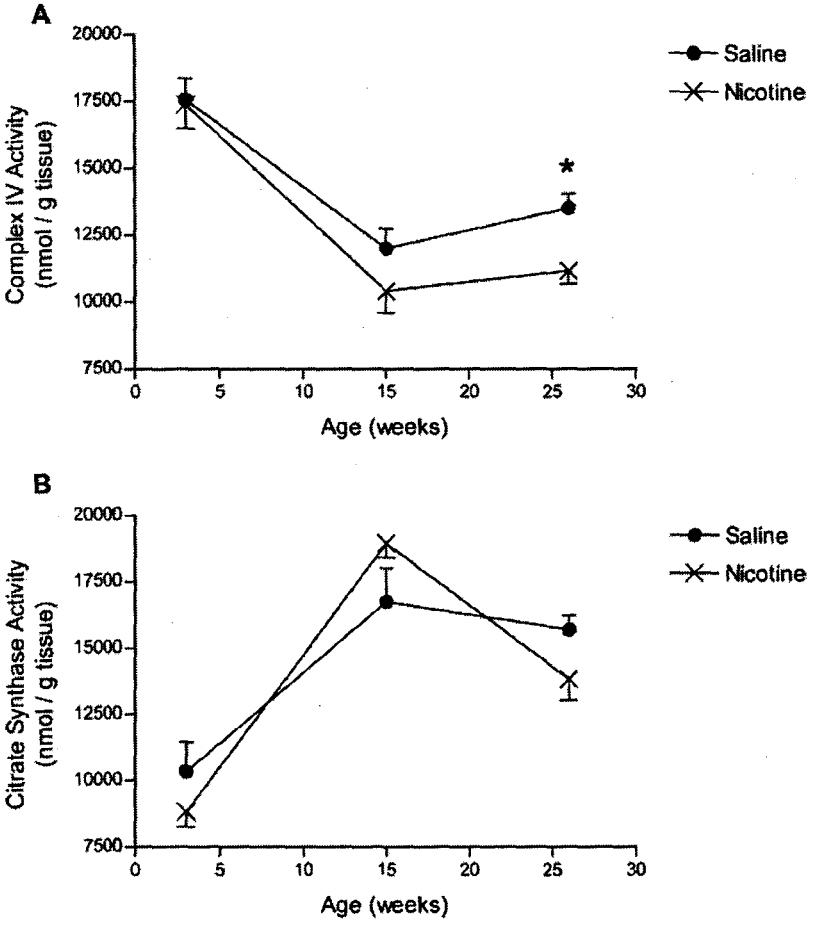


Figure 5.4: Mitochondrial enzyme activity during postnatal development.
A) Complex IV enzyme activity (an indicator of mitochondrial electron transport chain function) and B) citrate synthase enzyme activity (an indicator of mitochondrial mass) in the pancreas of saline- and nicotine-exposed offspring at 3, 15 and 26 weeks of age. All data are presented as mean ± SEM. Values with an asterisk are significantly ($p<0.05$) different from saline controls.

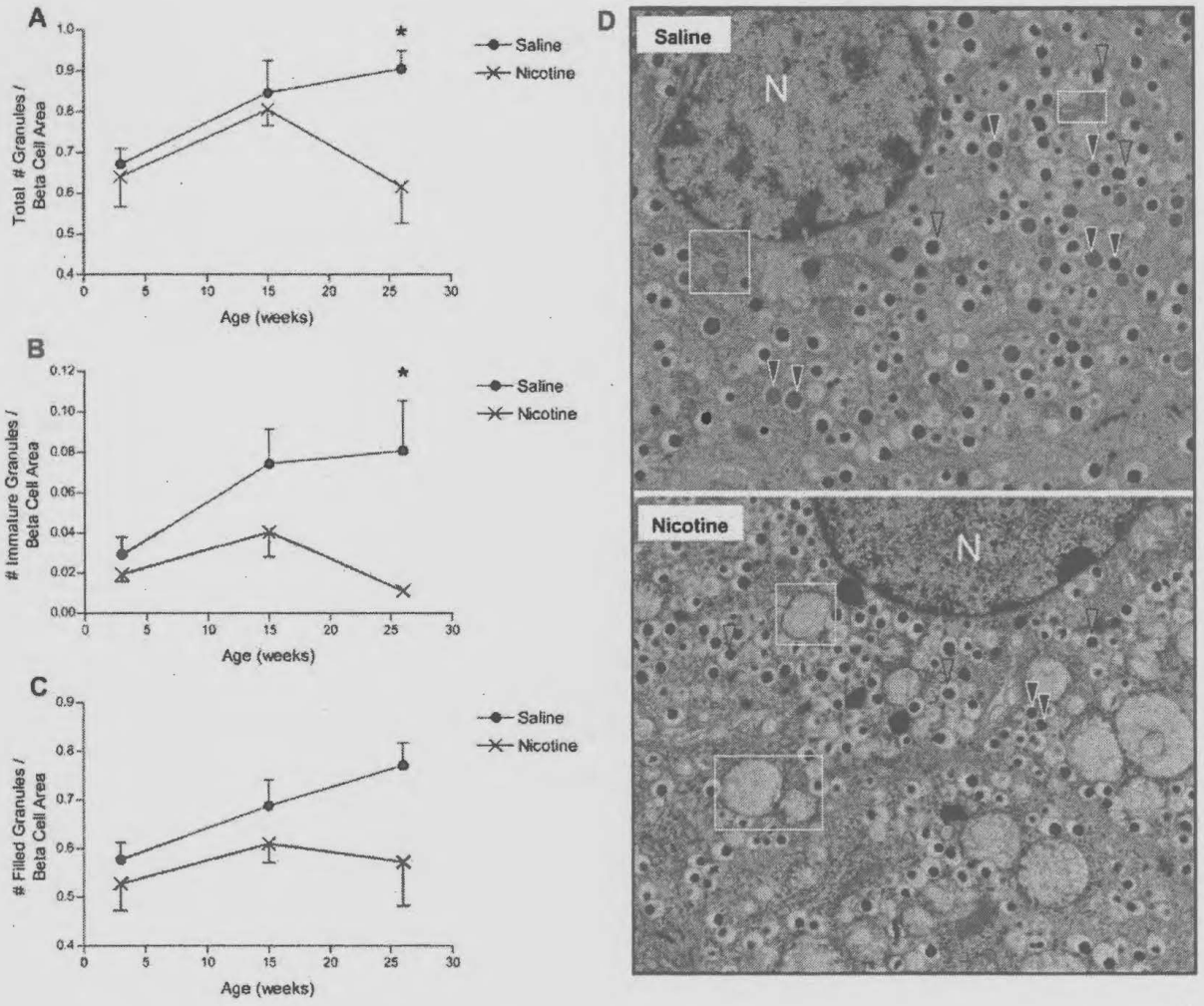


Figure 5.5: Insulin granule patterns during postnatal development.
The number per beta cell area of: A) total insulin granules (filled, immature and empty); B) immature insulin secretory granules (containing pale-staining proinsulin); and C) filled insulin granules (containing dense-core mature insulin). D) Representative electron microscopy photographs of saline and nicotine-exposed beta cells at 26 weeks of age illustrate both the typical insulin granule patterns (immature insulin granules are indicated by solid black arrows and mature insulin granules by striped arrows), and mitochondrial structures (indicated by white boxes); N = nucleus. All data are presented as mean ± SEM. Values with an asterisk are significantly ($p<0.05$) different from saline controls.

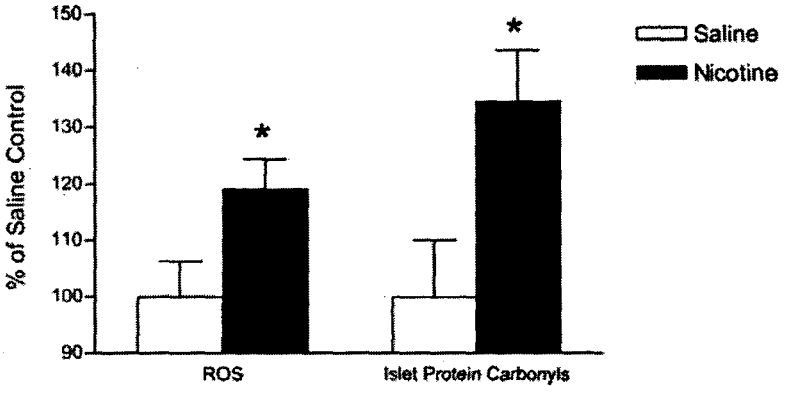


Figure 5.6: Oxidative stress at 26 weeks of age.
Reactive oxygen species (ROS) production and the incidence of protein carbonyl groups (an indication of oxidative damage to protein) in islets isolated from the pancreas of saline- (white bar) and nicotine-exposed (black bar) offspring at 26 weeks of age. All data are expressed as a percentage of the average saline control value and are presented as the mean ± SEM. Values with an asterisk are significantly ($p<0.05$) different from saline controls.

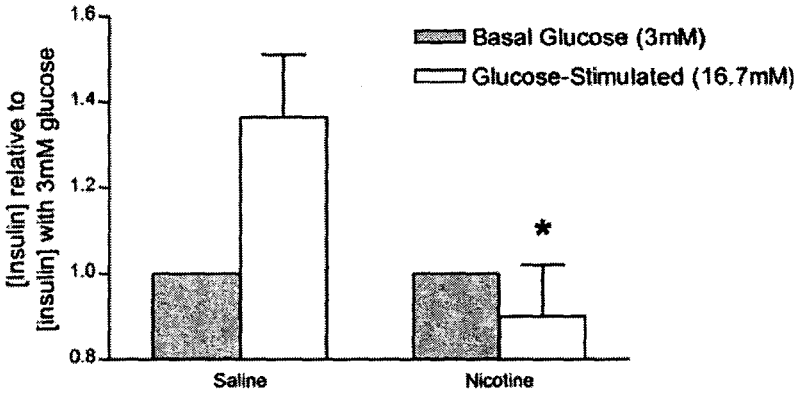


Figure 5.7: Glucose-stimulated insulin secretion at 26 weeks of age.
Insulin release into the media was measured following glucose stimulation (16.7mM; white bars) or basal glucose exposure (3.0mM; striped bars) in saline- and nicotine-expose offspring at 26 weeks of age. All data are expressed as the insulin concentration normalized to the insulin concentration under basal glucose conditions (3.0mM). Values with an asterisk indicate a significant difference in the stimulated / basal insulin release ratio for saline versus nicotine exposure ($p<0.05$). All data are presented as mean ± SEM.

CHAPTER 6

MATERNAL DIETARY ANTIOXIDANT SUPPLEMENTATION PREVENTS BETA CELL LOSS AND MITOCHONDRIAL DEFECTS FOLLOWING FETAL AND NEONATAL NICOTINE EXPOSURE.

BRUIN JE, WOYNILLOWICZ AK, HETTINGA BP, TARNOPOLSKY MA, GERSTEIN HC, HOLLOWAY AC. MATERNAL DIETARY ANTIOXIDANT SUPPLEMENTATION PREVENTS BETA CELL LOSS AND MITOCHONDRIAL DEFECTS FOLLOWING FETAL AND NEONATAL NICOTINE EXPOSURE. SUBMITTED TO *DIABETES*.

6.1 INTRODUCTION

Oxidative stress and redox signaling are essential processes for normal embryonic development (1). In particular, reactive oxygen species (ROS) are crucial for regulating cellular signaling and fate during healthy development (1). However, an increase in ROS that overwhelms the antioxidant defense systems can be detrimental, causing oxidative damage to proteins, DNA and lipids in susceptible tissues of the developing fetus. Pancreatic beta cells are known to be particularly sensitive to oxidative damage due to their relatively low expression of antioxidant enzymes compared to other cell types (2;3). We have previously shown that maternal exposure to nicotine (a known pro-oxidant), during pregnancy and lactation, causes increased islet ROS levels and pancreatic oxidative protein damage in the neonate (4). Furthermore, oxidative stress following nicotine exposure was associated with mitochondrial-mediated beta cell

apoptosis (5), loss of beta cell mass (6) and a progressive decline in mitochondrial function that coincided with the development of dysglycemia during adulthood in this animal model (7). Therefore, we hypothesized that maternal co-treatment with nicotine and an antioxidant cocktail during pregnancy and lactation would protect the developing beta cell mitochondria, thus preventing beta cell apoptosis and the consequent metabolic abnormalities in the adult offspring.

For this study we used an antioxidant diet containing coenzyme Q10 (CoQ), alpha lipoic acid (ALA) and vitamin E (VitE), which are known to act cooperatively as redox couples (8). Interventions containing different combinations of these antioxidants have been used successfully in various models of type 2 diabetes to improve markers of glucose homeostasis (9-13), and to protect beta cell mass and prevent beta cell apoptosis (9;13). Furthermore, CoQ, ALA and VitE have all been shown independently to prevent mitochondrial-mediated apoptosis in various adult cell types (14-19). However, to our knowledge this is the first study to test the ability of antioxidants to protect beta cells and their mitochondria from a toxic insult during fetal and neonatal development. Furthermore, although antioxidants have been shown to prevent dysglycemia and protect beta cell mass when treatment begins prior to the onset of symptoms (9;13), this is the first study to begin treatment during embryonic development. The goal of this study was to determine whether an antioxidant intervention during critical windows of fetal and neonatal pancreatic development could prevent the nicotine-induced beta cell defects in this animal model,

including loss of beta cell mass and impaired mitochondrial structure and function. We predicted that protection of beta cell mass and mitochondrial function would prevent the progression of postnatal mitochondrial defects which contribute to impaired beta cell function and dysglycemia during adulthood (6;7).

6.2 MATERIALS AND METHODS

6.2.1 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 200-250g female Wistar rats (Harlan, Indianapolis, IN, USA) 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 (n=10) or nicotine (n=20). Dams were injected with 1.0 mg/kg/day nicotine bitartrate (Sigma Aldrich, St. Louis, MO, USA) or saline subcutaneously for 14 days prior to mating, and during pregnancy until weaning (postnatal day 21). Nicotine-exposed dams received either normal chow (nicotine vehicle – NV; n=10) or diet containing antioxidants (nicotine antioxidant – NA; n=10) during mating, pregnancy and lactation. The antioxidant cocktail diet (Harlan Laboratories, Indianapolis, IN) contained coenzyme Q10 (0.25% w/w), α-lipoic acid (0.1% w/w) and vitamin E (1000 IU/kg). Saline-exposed dams received only the normal chow (saline vehicle - SV), as treatment with antioxidants may disrupt the healthy redox

balance, thus providing undesirable side effects. Dams were assessed throughout pregnancy and lactation to ensure that the antioxidant diet did not affect fertility, pre- and post-pregnancy maternal body weight, pregnancy weight gain or food consumption. For the fertility assessment, females were housed 1:1 with a male and monitored daily for confirmation of breeding (i.e. the presence of sperm in a vaginal flush). The day that a positive sign of copulation was observed was designated gestational day 0 (GD0). For each dam, time to pregnancy (days until detection of sperm in the vaginal flush), gestation length (GD0 to parturition), fecundity index (# pregnancies / # copulations x 100), and fertility index (# pregnancies / # females cohabited with male x 100) were determined.

At birth (postnatal day 1; PND1), litter size (total # pups per litter), live birth index (% live pups per litter), sex ratio (# male / # female pups), birth weight, total litter weight, number of LGA pups (large for gestational age; # pups born 2 standard deviations above the mean control body weight), and number of SGA pups (small for gestational age; # pups born 2 standard deviations below the mean control body weight) were assessed. Litter size was then culled to eight to ensure uniformity among treated and control litters. To eliminate any confounding effects of the female reproductive cycle, only male offspring were used in this study. At birth, 3, 7 and 26 weeks of age, animals were euthanized by CO2 asphyxiation, and pancreas tissue and fat pads (mesenteric, epididymal, and perirenal) were collected, weighed and processed for subsequent analysis.

6.2.2 Glucose Homeostasis

Glucose homeostasis was assessed at 26 weeks of age by oral glucose tolerance tests (OGTT; n=9 per group) and insulin tolerance tests (ITT; SV and NA: n=9, NV: n=8) using a maximum of one animal per litter. Briefly for the OGTT, after an overnight fast, rats were given 2g/kg glucose (Sigma-Aldrich, St. Louis, MO, USA) in water by gavage, as previously described (6;20). Insulin and glucose were then measured in saphenous vein samples, collected by repeated puncture, at baseline, 30 and 120 minutes. Glucose was measured using the Contour glucose meter (Bayer Inc., Toronto, ON). Following the glucose measurement, blood samples were collected, allowed to clot, centrifuged, and the serum stored at -80°C until analysis. Insulin concentrations were determined using an ultrasensitive rat insulin ELISA kit designed for small sample volumes (Crystal Chem Inc., Downers Grove, IL, USA). For the ITT, following an overnight fast, rats were given 1 IU/kg insulin (Novolin®ge Toronto, human biosynthetic insulin, Novo Nordisk, Mississauga ON) in saline by subcutaneous injection, as previously described (20;21). Glucose was measured using the Contour glucose meter (Bayer Inc., Toronto, ON) in saphenous vein samples, collected by repeated puncture, at baseline, 20, 40 and 60 minutes.

6.2.3 Electron Microscopy

Pancreas tissue from offspring at 3 weeks (SV: n=4, NA and NV: n=5 per group), 7 weeks (SV and NV: n=5 per group, NA: n=4), and 26 weeks (n=6 per

group) were collected and processed for electron microscopy as previously described (5). Grids were examined with a JEOL 1200EX transmission electron microscope (JEOL Ltd., Tokyo, Japan) and representative photographs were taken at 12000x magnification. All photographs (containing at least 500 mitochondria in the combined fields per animal) were analyzed by a single investigator blinded to the treatment groups using Image Pro Plus Version 5.1 software (Media Cybernetics, Inc., Silver Spring, MD, USA),

Mitochondrial morphology was assessed by quantifying the proportion of mitochondria in each of five defined stages of progressive deterioration (7). Stage 1 mitochondria were classified as structurally healthy, with dense, intact cristae. Stage 2 mitochondria had visible swelling, but maintained distinctive intact cristae structure. Stage 3 mitochondria had more severe swelling and minimal evidence of intact cristae. Stage 4 mitochondria displayed severe swelling, minimal cristae structure and formation of vacuoles. Stage 5 mitochondria were extremely large and swollen, with essentially complete loss of defined structure within the mitochondrial membrane. An example of mitochondria at each defined morphological stage is provided in Figure 6.3.

6.2.4 Beta Cell Fraction and Apoptosis

Beta cell fraction was measured in the offspring at birth, 3 and 26 weeks (n=6 per group at all ages). Immunohistochemical detection of insulin was performed on four paraffin-embedded sections (5μm) per animal, separated by a

minimum of 40μm, as previously described (6), using a polyclonal, guinea pig anti-swine insulin primary antibody (1:150 dilution) (DakoCytomation, Carpinteria, CA), the Vectastain anti-rabbit kit (Vector Laboratories, Burlinghame, CA), and diaminobenzadine (Sigma Aldrich, St. Louis, MO, USA) as the chromogen. For all sections, the whole pancreas was analyzed by combining measurements from up to 25 fields per section. Immunopositive cells were identified using Image Pro Plus Version 5.1 software (Media Cybernetics, Inc., Silver Spring MD) for automated cell counting. The beta cell fraction was calculated as the ratio of beta cell area (immunopositive staining) to total pancreas area (immunopositive staining plus pancreas counterstaining) x 100.

Beta cell apoptosis was measured in offspring at 3 (n=6 per group), 7 (n=6 per group) and 26 weeks of age (n=5 per group). Detection of apoptotic beta cells was performed using a triple immunofluorescent staining protocol as previously described (6). Briefly, insulin was detected using a polyclonal, guinea pig anti-swine insulin antibody (1:150 dilution, DakoCytomation, Carpinteria, CA), followed by an anti-rabbit Alexa Fluor 594 secondary antibody (1:400 dilution, Molecular Probes, Inc., Eugene OR). Next, tissues were subjected to the terminal deoxynucleotidyl transferase mediated dUTP nick end labelling (TUNEL) assay according to manufacturer's instructions (Roche Applied Science, Laval, Quebec). Finally, nuclei were counterstained with DAPI (Sigma Aldrich, St. Louis, MO) and tissue sections were imaged with a Leica DMRA2 microscope using Openlab software version 4.0.2 (Improvision, Waltham MA). Images were

analyzed with Image Pro Plus Version 5.1 software (Media Cybernetics, Inc., Silver Spring MD); five islets per section were quantified and reported as the percentage of TUNEL+ beta cells.

6.2.5 Reactive Oxygen Species in Isolated Islets

Reactive oxygen species (ROS) levels were quantified in isolated islets using 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Molecular Probes Inc., Eugene, OR, USA) fluorescence at 7 (n=5 per group) and 26 weeks (n=6 per group) as previously described (4). Briefly, pancreas tissue was digested with collagenase, and islets were manually picked using a small glass pipette and a dissecting microscope. Following an overnight equilibration period, 80 islets were hand-picked and incubated in 100µM H_2DCFDA for 3 h at 37°C. Fluorescence of the 2',7'-dichlorofluorescein product was determined using a SpectaMax Gemini XS (Molecular Devices Corp., Sunnyvale, CA, USA) microplate spectrofluorometer at excitation and emission wavelengths of 505 nm and 540 nm, respectively. Since H_2DCFDA must be made fresh immediately prior to use, islets isolated on different days were incubated in different batches of reagent. To account for day-to-day variability within the experiment, all measurements of islet ROS production were normalized to the average saline control value for each experiment.

6.2.6 Statistical Analysis

All statistical analyses were performed using Statistica software (version 7.0, StatSoft Inc.) and one-way ANOVA using orthogonal comparisons based on a priori hypotheses. Specifically, the planned comparisons included: 1) whether there was a difference between fetal and neonatal nicotine relative to saline exposure (SV versus NV); 2) whether the co-treatment of dams with nicotine and antioxidants prevented the nicotine-induced defects (NV versus NA); and 3) whether the maternal antioxidant treatment allowed for complete maintenance at saline control levels (NA versus SV). 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. Categorical variables (fertility and fecundity index) were compared using Fisher's exact test. For all outcomes at birth (litter size, live birth index, sex ratio, birth weight, # LGA, # SGA) the statistical unit was the litter. Area under the curve (AUC) for the offspring body weight (4-26 weeks) was assessed using the trapezoidal rule.

6.3 RESULTS

6.3.1 Maternal Outcomes

There were no differences in maternal food consumption or pregnancy weight gain between any of the three treatment groups (data not shown). Furthermore, fertility was unaffected by either nicotine treatment or the antioxidant diet; no differences were observed in time to pregnancy, gestation

length, fecundity index or fertility index between treatment groups (data not shown).

6.3.2 Litter Outcomes

No differences were observed between any of the three treatment groups for litter size, live birth index, sex ratio, total litter weight, or number of LGA pups (Table 6.1). However, exposure to the antioxidant diet did cause a significant reduction in the birth weight compared to pups exposed to nicotine and normal chow (Table 6.1). Birth weight for the SV offspring was not significantly different than either NA or NV (Table 6.1). Furthermore, the antioxidant-treated offspring also had a significant increase in the number of SGA pups per litter compared to both NV and SV (Table 6.1).

6.3.3 Postnatal Body Composition and Glucose Homeostasis

Maternal antioxidant exposure during fetal and neonatal development caused a transient, but significant reduction in body weight and proportion of body fat at weaning (Figure 6.1A and C) in the offspring. However, by 7 weeks of age (4 weeks post-treatment), the fat pad weight was no longer significantly different than the SV or NV groups (Figure 6.1D), although the body weight was still significantly lower (Figure 6.1B). By 26 weeks of age the body weight of the NA group was not significantly different from either the SV or NV animals (SV:

608.6 ± 10.87 g, NV: 629.5 ± 7.11 g, NA: 602.8 ± 12.01 g; $p>0.05$). Over the study period, nicotine-exposed offspring tended to be heavier than saline controls and were significantly heavier than the NA offspring, as measured by the AUC for body weight between 4 and 26 weeks of age (Figure 6.2C).

Fetal and neonatal nicotine exposure resulted in dysglycemia during adulthood. At 26 weeks of age, NV offspring had significantly increased fasting glucose levels relative to saline controls (Figure 6.2A), but no difference in fasting insulin (data not shown). There were no differences in the AUC for the glucose or insulin response to the OGTT (data not shown). However, at 120 minutes following an oral glucose challenge, NV offspring had a significantly reduced insulin:glucose ratio (Figure 6.2B), implying that there was an insufficient amount of insulin produced to maintain glucose homeostasis. There were no differences between the groups in the response to the ITT (data not shown). Taken together, the impaired fasting glucose, altered response to the OGTT and increased body weight indicate altered metabolic homeostasis in the NV offspring compared to saline controls. Treatment with antioxidants partially prevented the nicotine-induced increase in fasting glucose levels and altered post-GTT insulin:glucose ratio (Figure 6.2A and B).

6.3.4 Beta Cell Defects

Fetal and neonatal nicotine exposure caused mitochondrial defects in the beta cells beginning at 3 weeks of age, as indicated by a significant reduction in

the proportion of stage 1 mitochondria and an increase in stage 2 mitochondria (Figure 6.3). Co-treatment of the dams with nicotine and the antioxidant cocktail completely prevented this early loss of structural integrity in the offspring (Figure 6.3). Beta cell mitochondrial ultrastructure in the nicotine-exposed offspring continued to deteriorate with advancing age, despite discontinuation of nicotine exposure at weaning (Figure 6.4). At all ages examined, more than 75% of mitochondria in saline-exposed offspring were in stages 1-2 (Figure 6.4A). In contrast, by 26 weeks of age, over half of the NV mitochondria were in stages 3-5 (Figure 6.4B). Fetal and neonatal treatment with antioxidants completely prevented this decline in mitochondrial structure, such that the proportion of mitochondria in either stages 1-2 or 3-5 in NA beta cells was not significantly different than SV at any age (Figure 6.4A-B).

Fetal and neonatal nicotine exposure caused a significant increase in postnatal beta cell apoptosis at all ages examined (3, 7 and 26 weeks) relative to saline exposure. This effect was completely prevented by co-treatment with the antioxidant cocktail (Figure 6.5).

Nicotine exposure resulted in a permanent loss of the pancreatic beta cell fraction beginning at birth and persisting until 26 weeks of age relative to saline controls (Figure 6.6). Co-treatment with antioxidants completely prevented the beta cell loss at birth and 3 weeks of age, but by 26 weeks, the beta cell fraction in NA offspring was not significantly different than either SV or NV offspring (Figure 6.6).

Isolated islets from nicotine-exposed offspring had significantly higher levels of reactive oxygen species (ROS) at both 7 and 26 weeks relative to saline controls (Figure 6.7). The antioxidant intervention completely prevented the increased ROS levels at 7 weeks, but not at 26 weeks (Figure 6.7).

6.4 DISCUSSION

We have previously shown that fetal and neonatal nicotine exposure results in postnatal pancreatic oxidative stress and mitochondrial defects, which subsequently leads to adult-onset dysglycemia and related metabolic abnormalities (4;5;7). Results from the current study demonstrate that developmental exposure to an antioxidant cocktail containing coenzyme Q10 (CoQ), alpha lipoic acid (ALA) and vitamin E (VitE) prevents nicotine-induced beta cell apoptosis, loss of beta cell fraction, mitochondrial swelling and increased reactive oxygen species (ROS) levels. Although the antioxidant intervention was provided only until weaning (postnatal day 21), the protective effects of this treatment on beta cell apoptosis and mitochondria ultrastructure persisted until adulthood (26 weeks). Furthermore, the dysglycemia in nicotine-exposed adult offspring was partially improved by the antioxidant intervention.

For this study we opted to treat only the nicotine-exposed dams with the antioxidant cocktail, since maintenance of a healthy oxidative balance is particularly important during pregnancy (1). We predicted that an antioxidant intervention in healthy, saline-treated dams without the presence of a pro-oxidant

would cause undesirable side effects. Indeed, ALA has been shown to protect beta cells, but only in the presence of a pro-oxidant (22;23); ALA treatment of healthy, unstressed beta cells led to *decreased* beta cell function and viability (22;23). Similarly, diabetic rats treated with ALA showed improved albuminuria and kidney pathology, whereas in healthy rats ALA acted as a pro-oxidant and contributed to renal dysfunction (24).

The antioxidant cocktail used in this study was designed carefully to contain compounds that are known to: a) improve mitochondrial dysfunction, b) work cooperatively as redox couples and c) prevent mitochondrial-mediated apoptosis (8;14-19). CoQ protects the mitochondria through a number of mechanisms, including its ability to act as a free radical scavenger (25), inhibit mitochondrial depolarization independent of its free radical scavenging property (17), and function as a redox couple (8;26). Vitamin E (α-tocopherol) is a lipid soluble, chain breaking, terminating antioxidant that acts primarily to scavenge lipid peroxyl radicals (8), and has also been shown to protect cells from mitochondrial damage (19). ALA functions as an essential co-factor for several mitochondrial enzyme complexes, a free radical scavenger, and an important redox couple for CoQ and VitE (8). We propose that the free radical scavenging properties of these antioxidants likely prevented nicotine from triggering the initial oxidative insult to mitochondrial proteins, and the subsequent cycle of electron leakage by dysfunctional respiratory chain enzymes which causes further ROS formation. Indeed, at 7 weeks of age (4 weeks after cessation of all treatments),

nicotine-exposed offspring had increased islet ROS levels and further deterioration of beta cell mitochondrial structure, suggesting that the cycle of mitochondrial damage / ROS formation was initiated during fetal and neonatal development. Importantly, both the increased ROS levels and decline in mitochondrial structure were completely prevented at 7 weeks of age by the developmental intervention with antioxidants. Furthermore, while the mitochondria continued to deteriorate in the beta cells of NV offspring, the protective effect of antioxidants on the mitochondria persisted until 26 weeks, despite only transient maintenance of ROS levels.

The observed protection from nicotine-induced beta cell apoptosis in this study following maternal antioxidant consumption is likely related to the prevention of nicotine-induced mitochondrial damage. Mitochondrial swelling is a key initiating event for the mitochondrial-mediated apoptotic pathway, resulting in release of proteins such as cytochrome *c* into the cytosol and a caspase-mediated signaling cascade (27). We have shown previously that fetal and neonatal nicotine exposure causes mitochondrial swelling and triggers the mitochondrial-mediated apoptotic pathway at weaning (5). Because the antioxidants chosen for this study can prevent mitochondrial-mediated apoptosis (14-19), we predicted that maternal administration of this cocktail would prevent the nicotine-induced mitochondrial swelling, beta cell apoptosis and loss of beta cell mass we have previously observed in this model (5-7). Indeed, co-administration of the antioxidant cocktail with nicotine completely protected the

offspring from nicotine-induced mitochondrial swelling (Figure 6.3 and 6.4), beta cell apoptosis (Figure 6.5), and loss of beta cell fraction (Figure 6.6), such that the NA offspring were not different from the saline-exposed control offspring at any age examined. Furthermore, the prevention of nicotine-induced beta cell defects by the antioxidant treatment was also associated with improved regulation of glucose homeostasis in adulthood. In humans, a 40–60% reduction in beta-cell mass has been observed in patients with type 2 diabetes prior to diagnosis compared to weight-matched controls (28-30). Furthermore, the inability to produce sufficient insulin for maintenance of glucose homeostasis has been observed prior to the diagnosis of type 2 diabetes (31) and is thought to be attributed to this reduction in beta cell mass (32;33). Therefore, the preservation of beta cell mass in this animal model with the use of an antioxidant cocktail may have implications for the maintenance of beta cell mass and thus prevention of type 2 diabetes in humans.

However, there may be some potential limitations to the protective effects of this antioxidant cocktail in our animal model. First, it appears that at least some of the protective effects of the antioxidants may not be permanent. By 26 weeks, although the beta cell fraction in the NA offspring was 40% higher than the NV offspring, it was no longer significantly different from the nicotine-exposed animals. In addition, the NA and NV offspring both had increased islet ROS levels at 26 weeks compared to SV offspring. Secondly, the antioxidant intervention caused a reduction in body weight at birth, which persisted for

approximately 15 weeks after cessation of the antioxidant treatment, at which point the body weight recovered to control levels. Although this finding did not adversely influence the protective effects of the antioxidant diet on beta cells, it is certainly of concern given the association of intrauterine growth restriction with adult-onset diseases, including the metabolic syndrome (34). We predict that the low birth weight following antioxidant treatment in our study may be attributed to the presence of vitamin E in the diet. CoQ and ALA have both been safely used during pregnancy with no reported adverse effects on birth weight (35;36). On the contrary, concerns have been raised about the use of VitE during pregnancy for the treatment of pre-eclampsia, due to an increased incidence of low birth weight babies born to women supplemented with vitamin E and vitamin C (37). Knowing the effects of our antioxidant cocktail on low birth weight in this study, we would propose a follow-up study to compare the protective effects of the maternal diet with and without vitamin E, and to determine whether CoQ and ALA alone could protect the beta cell mitochondria from nicotine without affecting birth weight.

Results from this study demonstrate that an antioxidant intervention during pregnancy and lactation can protect developing beta cell mitochondria from the damaging pro-oxidant effects of nicotine, and thus preserve beta cell survival and function. These findings have several important clinical implications. First, we have shown that this antioxidant cocktail may be a useful tool for protecting beta cells from oxidative stress during critical windows of fetal and neonatal

development. Aside from nicotine exposure, other common conditions that are known to be associated with oxidative stress during pregnancy include: preeclampsia, diabetes, smoking, malnutrition or excessive nutrition, infection and inflammation (38). Secondly, this study has provided insight into a potential harm-reduction strategy for women who are highly dependent on nicotine, the addictive component of cigarettes, and rely on nicotine replacement therapy (NRT) as a safer alternative to smoking during pregnancy. We propose that for women who are unable to quit smoking by other means, the use of NRT supplemented with an antioxidant cocktail may be a safer alternative for the developing fetus. However, the efficacy of an antioxidant intervention would have to be carefully evaluated before recommendation for pregnant women, especially considering the potential adverse effects of vitamin E on birth weight.

6.5 REFERENCES

1. Dennery,PA: Effects of oxidative stress on embryonic development. *Birth Defects Res C Embryo Today* 81:155-162, 2007

2. Lenzen,S, Drinkgern,J, Tiedge,M: Low antioxidant enzyme gene expression in pancreatic islets compared with various other mouse tissues. *Free Radic Biol Med* 20:463-466, 1996

3. Tiedge,M, Lortz,S, Drinkgern,J, Lenzen,S: Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells. *Diabetes* 46:1733-1742, 1997

4. Bruin,JE, Petre,MA, Lehman,MA, Raha,S, Gerstein,HC, Morrison,KM, Holloway,AC: Maternal nicotine exposure increases oxidative stress in the offspring. *Free Radic Biol Med* 44:1919-1925, 2008

5. Bruin,JE, Gerstein,HC, Morrison,KM, Holloway,AC: Increased pancreatic beta cell apoptosis following fetal and neonatal exposure to nicotine is mediated via the mitochondria. *Toxicol Sci* 103:362-370, 2008

6. Bruin,JE, Kellenberger,LD, Gerstein,HC, Morrison,KM, Holloway,AC: Fetal and neonatal nicotine exposure and postnatal glucose homeostasis: identifying critical windows of exposure. *J Endocrinol* 194:171-178, 2007

7. Bruin,JE, Petre,MA, Raha,S, Morrison,KM, Gerstein,HC, Holloway,AC: Fetal and neonatal nicotine exposure in Wistar rats causes progressive pancreatic mitochondrial damage and beta cell dysfunction. *PLoS One* 3:e3371, 2008

8. Tarnopolsky,MA: The mitochondrial cocktail: rationale for combined nutraceutical therapy in mitochondrial cytopathies. *Adv Drug Deliv Rev* 60:1561-1567, 2008

9. Kaneto,H, Kajimoto,Y, Miyagawa,J, Matsuoka,T, Fujitani,Y, Umayahara,Y, Hanafusa,T, Matsuzawa,Y, Yamasaki,Y, Hori,M: Beneficial effects of antioxidants in diabetes: possible protection of pancreatic beta-cells against glucose toxicity. *Diabetes* 48:2398-2406, 1999

10. Sena,CM, Nunes,E, Gomes,A, Santos,MS, Proenca,T, Martins,MI, Seica,RM: Supplementation of coenzyme Q10 and alpha-tocopherol lowers glycated hemoglobin level and lipid peroxidation in pancreas of diabetic rats. *Nutr Res* 28:113-121, 2008

11. Ihara,Y, Yamada,Y, Toyokuni,S, Miyawaki,K, Ban,N, Adachi,T, Kuroe,A, Iwakura,T, Kubota,A, Hiai,H, Seino,Y: Antioxidant alpha-tocopherol ameliorates glycemic control of GK rats, a model of type 2 diabetes. *FEBS Lett* 473:24-26, 2000

12. Slyvka,Y, Inman,SR, Malgor,R, Jackson,EJ, Yee,J, Oshogwemoh,O, Adame,J, Nowak,FV: Protective effects of antioxidant-fortified diet on renal function and metabolic profile in obese Zucker rat. *Endocrine* 35:89-100, 2009

13. Song,KH, Lee,WJ, Koh,JM, Kim,HS, Youn,JY, Park,HS, Koh,EH, Kim,MS, Youn,JH, Lee,KU, Park,JY: alpha-Lipoic acid prevents diabetes mellitus in diabetes-prone obese rats. *Biochem Biophys Res Commun* 326:197-202, 2005

14. Tharakan,B, Holder-Haynes,JG, Hunter,FA, Childs,EW: Alpha lipoic acid attenuates microvascular endothelial cell hyperpermeability by inhibiting the intrinsic apoptotic signaling. *Am J Surg* 195:174-178, 2008

15. Naderi,J, Somayajulu-Nitu,M, Mukerji,A, Sharda,P, Sikorska,M, Borowy-Borowski,H, Antonsson,B, Pandey,S: Water-soluble formulation of Coenzyme Q10 inhibits Bax-induced destabilization of mitochondria in mammalian cells. *Apoptosis* 11:1359-1369, 2006

16. Moon,Y, Lee,KH, Park,JH, Geum,D, Kim,K: Mitochondrial membrane depolarization and the selective death of dopaminergic neurons by rotenone: protective effect of coenzyme Q10. *J Neurochem* 93:1199-1208, 2005

17. Papucci,L, Schiavone,N, Witort,E, Donnini,M, Lapucci,A, Tempestini,A, Formigli,L, Zecchi-Orlandini,S, Orlandini,G, Carella,G, Brancato,R, Capaccioli,S: Coenzyme q10 prevents apoptosis by inhibiting mitochondrial depolarization independently of its free radical scavenging property. *J Biol Chem* 278:28220-28228, 2003

18. Moreira,PI, Santos,MS, Sena,C, Nunes,E, Seica,R, Oliveira,CR: CoQ10 therapy attenuates amyloid beta-peptide toxicity in brain mitochondria isolated from aged diabetic rats. *Exp Neurol* 196:112-119, 2005

19. de Arriba,G, de Hornedo,JP, Rubio,SR, Fernandez,MC, Martinez,SB, Camarero,MM, Cid,TP: Vitamin E protects against the mitochondrial damage caused by cyclosporin A in LLC-PK1 cells. *Toxicol Appl Pharmacol* 2009

20. Simmons,RA, Templeton,LJ, Gertz,SJ: Intrauterine growth retardation leads to the development of type 2 diabetes in the rat. *Diabetes* 50:2279-2286, 2001

21. Durham,HA, Truett,GE: Development of insulin resistance and hyperphagia in Zucker fatty rats. *Am J Physiol Regul Integr Comp Physiol* 290:R652-R658, 2006

22. Schroeder,MM, Belloto,RJ, Jr., Hudson,RA, McInerney,MF: Effects of antioxidants coenzyme Q10 and lipoic acid on interleukin-1 beta-mediated inhibition of glucose-stimulated insulin release from cultured mouse pancreatic islets. *Immunopharmacol Immunotoxicol* 27:109-122, 2005

23. Lee,BW, Kwon,SJ, Chae,HY, Kang,JG, Kim,CS, Lee,SJ, Yoo,HJ, Kim,JH, Park,KS, Ihm,SH: Dose-related cytoprotective effect of alpha-lipoic acid on hydrogen peroxide-induced oxidative stress to pancreatic beta cells. *Free Radic Res* 43:68-77, 2009

24. Bhatti,F, Mankhey,RW, Asico,L, Quinn,MT, Welch,WJ, Maric,C: Mechanisms of antioxidant and pro-oxidant effects of alpha-lipoic acid in the diabetic and nondiabetic kidney. *Kidney Int* 67:1371-1380, 2005

25. Crane,FL: Biochemical functions of coenzyme Q10. *J Am Coll Nutr* 20:591-598, 2001

26. Lass,A, Sohal,RS: Electron transport-linked ubiquinone-dependent recycling of alpha-tocopherol inhibits autooxidation of mitochondrial membranes. *Arch Biochem Biophys* 352:229-236, 1998

27. Wallace,DC: A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. *Annu Rev Genet* 39:359-407, 2005

28. Butler,AE, Janson,J, Bonner-Weir,S, Ritzel,R, Rizza,RA, Butler,PC: Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. *Diabetes* 52:102-110, 2003

29. Sakuraba,H, Mizukami,H, Yagihashi,N, Wada,R, Hanyu,C, Yagihashi,S: Reduced beta-cell mass and expression of oxidative stress-related DNA damage in the islet of Japanese Type II diabetic patients. *Diabetologia* 45:85-96, 2002

30. Yoon,KH, Ko,SH, Cho,JH, Lee,JM, Ahn,YB, Song,KH, Yoo,SJ, Kang,MI, Cha,BY, Lee,KW, Son,HY, Kang,SK, Kim,HS, Lee,IK, Bonner-Weir,S: Selective beta-cell loss and alpha-cell expansion in patients with type 2 diabetes mellitus in Korea. *J Clin Endocrinol Metab* 88:2300-2308, 2003

31. Kahn,SE: The relative contributions of insulin resistance and beta-cell dysfunction to the pathophysiology of Type 2 diabetes. *Diabetologia* 46:3-19, 2003

32. Leahy,JL: Pathogenesis of type 2 diabetes mellitus. *Arch Med Res* 36:197-209, 2005

33. Rhodes,CJ: Type 2 diabetes-a matter of beta-cell life and death? *Science* 307:380-384, 2005

34. Kanaka-Gantenbein,C, Mastorakos,G, Chrousos,GP: Endocrine-related causes and consequences of intrauterine growth retardation. *Ann N Y Acad Sci* 997:150-157, 2003

35. Teran,E, Hernandez,I, Nieto,B, Tavara,R, Ocampo,JE, Calle,A: Coenzyme Q10 supplementation during pregnancy reduces the risk of pre-eclampsia. *Int J Gynaecol Obstet* 105:43-45, 2009

36. Al Ghafli,MH, Padmanabhan,R, Kataya,HH, Berg,B: Effects of alpha-lipoic acid supplementation on maternal diabetes-induced growth retardation and congenital anomalies in rat fetuses. *Mol Cell Biochem* 261:123-135, 2004

37. Poston,L, Briley,AL, Seed,PT, Kelly,FJ, Shennan,AH: Vitamin C and vitamin E in pregnant women at risk for pre-eclampsia (VIP trial): randomised placebo-controlled trial. *Lancet* 367:1145-1154, 2006

38. Luo,ZC, Fraser,WD, Julien,P, Deal,CL, Audibert,F, Smith,GN, Xiong,X, Walker,M: Tracing the origins of "fetal origins" of adult diseases: programming by oxidative stress? *Med Hypotheses* 66:38-44, 2006

6.6 TABLES AND FIGURES

Table 6.1: Litter outcomes at birth (postnatal day 1).

	SV	NV	NA
# Live pups / litter	13.90 ± 1.069	13.58 ± 1.026	14.67 ± 0.972
# Dead pups / litter	0.00 ± 0.000	0.08 ± 0.083	0.67 ± 0.667
Litter size	13.90 ± 1.069	13.67 ± 1.047	15.33 ± 0.972
Live Birth Index (%)	100.00 ± 0.000	99.51 ± 0.490	96.30 ± 3.704
Sex Ratio (M to F)	0.96 ± 0.227	1.08 ± 0.199	1.49 ± 0.207
Average body weight (g)	6.00 ± 0.279[ab]	6.14 ± 0.135[a]	5.45 ± 0.197[b]
Total litter weight (g)	80.01 ± 4.064	81.84 ± 5.158	78.93 ± 3.745
# LGA	0.11 ± 0.110	0.17 ± 0.167	0.11 ± 0.111
# SGA	0.10 ± 0.100[a]	0.00 ± 0.000[a]	0.50 ± 0.189[b]

SV: saline vehicle. NV: nicotine vehicle. NA: nicotine antioxidant. LGA: large for gestational age. SGA: small for gestational age. M: male, F: female. All data are expressed as the mean ± SEM.

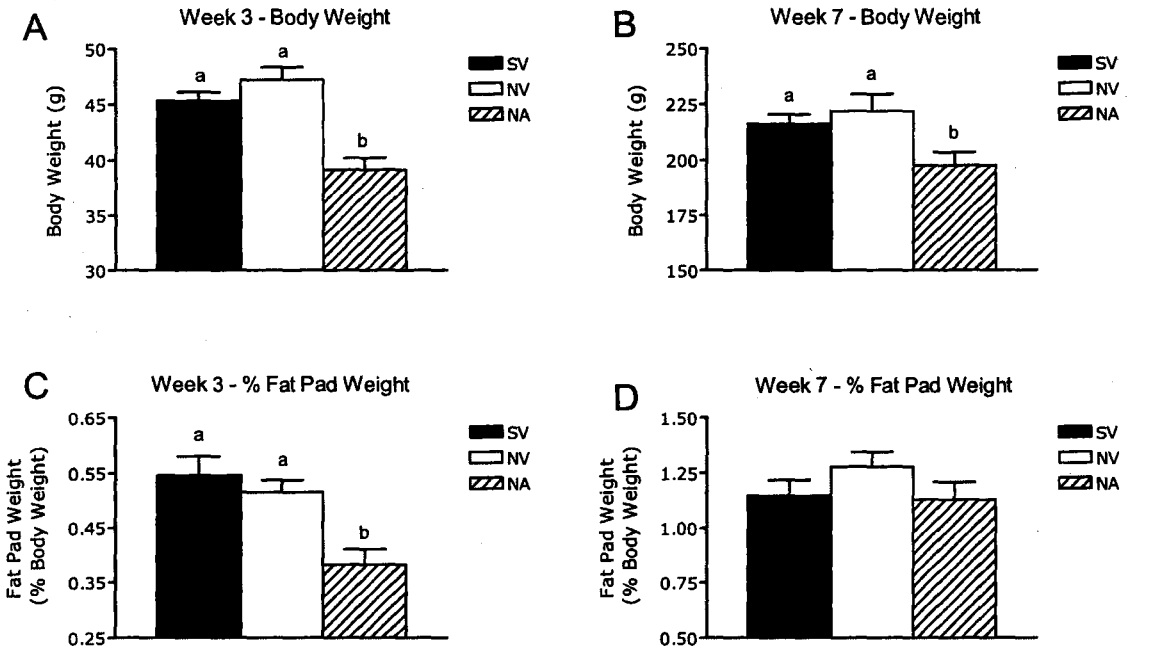


Figure 6.1: Offspring body weight and fat weight (epididymal, mesenteric and perirenal fat pads).
Total fat pad weights are expressed as a proportion of body weight at 3 and 7 weeks of age. All data are presented as the mean ± SEM.

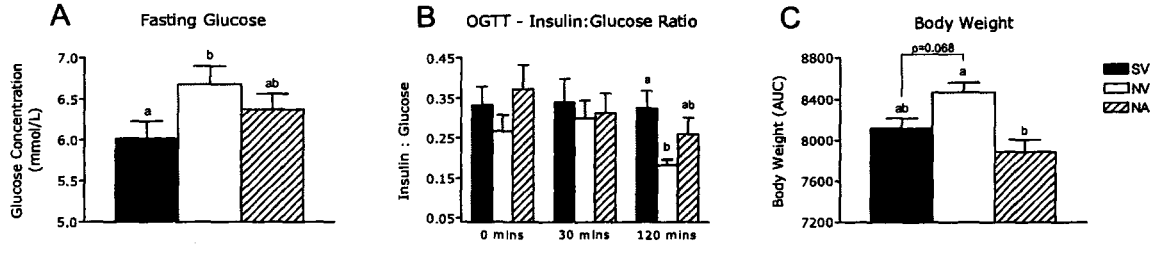


Figure 6.2: Metabolic phenotype.
A) fasting glucose (26 weeks), B) insulin:glucose ratio following an oral glucose challenge (26 weeks), and C) area under the curve (AUC) for body weight (4-26 weeks). All data are presented as the mean ± SEM.

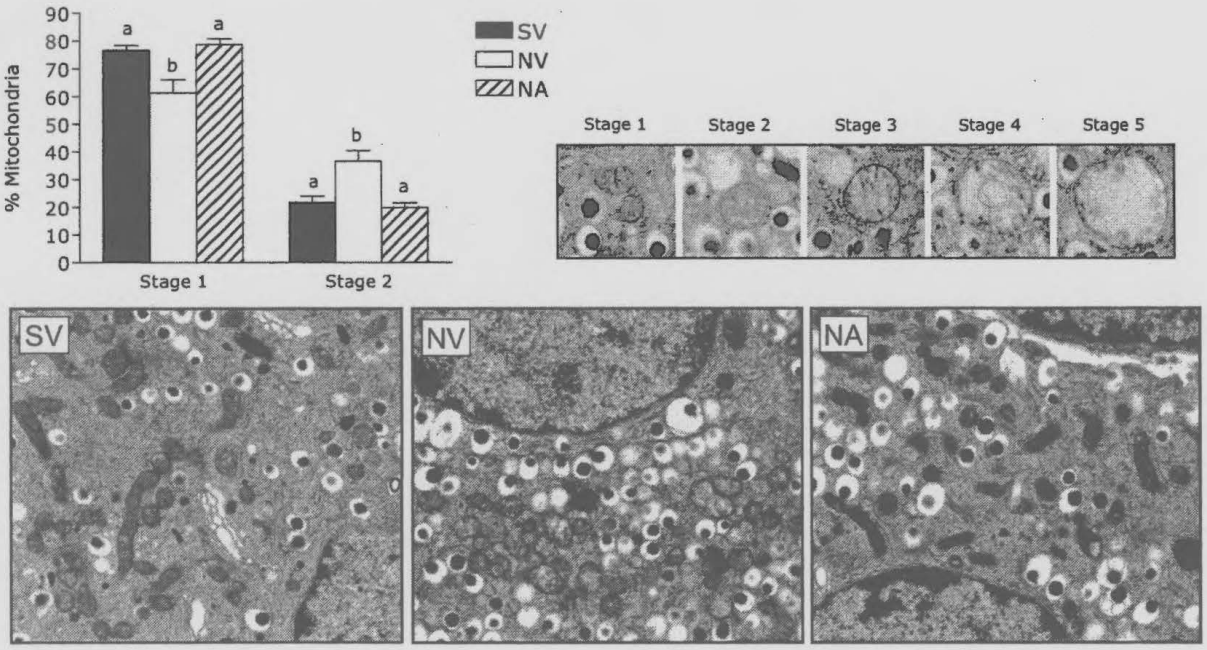


Figure 6.3: Mitochondria ultrastructure at 3 weeks of age.
Mitochondria ultrastructure was measured as the percentage of mitochondria in each of five stages of morphological health (an example of each is provided). Representative electron microscopy photographs (12,000x magnification) are shown for SV, NV and NA beta cells at 3 weeks. All data are presented as the mean ± SEM.

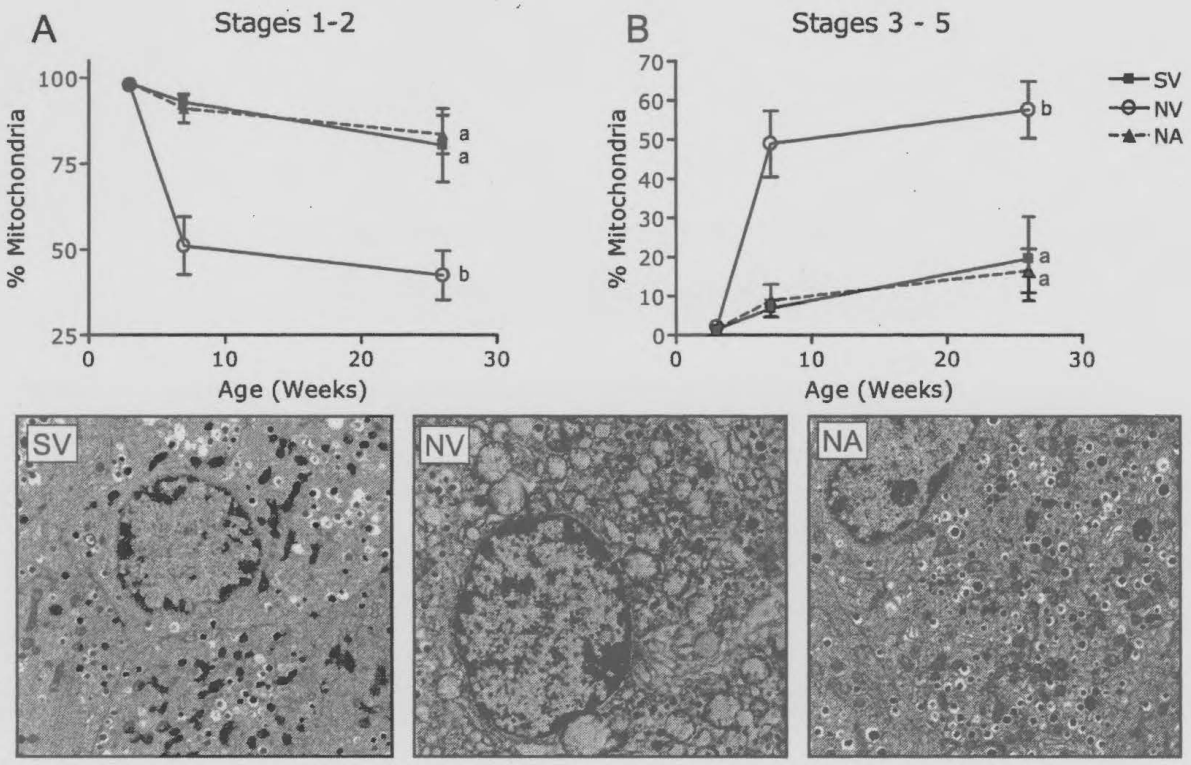


Figure 6.4: Mitochondria ultrastructure during postnatal development (3, 7 and 26 weeks).
Data are expressed as the percentage of mitochondria in each of five stages of morphological health. Representative electron microscopy photographs (12,000x magnification) are shown for SV, NV and NA beta cells at 26 weeks. All data are presented as the mean ± SEM.

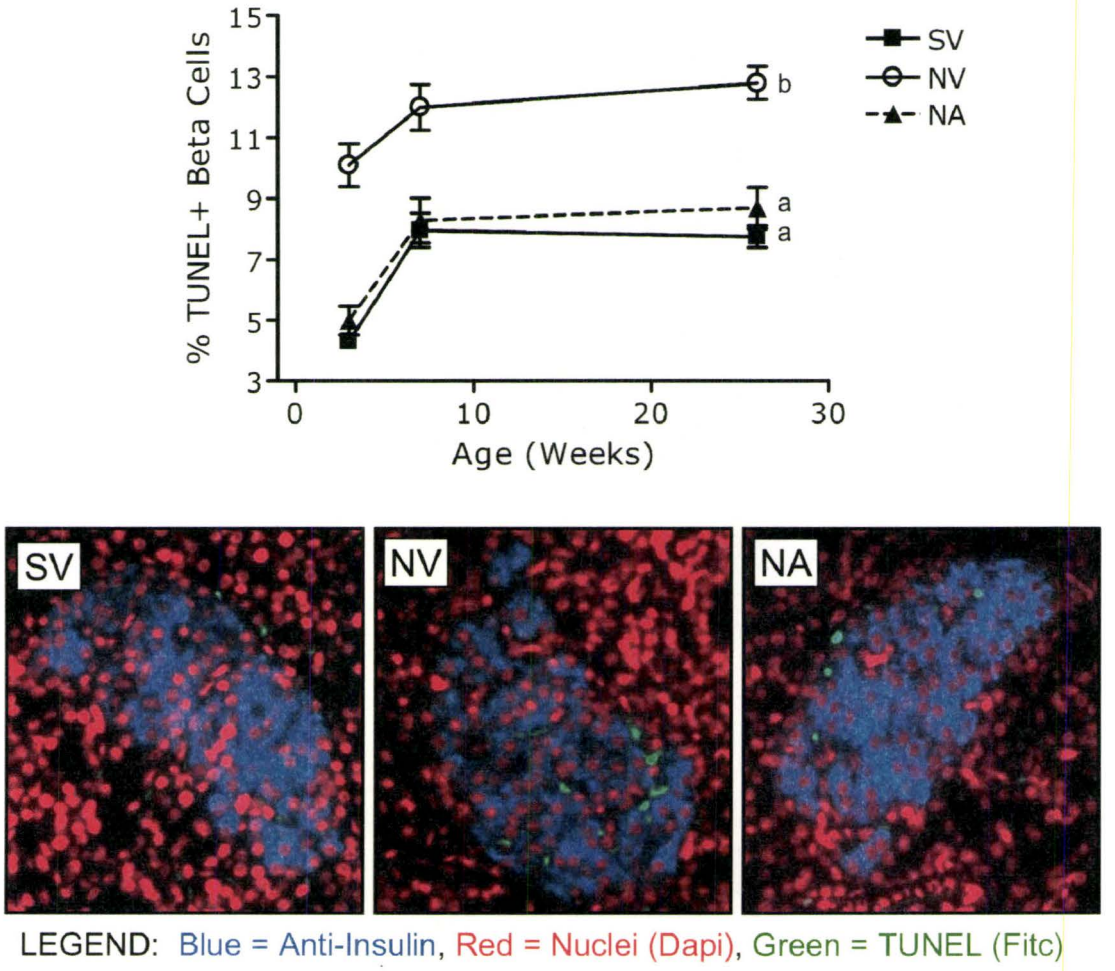


Figure 6.5: Beta cell apoptosis at 3, 7 and 26 weeks of age.
Apoptosis was measured as the percentage of TUNEL-positive beta cells; TUNEL = terminal dUTP-mediated nick end labelling. Representative immunofluorescent photographs (400x magnification) are provided for SV, NV and NA beta cells. Blue staining represents beta cells (anti-insulin with alexa fluor 594 secondary antibody); red staining reperesents nuclei (dapi counterstain) and green represents TUNEL-positive cells (fitc-labeled). All data are presented as the mean ± SEM.

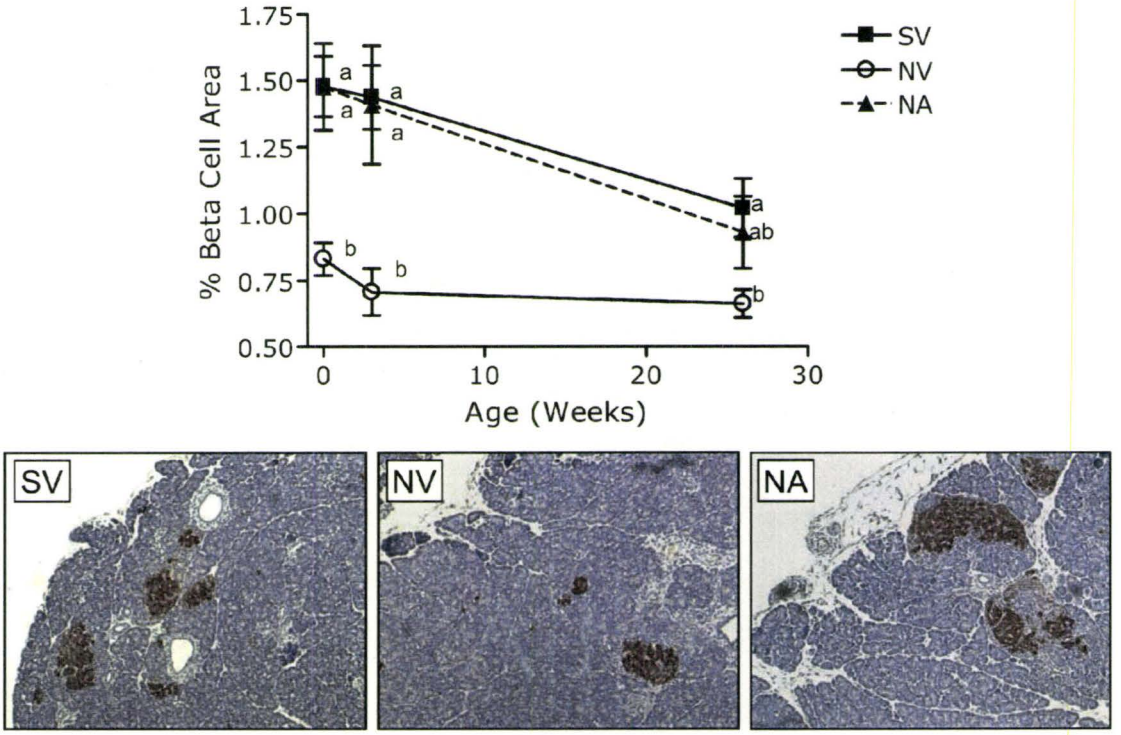


Figure 6.6: Beta cell fraction at birth, 3 and 26 weeks of age.
Data are expressed as the beta cell area / total pancreas area x 100. Representative photographs of immunohistochemical staining are provided for SV, NV and NA offspring at 3 weeks of age. Brown staining indicates insulin-positive beta cells. All data are presented as the mean ± SEM.

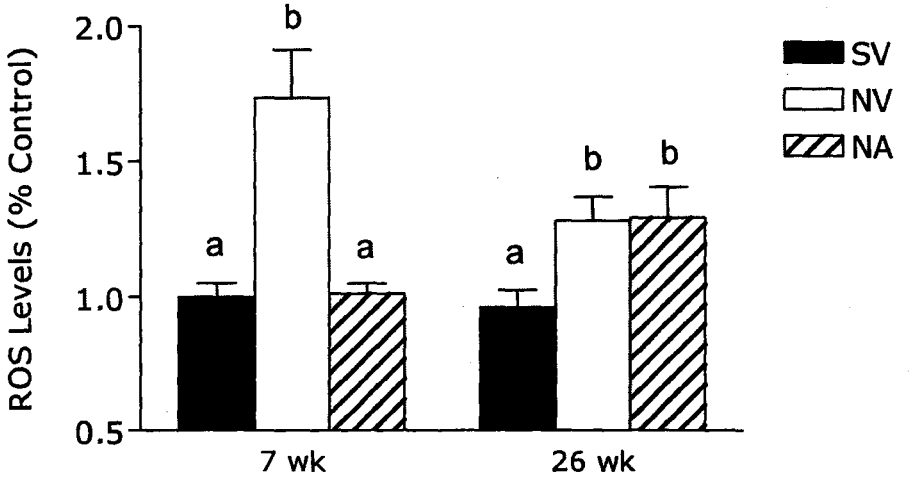


Figure 6.7: Reactive oxygen species levels in isolated pancreatic islets at 7 and 26 weeks of age.
Reactive oxygen species were measured by H_2DCFDA fluorescence and expressed as a percentage of the average saline control. All data are presented as mean ± SEM.

CHAPTER 7

GENERAL DISCUSSION

7.1 PROPOSED MECHANISM FOR NICOTINE-INDUCED BETA CELL DEFECTS

The overall goal of this thesis was to determine the mechanisms underlying the development of dysglycemia during adulthood following fetal and neonatal nicotine exposure, with a specific focus on the effects of nicotine on pancreatic development. Based on the combined studies from this thesis, I have developed a model of the mechanism through which maternal nicotine adversely alters fetal and neonatal beta cells (Figure 7.1).

I propose that nicotine binds to the nicotinic acetylcholine receptor (nAChR) on the developing beta cell (chapter 4; (173)), causing an increase in the production of intracellular reactive oxygen species (ROS) (chapter 4; (173)). Since the antioxidant defense system in the beta cells is known to be relatively low compared to other cell types (130;131), beta cells are particularly susceptible to oxidative stress. Indeed, following nicotine exposure the beta cell antioxidant enzymes, although upregulated, are insufficient to compensate fully for the increased ROS levels (chapter 4; (173)). Consequently, susceptible pancreatic proteins, including those located in the mitochondria, are damaged by these highly reactive molecules (chapter 4; (173)).

The implications of this mitochondrial damage are two-fold. First, loss of mitochondrial membrane integrity allows for swelling and release of proteins from

the inner membrane space into the cell cytosol, thus triggering beta cell apoptosis (chapter 3; (174)). Since the majority of plasticity in rodent beta cell mass is confined to the fetal and neonatal periods of development, any toxic insult which influences beta cell survival during these critical windows may permanently alter beta cell mass. Indeed, fetal and neonatal nicotine exposure causes a permanent loss of beta cell mass beginning at birth, which was attributed primarily to increased beta cell apoptosis and an impaired capacity for beta cell proliferation relative to saline controls (chapter 2; (172)). As in humans, the loss of beta cell mass in this animal model precedes the onset of dysglycemia and likely contributes to the deterioration of beta cell function in the remaining cells.

The second implication of nicotine-induced mitochondrial damage in this animal model is the ability of defective mitochondria to perpetuate oxidative stress in the cell long after cessation of the toxicant exposure. When proteins in the electron transport chain (ETC) are damaged by ROS, electron leakage occurs from the oxidation-reduction reactions in the inner mitochondrial membrane, thus causing further ROS formation (119). It is this vicious cycle that is likely activated in the beta cells by developmental nicotine exposure, and is responsible for the ongoing deterioration of mitochondrial structure and function after cessation of nicotine treatment (chapter 5; (175)). Since mitochondria are central for glucose-stimulated insulin secretion (GSIS), we predicted that nicotine-induced mitochondrial defects would influence beta cell function in addition to beta cell survival. Indeed, the progressive decline in mitochondrial structure and

function following nicotine exposure was associated with impaired GSIS in isolated islets and reduced insulin granule biosynthesis (chapter 5; (175)), both of which likely contributed to the altered glucose homeostasis in this animal model.

Finally, to confirm the involvement of oxidative stress in triggering the beta cell defects in this animal model, dams were co-treated with nicotine and an antioxidant cocktail (chapter 6; (176)). Indeed, the nicotine-induced increase in ROS production and mitochondrial defects were both prevented by antioxidant exposure, and as was predicted, this also protected the developing beta cell population from increased beta cell apoptosis and loss of beta cell mass and function (chapter 6; (176)).

7.2 DISCUSSION OF PROPOSED MECHANISM IN BETA CELLS

Although the studies from this thesis allow for solid, evidence-based deduction of the mechanism though which fetal and neonatal nicotine exposure causes beta cell defects and altered glucose homeostasis, the story is by no means complete. There are several unanswered questions about the proposed mechanism which would require substantial work to answer, but may provide interesting future directions for this animal model.

7.2.1 Nicotinic Acetylcholine Receptors

The topic of the nAChR was examined only briefly in this thesis and

remains an area with considerable potential for future research. Based on the mRNA expression of the various nAChR subunits in the pancreas at birth and weaning, it was concluded that nicotine could potentially be acting directly on the developing beta cell by binding to its receptor. Indeed, nicotine has been shown to exert its toxic effects in other developing tissues via the nAChR. For instance, binding of nicotine to the nAChR during development is known to be a necessary step in mediating the neurotoxic effects of nicotine (177). Following receptor binding, the adverse effects of nicotine in the developing brain are attributed to reduced DNA synthesis and altered cell signaling which cause neuronal cell death (177). Indeed, prenatal nicotine exposure elicits long-lasting changes in neuronal nAChR expression and binding capacity (178). Altered pulmonary function following maternal smoke exposure is also thought to be mediated by the direct effects of maternal nicotine on the nAChR in developing lung (179). Similarly, the adverse effects of fetal nicotine exposure in our animal model on hypoxia-sensing by adrenal chromaffin cells have been attributed to stimulation of the α7 nAChR (83).

However, the presence of nAChR gene expression in the developing pancreas does not provide conclusive evidence that the beta cells are directly affected by nicotine. First, the presence of the nAChR was never localized to the beta cells specifically, but rather to the whole pancreas since good antibodies for immunohistochemical staining were not available. For the same reason, protein expression could not be confirmed for all of the nAChR subunits in the whole

pancreas, although preliminary data demonstrates that the α2 subunit is expressed at the protein level in the neonatal pancreas (Figure 7.2). However, it is reasonable to predict that mRNA transcription likely corresponds to translation into the corresponding protein, so I hypothesize that protein expression occurs for the other subunits as well. Regardless, the presence of mRNA and/or protein expression for the nAChR subunits does not necessarily imply that the hetero- or homopentameric receptors are actually assembled and functional, or that nicotine actually binds to the receptor to produce a downstream effect in the pancreas. To conclusively assess the involvement of the nAChR in mediating the effects of nicotine on developing beta cells, a future study could be performed using a competitive nAChR antagonist such as α-Lobeline, mecamylamine or dihydro-β-erythroidine (180). Co-treatment of nicotine with a nAChR antagonist would prevent binding of nicotine to the receptor, and assuming that this interaction is required for inducing oxidative stress, would also prevent the beta cell apoptosis and mitochondrial defects.

Presuming that nicotine does bind to its receptor in the developing pancreas, the next relevant topic would be the downstream effects of this interaction and their potential involvement in beta cell apoptosis. The nAChR is activated by the binding of acetylcholine or nicotine to the extracellular region of the receptor; homomeric receptors contain five identical binding sites, whereas heteromeric receptors have two binding sites at the α/β interfaces (181). Substrate binding to the nAChR leads to three possible functional states: resting

(closed - in the absence of any agonist), active (open - in the presence of agonist) and desensitized (closed - after prolonged exposure to agonist) (181). In the active state, this receptor is highly permeable to calcium, meaning that binding of nicotine to its receptor results in an increase in intracellular calcium levels (113;182) (Figure 7.3). This can occur through a combination of events, including: increased ion flux through the active nAChR, nAChR-mediated activation of voltage-operated calcium channels, and calcium-induced calcium release from intracellular stores (113;182) (Figure 7.3). Calcium is thought to be a key mediator of nAChR signaling in nonexcitable cells, including calcium-dependent apoptosis (182). In addition, calcium plays a crucial role in regulating glucose-stimulated insulin secretion by beta cells (122;183). Therefore, I predict that calcium may be involved in mediating the effects of nicotine in this animal model. As described in the introduction, mitochondrial ATP production stimulates closure of ATP-sensitive potassium channels, depolarization of the beta cell membrane, opening of the voltage-gated calcium channels and finally a rise in intracellular calcium levels, which triggers insulin exocytosis (146). Therefore, an unexpected rise in intracellular calcium levels in response to nicotine binding to the nAChR during fetal and neonatal development could alter this tightly regulated calcium homeostasis which controls beta cell function and cell signaling. Furthermore, the mitochondrial permeability transition pore (mtPTP) can be stimulated to open in response to mitochondrial uptake of excessive calcium (119), meaning that increased calcium influx following nicotine exposure

could contribute to the mitochondrial swelling and subsequent apoptosis observed in this animal model. However, the involvement of calcium in the beta cell defects following fetal and neonatal nicotine exposure remains to be determined.

7.2.2 Beta Cell Neogenesis

As discussed previously, the regulation of beta cell mass is determined by a balance of beta cell size, replication, neogenesis and apoptosis (95;99;102). In the critical windows of exposure study (chapter 2), we demonstrated that fetal and neonatal nicotine exposure caused an irreversible reduction in beta cell mass beginning at birth, which was attributed to increased levels of beta cell apoptosis and a decreased capacity for islet cell proliferation compared to saline controls (172). This is similar to humans with type 2 diabetes, in which the primary cause of impaired beta cell mass is increased apoptosis (7). Other animal models of metabolic fetal programming demonstrate loss of beta cell mass caused by: a) both increased apoptosis and reduced proliferation (107-109), b) reduced proliferation and no change in apoptosis (31;111), or c) altered neogenesis and no change in either proliferation or apoptosis (109;110). Since impaired beta cell neogenesis from progenitor cells is an additional mechanism through which beta cell mass may be regulated, it would be interesting to examine the effect of fetal and neonatal nicotine exposure on this pathway.

The differentiation of ductal epithelium into mature pancreatic beta cells is regulated by a complex cascade of transcription factors (184) (Figure 7.4). Typically, beta cell neogenesis is assessed by measuring expression of the transcription factors which are known to be present in beta cell progenitors. Specifically, pancreatic duodenal homeobox gene-1 (Pdx-1) is a key regulator of pancreatic development and beta cell differentiation, as well as a glucose-responsive regulator of insulin gene expression in adult beta cells (184). Neurogenin3 (Ngn3) is required for the development of all endocrine cell lineages in the pancreas, and expression of Beta2/NeuroD, Pax4, Nkx2.2, Nkx6.1 are all important for specific determination of beta cells (184) (Figure 7.4). Although the beta cell neogenesis pathway was not examined in this thesis, Somm and colleagues have assessed the effects of prenatal nicotine exposure on beta cell neogenesis in a similar animal model (93). In their study, female rats were exposed to nicotine at a dose of 3 mg/kg/day using an osmotic minipump between day 4 and 18 of gestation (compared to 1 mg/kg/day via daily subcutaneous injection throughout gestation *and* lactation in our animal model). The treatment protocol used by Somm et al resulted in reduced islet size and number at postnatal day 7, which was accompanied by a reduction in islet gene expression of Pdx-1, Pax-6, Nkx6.1 and insulin (93). Furthermore, the nicotine-exposed offspring with impaired beta cell neogenesis subsequently developed glucose and insulin intolerance, hyperinsulinemia, and increased body weight during adulthood (93). Therefore, the study by Somm and colleagues suggests

that in addition to increased beta cell apoptosis and an impaired capacity for beta cell proliferation, fetal and neonatal exposure to maternal nicotine may also impair beta cell neogenesis. However, this question remains to be answered in our animal model.

7.2.3 Mitochondrial Function

Impaired mitochondrial structure and function was demonstrated in nicotine-exposed offspring through a number of different techniques, including electron microscopy (beta cell specific), complex IV enzyme activity (whole pancreas homogenates), and the glucose-stimulated insulin secretion assay (islet specific). However, there are several other experiments that could be performed to expand on these findings.

First, it would be interesting to perform the mitochondrial enzyme activity assays in isolated islets rather than whole pancreas homogenates. Although a small reduction in complex IV activity was observed at 26 weeks of age in the whole tissue, it is likely that this effect would be more dramatic and perhaps detectable at an earlier age in the isolated islets. It is certainly feasible to perform these assays in the isolated islets (150). However, a large number of islets would be required for these experiments and only a limited number of islets could be isolated in our studies. This would be an interesting question for a future cohort where isolated islets from numerous animals could be pooled.

Another mechanism through which mitochondrial dysfunction can occur is via uncoupling of the electron transport chain to ATP synthesis. In addition to the ROS-induced damage to the ETC enzyme complexes, induction of uncoupling protein 2 (UCP2) has also been implicated in loss of mitochondrial function and impaired glucose-stimulated insulin release (147;148;185). UCP2 is an inner mitochondrial membrane protein that when activated, leaks protons into the matrix, thus diminishing the proton gradient generated by the respiratory chain (Figure 1.3; (147;185)). UCP2 expression in beta cells can be induced by hyperglycaemia (186) and superoxide (187), ultimately leading to loss of GSIS. Indeed, in human patients with type 2 diabetes, UCP2 protein expression was significantly increased in islet cells compared to control patients (149). Therefore, I predicted that upregulation of UCP2 would contribute to pancreatic mitochondrial dysfunction following fetal and neonatal nicotine exposure. However, numerous attempts to measure UCP2 expression in the pancreas were unsuccessful. By Western blotting, four different antibodies from four different companies were used, but no band was ever detected at the correct molecular weight in whole pancreas homogenates. Even in the purified mitochondrial fraction, where UCP2 expression should be localized, the correct band could not be detected with any antibody. Similarly, real-time PCR was used to detect UCP2 mRNA, but the Ct values for UCP2 were barely below the blank values, suggesting that mRNA expression in the pancreas is incredibly low. It is also important to note that the Ct values for beta actin were substantially lower than

the blank Ct, meaning the RNA quality was not an issue in these experiments. Therefore, the relative expression of UCP2 in the beta cell mitochondria of nicotine- and saline-exposed offspring remains to be determined in this animal model.

The majority of the proteins required for mitochondrial function are encoded by nuclear DNA (nDNA), translated by cytosolic ribosomes and imported to the mitochondria (119). However, 37 genes that are crucial to mitochondrial function are encoded by mitochondrial DNA (mtDNA), including genes for rRNA, tRNA and 13 polypeptides of the oxidative phosphorylation protein complexes (119). MtDNA differs significantly from nDNA because it is maternally inherited, contains only exons (coding regions of DNA), and shows substantial regional variation as a result of its susceptibility to ROS-induced mutations (119). In fact, the rate of mtDNA mutations is thought to be almost entirely modulated by the extent of oxidative stress in the mitochondria (119). These mutations will eventually interfere with the transcription of genes that are central to mitochondrial function, and consequently, may impact energy production and cell function. In animal models, mtDNA defects, induced either through random mutations or dysregulation of gene expression, are associated with impaired beta cell function and diabetes (150;188). Moreover, clinical studies have demonstrated a relationship between mtDNA mutations in diabetic patients and impaired insulin release following a glucose challenge (189;190). Pancreatic mtDNA defects were briefly examined in our animal model, although

not presented in this thesis. Indeed, a small but statistically significant increase in the rate of mtDNA deletions was detected in the whole pancreas from nicotine-exposed versus saline-exposed offspring by 26 weeks of age (Figure 7.5; (191)). An attempt was also made to measure mtDNA mutations in the whole pancreas. For this experiment, primers were designed to amplify the D-loop, ATPase-6, complex I and II regions of the mtDNA genome (Figure 7.5A). The amplified DNA products were then sequenced by the MOBIX lab at McMaster University and compared to the known rat sequence for these mtDNA regions. However, due to mitochondria heteroplasmy, mtDNA mutations would likely only be present in a very small proportion of the mitochondria, meaning that the sequencing technology would need to be sensitive enough to detect mutations in only 1-2% of the mtDNA. No differences were detected in the nicotine versus saline-exposed offspring at 26 weeks of age, but this may be attributed to either the sensitivity of the sequencing technology and/or the fact that the majority of mtDNA mutations would be localized in the beta cells rather than the whole pancreas. It would be interesting to examine both mtDNA deletions and mutation in the isolated islets if enough cells could be pooled for DNA extraction.

Finally, mitochondrial dysfunction can also be assessed by examining the mitochondrial membrane potential and the ATP/ADP ratio, preferentially in isolated islets. The formation of ATP in the mitochondria depends on the electrochemical gradient created by pumping protons across the inner mitochondrial membrane at complex I, III and IV in the electron transport chain

(119). Therefore, a defect in complex IV activity, as observed in nicotine-exposed offspring, could interfere with this membrane proton gradient and thus the efficiency of ATP production. Furthermore, proton leakage caused by increased UCP2 would also contribute to alterations in the mitochondrial membrane potential, and subsequently a lower ATP/ADP ratio (149). Indeed, both the mitochondrial membrane potential and ATP/ADP ratio are reduced in glucose-stimulated islets from humans with type 2 diabetes (149). The effect of fetal and neonatal nicotine exposure on these outcomes in isolated islets remains to be determined.

7.2.4 Insulin-Like Growth Factor Signaling

This thesis has demonstrated clearly that oxidative stress and mitochondrial dysfunction play a crucial role in the regulation of beta cell mass and function following fetal and neonatal nicotine exposure. These findings are supported by numerous other groups that have shown the presence of oxidative stress and mitochondrial defects in the beta cells of both animals (150;192;193) and humans (149;194) with type 2 diabetes. However, there are numerous other pathways that regulate beta function and mass, such as the insulin-like growth factor (IGF) system, which were not examined in this thesis and may also contribute to the altered glucose homeostasis in our animal model. The pancreatic IGF system includes the ligands, IGF1 and IGF2, and receptors, IGF1R, IGF2R and the insulin receptor (IR). Mice lacking either IR or IGF1R in

beta cells develop glucose intolerance associated with defective glucose-stimulated insulin secretion (195;196). Furthermore, when both IR and IGF1R are absent in beta cells, mice are born with normal beta cell mass, but display increased postnatal beta cell apoptosis and a gradual age-dependent decline in beta cell mass (197). Furthermore the ligands IGF1 and IGF2 have been shown to protect beta cell mass and function, as well as glucose homeostasis in animal and human studies (198-200). In humans, circulating IGF1 levels are significantly lower in patients with metabolic syndrome (201), and treatment with recombinant IGF1 has been shown to lower glucose levels in both healthy and diabetic individuals (reviewed in (202)). Therefore, in addition to mitochondrial dysfunction, the IGF signaling pathway could also be involved in the loss of beta cell mass and function in this animal model. Although the IGF pathway was not studied in this thesis, we have demonstrated that fetal and neonatal nicotine exposure results in loss of IR and IGF1R protein expression in the adult pancreas (Figure 7.6; (191)), as well as a reduction in islet IGF1 expression (Figure 7.7; (191)). These results are consistent with the Goto-Kakizaki rat model of type 2 diabetes which also displays defects in both beta cell mitochondrial structure and function (203), as well as the pancreatic IGF axis (204). Therefore, mitochondrial dysfunction is likely not the only defect contributing to loss of beta cell mass and function in our animal model.

7.3 DISCUSSION OF ALTERNATIVE MECHANISMS IN PERIPHERAL TISSUES

7.3.1 Regulation of Glucose Homeostasis

Although loss of beta cell mass and function are known to be crucial in the pathogenesis of type 2 diabetes, it is also important to consider the involvement of a reduced insulin effect at peripheral tissues in regulating glucose homeostasis. For instance, patients with type 2 diabetes exhibit increased hepatic glucose production and impaired glucose uptake by the skeletal muscle, both of which contribute to hyperglycemia (6). In addition, type 2 diabetes is associated with high levels of circulating triglycerides and free fatty acids, which are thought to contribute to organ abnormalities due to lipotoxicity (6). This thesis focused on the effects of fetal and neonatal nicotine on the beta cell, but peripheral tissues likely also contribute to the glucose intolerance in this animal model. Interestingly, our lab has demonstrated a widespread reduction in insulin uptake, not only in the pancreas from this animal model, but also in numerous other glucose-sensitive tissues, including the liver, skeletal muscle and adipose, as well as non-metabolic tissues such as the heart, kidney and brain (205). The impaired peripheral insulin uptake was associated with reduced IR protein expression in both the skeletal muscle and pancreas of nicotine-exposed offspring (205), but not in the liver (data not shown). Interestingly, there was also no change in the protein expression of gluconeogenic enzymes, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphate dehydrogenase, in the liver (data not shown), although the activity of these

enzymes has not been assessed. The observed reduction in fat pad insulin uptake was associated with increased adiposity, hypertriglyceridemia, obesity, and altered perivascular adipose tissue in the nicotine-exposed offspring relative to saline controls from our animal model (88-90). This altered adiposity has also been confirmed in the offspring from other animal models of maternal nicotine exposure (91-93). These data suggest that peripheral tissues, such as skeletal muscle and adipose (but perhaps not the liver) may contribute to the impaired glucose homeostasis in this animal model. However, the effects of fetal and neonatal nicotine exposure on the function of these peripheral tissues has not yet been examined in this animal model, but would be of great interest to further understanding the metabolic defects observed in our studies.

7.3.2 Mitochondrial Dysfunction in Other Tissues

Given the involvement of mitochondria in the beta cells defects following developmental nicotine exposure, it would be interesting to examine mitochondrial structure and function in other metabolic tissues in this animal model. Humans with type 2 diabetes are known to have mitochondrial defects in skeletal muscle (206;207) and elderly patients with insulin resistance have impaired mitochondrial oxidative phosphorylation compared to young, healthy controls (208). Furthermore, animal models of type 2 diabetes have impaired mitochondria in adipocytes (209) and liver (210). Nicotine treatment in adult rats causes alterations to the mitochondrial electron transport chain in multiple rat

brain regions (211) and fetal nicotine exposure resulted in mitochondrial swelling in the brain (212), suggesting that beta cell mitochondria are not the only susceptible target of nicotine. Therefore, mitochondrial dysfunction may also be involved in other metabolic tissues in this animal model and may also contribute to the observed glucose intolerance following fetal and neonatal nicotine exposure.

7.4 STRENGTHS OF THE THESIS

7.4.1 Nicotine Dose and Mode of Administration

There are a number of strengths to this animal model as an applicable tool for studying the effects of fetal and neonatal nicotine exposure in humans. First there is the question of dose, with respect to both mode of administration and concentration of nicotine. In our animal model, female Wistar rats are treated daily with 1.0 mg/kg/day of nicotine via subcutaneous (s.c.) injection for 2 weeks prior to pregnancy, throughout gestation and lactation (89). In contrast, other studies deliver nicotine at doses generally ranging from 2.0 – 6.0 mg/kg/day via drinking water, i.v. self-administration, or most commonly, an osmotic minipump (213;214). While the osmotic minipump method is certainly convenient, as it eliminates the need for daily injections, there are a number of downsides to this commonly used mode of nicotine delivery. First, the trauma of the surgical procedure and anesthesia which are required for both implantation and removal of the minipump is certainly more substantial than a daily s.c. injection. Although

the process of a daily injection may be somewhat stressful for the animals at first, the dams are easily trainable and quickly become accustomed to the daily injection routine. Another major issue with the osmotic minipump is that it achieves constant blood levels of the drug, but the plasma concentrations decline gradually as the pregnancy progresses and the dam body weight increases (213). An approach that several groups use to deal with this issue is to provide a dose of nicotine based on the projected body weight of the dam towards the end of pregnancy (214). However, this method has been shown to deliver extremely high levels of nicotine to the developing fetus that are not biologically plausible or relevant to humans (214). An advantage of daily nicotine injections is that the concentration of nicotine can be adjusted according to the dam weight gain during pregnancy such that the daily dose is *actually* 1.0 mg/kg/day throughout the entire treatment period rather than just in the first or last stage of gestation. Unfortunately, neither the osmotic minipump or daily s.c. injection method truly mimics the delivery of nicotine via cigarette smoking or NRT in a pregnant woman. In humans, nicotine levels drop overnight, and then spike after the first cigarette in the morning (most similar to s.c. injection), at which point the nicotine levels generally remain fairly constant throughout the day based on self-administration of cigarettes (most similar to osmotic minipump).

An extremely important consideration for our animal model is selection of 1.0 mg/kg/day as the daily dose of nicotine prior to pregnancy, throughout gestation (fetal development) and lactation (neonatal development). As

discussed previously, this dose was selected because of its biological relevance to human nicotine exposure either via cigarette smoking or NRT use during pregnancy (213;214). Our treatment protocol results in maternal serum cotinine concentrations of 136 ng/ml (85), which is within the range of cotinine levels reported in women who are considered "moderate smokers" (80 to 163 ng/mL) (86). In addition, this dose of nicotine results in serum cotinine concentrations of 26 ng/ml in the nicotine-exposed offspring at birth (85), which is also within the range (5 to 30 ng/ml) observed in infants nursed by smoking mothers (87). Most commonly, a dose of nicotine between 2.0 and 6.0 mg/kg/day is used to mimic nicotine exposure during pregnancy (213;214). Recently, Hussein and colleagues examined the relevance of these nicotine dosages in pregnant rats and concluded that a dose of 6.0 mg/kg/day (delivered by osmotic minipump using the projected maternal weight gain model) is equivalent to smoking 560 cigarettes per day by a 70 kg pregnant woman (214). It was concluded that studies using high doses of nicotine to achieve toxicity are highly irrelevant and their validity should be questioned (214). Importantly, in a recent review of nicotine dose selection for *in vivo* research by Matta and colleagues, 1.0 mg/kg/day was discussed as the nicotine dose in rats that most closely approximated nicotine levels in habitual smokers (213). Therefore, the toxic effects of nicotine on the developing pancreas in this thesis are extremely relevant to humans exposed to nicotine via cigarette smoking or NRT.

7.4.2 Reproducibility

An important requirement to strengthen any scientific work is evidence of reproducibility. Results from this thesis have been reproduced not only within our lab, but also by groups at other institutions. For example, the observed loss of beta cell mass and increased apoptosis were reproduced in two different animal cohorts during my PhD (172;176). Furthermore, I have confirmed the presence of mitochondrial defects in the beta cells of saline- and nicotine-exposed offspring from three different cohorts (175;176;191) and the increased production of reactive oxygen species by isolated islets in two cohorts (173;176). Dr. Jim Petrik at the University of Guelph has also independently confirmed in our animal model that fetal and neonatal nicotine exposure causes a significant reduction in beta cell mass between birth and 26 weeks of age, which is mediated by increased beta cell apoptosis (215). In addition, similar results have been shown in a different animal model of prenatal nicotine exposure. Somm and colleagues from Geneva, Switzerland demonstrated a reduction in islet proportion, size and number at postnatal day 7 following fetal exposure to nicotine, which was associated with altered glucose homeostasis at 26 weeks of age compared to saline controls (93). However, contrary to results from our critical windows study where nicotine exposure was required during both pregnancy and lactation (172), Somm et al found that gestational exposure alone was sufficient to cause glucose intolerance during adulthood (93). These differences can likely be attributed to the differing treatment protocols between animal models; Somm and colleagues

performed their work in a different rodent strain (Sprague Dawley versus Wistar rats), and with a relatively high dose of nicotine (3.0 mg/kg/day versus 1.0 mg/kg/day) by osmotic minipump (versus daily injections) between gestational days 4 and 18 (93). Although comparisons between the two models are limited, this study does confirm that fetal nicotine exposure can irreversibly damage the developing beta cells and predispose offspring to abnormal glucose homeostasis later in life.

7.5 LIMITATIONS OF THE THESIS

There were numerous technical challenges that limited the depth to which certain questions could be answered in this thesis. One of the consistent challenges throughout my thesis was the issue of using whole pancreas homogenates to gain insight into beta cell specific questions. Western blotting was used in this thesis for the majority of protein quantification because it is more quantitative than immunohistochemistry, but it does not allow for localization of the protein expression within the tissue. There are alternative methods for teasing out the fetal and neonatal effects of nicotine on protein expression in adult beta cells, but each have their disadvantages. First, expression of a protein-of-interest can be co-localized with insulin protein expression by dual immunofluorescent staining of pancreas tissue sections. Unfortunately, this technique proved to be difficult because the most effective insulin antibody reacts with both anti-mouse *and* anti-rabbit secondary antibodies. Alternatively, protein

expression can be localized to the islets by immunohistochemical staining with hematoxylin as a counterstain. This technique is "endocrine" rather than "beta" cell specific, but is more specific than whole pancreas homogenates. For instance, in the Toxicological Sciences manuscript, we demonstrated that expression of active caspase-3 (the final "executioner" caspase) protein expression was localized to the pancreatic islets by immunohistochemical staining, indicating that the observed activation of the mitochondrial-mediated apoptotic signalling pathway was specific to endocrine cells (chapter 3; (174)). This technique was useful for localization of the protein, but not for quantification of expression. The gold standard for the research questions in this thesis would be western blotting of isolated pancreatic islets. This was attempted with numerous different antibodies, but since such a small number of cells can be used, only very strong antibodies were able to detect a signal. An example of how western blotting of isolated islets was used successfully in this thesis, was the detection of increased protein carbonyls at 26 weeks of age (chapter 5; (175)). There was no difference in protein carbonyl levels in the whole pancreas (data not shown), but a significant increase was observed in the isolated adult islets following fetal and neonatal nicotine exposure. In general, when no difference was detected in the whole pancreas for various outcomes in this thesis (e.g. antioxidant gene expression, citrate synthase enzyme activity), it is possible that effects were masked by the overwhelming number non-endocrine cells in the pancreas. In contrast, when a treatment effect was seen at the whole pancreas

level (e.g. antioxidant protein expression, markers of the mitochondrial-mediated apoptotic signaling cascade), we propose that the observed effect is likely more modest than what would have been detected in isolated islets if this were possible.

Another limitation to this thesis was the lack of an appropriate beta cell line to mimic the *in vivo* animal model. For instance, two beta cell lines that are commonly used for basic science research include the beta-TC6 and INS-1E cells (mouse and rat adult insulinoma cells, respectively). The studies in this animal model all examine the beta cells between birth and 26 weeks of age following fetal and neonatal treatment, which is impossible to mimic in an available cell line. Although the effects of nicotine treatment on adult beta cells is certainly interesting, this is a very different question than the long term effects of fetal and neonatal nicotine exposure on postnatal beta cell health. That being said, the effects of nicotine on adult beta cell function *in vitro* may provide some insights into the ability of nicotine to directly affect the beta cell (always keeping in mind of course that adult beta cells substantially differ from developing beta cells). For instance, nicotine treatment has been shown to inhibit insulin secretion in INS-1E cells (116;216) and mitochondrial enzyme activity in beta-TC6 cells (Figure 7.8). Similarly, fetal and neonatal nicotine exposure resulted in impaired glucose-stimulated insulin secretion in adult islets, which coincided with beta cell mitochondrial dysfunction (175).

7.6 CLINICAL IMPLICATIONS

To evaluate the safety of nicotine replacement therapy for use during pregnancy, it is important to consider the effects of fetal and neonatal nicotine exposure on other tissues in this animal model in addition to the pancreas. Briefly, nicotine-exposed female offspring have impaired fertility and altered ovarian steroid hormone levels at 26 weeks of age (85). Furthermore, developmental nicotine exposure resulted in reduced granulosa cell proliferation, increased ovarian cell apoptosis, and decreased ovarian vascularity during adulthood compared to saline controls (217). The reproductive organs in the male offspring do not appear to be affected to the same degree, although some differences in histopathology of the testes were observed between saline and nicotine-exposed offspring (218;219). Another major defect in this animal model is the impaired ability of adrenomedullary chromaffin cells (AMCs) to respond to hypoxic stress following fetal nicotine exposure (220). This study suggested that the increased risk of sudden infant death syndrome associated with maternal cigarette smoking (51) may be attributable to the ability of nicotine to suppress acute hypoxic sensitivity in adrenal chromaffin cells (220). Maternal nicotine exposure may also be involved in the increased risk of asthma in offspring associated with maternal smoking during pregnancy (221;222). In our animal model, fetal and neonatal nicotine exposure caused an increase in alveolar size at weaning which was associated with increased glucocorticoid receptor and decreased vascular endothelial growth factor receptor-2 protein expression in the

lung compared to saline controls (223). Finally, nicotine-exposed offspring have increased levels of perivascular adipose tissue, which were associated with an impaired ability to induce vascular relaxation and increased blood pressure (88;90). Therefore, this animal model has demonstrated widespread adverse effects of fetal and neonatal nicotine exposure in the pancreas, perivascular adipose tissue, chromaffin cells, ovaries, testes and lungs of the offspring compared to saline exposure. Taken together with other studies from our animal model, the results from this thesis have significant clinical implications and should be considered by health associations when making policy decisions about the use of NRT during pregnancy.

The Ontario Medical Association (OMA) currently recommends nicotine replacement therapy as a safe alternative to cigarette smoking for pregnant women and suggests that Health Canada should modify their labeling requirements to include NRT use among pregnant women (72). The rationale for this recommendation is that the nicotine patch and gum are safer than smoking for the pregnant woman and her fetus (72). However, these conclusions do not consider recent evidence of the long term adverse effects of maternal nicotine exposure on metabolism (88-90;93;172;175), fertility (85), and neurological outcomes in the adult offspring (59;60). Indeed, in light of this more recent evidence, a 2007 critical review by Ginzel and colleagues recommended that exposure to nicotine during pregnancy and lactation be strictly avoided due to concerns about fetotoxicity and neuroteratogenicity (73). Furthermore, this

recommendation was made without consideration of the long term consequences of maternal nicotine exposure on the offspring metabolism. Therefore, based on the studies from this thesis, combined with other evidence from our animal model, I highly recommend that the safety of NRT use during pregnancy and lactation be re-evaluated by policy decision makers.

Further research is required to assess alternative harm reduction strategies for pregnant women who are highly dependent on nicotine and have been unable to quit smoking by traditional means. The final study from this thesis evaluated the efficacy of maternal co-treatment with antioxidants and nicotine during pregnancy and lactation. Results from our study demonstrated that co-treatment of dams with nicotine and a dietary antioxidant cocktail effectively protected beta cells in the developing fetus and neonate from the damaging pro-oxidant effects of nicotine (Chapter 6; (176)). Therefore, an antioxidant intervention may be a promising harm reduction strategy for women who require NRT to quit smoking during pregnancy. However, the antioxidant cocktail formulation would have to be carefully evaluated before recommendation for pregnant women, considering the adverse effects of some antioxidants (e.g. Vitamin E) on birth weight. The safety of other pharmacological smoking cessation aids such as bupropion (Zyban®, a noncompetitive antagonist at the nicotinic receptor) and varenicline (CHANTIX®, a partial nicotinic receptor agonist) should also be evaluated as potentially safer harm reduction strategies compared to NRT use for pregnant smokers.

7.7 REFERENCES FOR GENERAL INTRODUCTION AND DISCUSSION

1. Wild,S, Roglic,G, Green,A, Sicree,R, King,H: Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes Care* 27:1047-1053, 2004

2. Gaster,B, Hirsch,IB: The effects of improved glycemic control on complications in type 2 diabetes. *Arch Intern Med* 158:134-140, 1998

3. Lipscombe,LL, Hux,JE: Trends in diabetes prevalence, incidence, and mortality in Ontario, Canada 1995-2005: a population-based study. *Lancet* 369:750-756, 2007

4. Unwin,N, Shaw,J, Zimmet,P, Alberti,KG: Impaired glucose tolerance and impaired fasting glycaemia: the current status on definition and intervention. *Diabet Med* 19:708-723, 2002

5. Kahn,SE: The relative contributions of insulin resistance and beta-cell dysfunction to the pathophysiology of Type 2 diabetes. *Diabetologia* 46:3-19, 2003

6. Leahy,JL: Pathogenesis of type 2 diabetes mellitus. *Arch Med Res* 36:197-209, 2005

7. Butler,AE, Janson,J, Bonner-Weir,S, Ritzel,R, Rizza,RA, Butler,PC: Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. *Diabetes* 52:102-110, 2003

8. Prentki,M, Nolan,CJ: Islet beta cell failure in type 2 diabetes. *J Clin Invest* 116:1802-1812, 2006

9. Faerch,K, Borch-Johnsen,K, Holst,JJ, Vaag,A: Pathophysiology and aetiology of impaired fasting glycaemia and impaired glucose tolerance: does it matter for prevention and treatment of type 2 diabetes? *Diabetologia* 2009

10. Eliasson,B: Cigarette smoking and diabetes. *Prog Cardiovasc Dis* 45:405-413, 2003

11. Willi,C, Bodenmann,P, Ghali,WA, Faris,PD, Cornuz,J: Active smoking and the risk of type 2 diabetes: a systematic review and meta-analysis. *JAMA* 298:2654-2664, 2007

12. Tonstad,S: Cigarette smoking, smoking cessation, and diabetes. *Diabetes Res Clin Pract* 85:4-13, 2009

13. Barker D.J., Osmond C.: Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. *Lancet* 1:1077-1081, 1986

14. Barker D.J., Hales C.N., Fall C.H., Osmond C., Phipps K., Clark P.M.: Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidaemia (syndrome X): relation to reduced fetal growth. *Diabetologia* 36:62-67, 1993

15. Barker D.J., Eriksson J.G., Forsen T., Osmond C.: Fetal origins of adult disease: strength of effects and biological basis. *Int J Epidemiol* 31:1235-1239, 2002

16. Ravelli,GP, Stein,ZA, Susser,MW: Obesity in young men after famine exposure in utero and early infancy. *N Engl J Med* 295:349-353, 1976

17. Rich-Edwards,JW, Colditz,GA, Stampfer,MJ, Willett,WC, Gillman,MW, Hennekens,CH, Speizer,FE, Manson,JE: Birthweight and the risk for type 2 diabetes mellitus in adult women. *Ann Intern Med* 130:278-284, 1999

18. Barker D.J., Winter P.D., Osmond C., Margetts B., Simmonds S.J.: Weight in infancy and death from ischaemic heart disease. *Lancet* 2:577-580, 1989

19. Hales,CN, Barker,DJ, Clark,PM, Cox,LJ, Fall,C, Osmond,C, Winter,PD: Fetal and infant growth and impaired glucose tolerance at age 64. *BMJ* 303:1019-1022, 1991

20. Osmond,C, Barker,DJ, Winter,PD, Fall,CH, Simmonds,SJ: Early growth and death from cardiovascular disease in women. *BMJ* 307:1519-1524, 1993

21. Leon,DA, Lithell,HO, Vagero,D, Koupilova,I, Mohsen,R, Berglund,L, Lithell,UB, McKeigue,PM: Reduced fetal growth rate and increased risk of death from ischaemic heart disease: cohort study of 15 000 Swedish men and women born 1915-29. *BMJ* 317:241-245, 1998

22. Roseboom,TJ, van der Meulen,JH, Ravelli,AC, Osmond,C, Barker,DJ, Bleker,OP: Effects of prenatal exposure to the Dutch famine on adult disease in later life: an overview. *Twin Res* 4:293-298, 2001

23. Stein,CE, Fall,CH, Kumaran,K, Osmond,C, Cox,V, Barker,DJ: Fetal growth and coronary heart disease in south India. *Lancet* 348:1269-1273, 1996

24. Levitt,NS, Lambert,EV, Woods,D, Hales,CN, Andrew,R, Seckl,JR: Impaired glucose tolerance and elevated blood pressure in low birth weight, nonobese, young south african adults: early programming of cortisol axis. *J Clin Endocrinol Metab* 85:4611-4618, 2000

25. Gluckman,PD, Pinal,CS: Regulation of fetal growth by the somatotrophic axis. *J Nutr* 133:1741S-1746S, 2003

26. Kanaka-Gantenbein,C, Mastorakos,G, Chrousos,GP: Endocrine-related causes and consequences of intrauterine growth retardation. *Ann N Y Acad Sci* 997:150-157, 2003

27. Bispham,J, Gopalakrishnan,GS, Dandrea,J, Wilson,V, Budge,H, Keisler,DH, Broughton,PF, Stephenson,T, Symonds,ME: Maternal endocrine adaptation throughout pregnancy to nutritional manipulation: consequences for maternal plasma leptin and cortisol and the programming of fetal adipose tissue development. *Endocrinology* 144:3575-3585, 2003

28. Woodall,SM, Bassett,NS, Gluckman,PD, Breier,BH: Consequences of maternal undernutrition for fetal and postnatal hepatic insulin-like growth factor-I, growth hormone receptor and growth hormone binding protein gene regulation in the rat. *J Mol Endocrinol* 20:313-326, 1998

29. Hawkins,P, Steyn,C, McGarrigle,HH, Calder,NA, Saito,T, Stratford,LL, Noakes,DE, Hansona,MA: Cardiovascular and hypothalamic-pituitary-adrenal axis development in late gestation fetal sheep and young lambs following modest maternal nutrient restriction in early gestation. *Reprod Fertil Dev* 12:443-456, 2000

30. Langley-Evans,SC, Gardner,DS, Jackson,AA: Maternal protein restriction influences the programming of the rat hypothalamic-pituitary-adrenal axis. *J Nutr* 126:1578-1585, 1996

31. Simmons,RA, Templeton,LJ, Gertz,SJ: Intrauterine growth retardation leads to the development of type 2 diabetes in the rat. *Diabetes* 50:2279-2286, 2001

32. Baserga M., Hale M.A., McKnight R.A., Yu X., Callaway C.W., Lane R.H.: Uteroplacental insufficiency alters hepatic expression, phosphorylation, and activity of the glucocorticoid receptor in fetal IUGR rats. *Am J Physiol Regul Integr Comp Physiol* 289:R1348-R1353, 2005

33. Ain R., Canham L.N., Soares M.J.: Dexamethasone-induced intrauterine growth restriction impacts the placental prolactin family, insulin-like growth factor-II and the Akt signaling pathway. *J Endocrinol* 185:253-263, 2005

34. Moss,TJ, Sloboda,DM, Gurrin,LC, Harding,R, Challis,JR, Newnham,JP: Programming effects in sheep of prenatal growth restriction and glucocorticoid exposure. *Am J Physiol Regul Integr Comp Physiol* 281:R960-R970, 2001

35. Cleasby,ME, Livingstone,DE, Nyirenda,MJ, Seckl,JR, Walker,BR: Is programming of glucocorticoid receptor expression by prenatal dexamethasone in the rat secondary to metabolic derangement in adulthood? *Eur J Endocrinol* 148:129-138, 2003

36. Lindsay,RS, Lindsay,RM, Edwards,CR, Seckl,JR: Inhibition of 11-beta-hydroxysteroid dehydrogenase in pregnant rats and the programming of blood pressure in the offspring. *Hypertension* 27:1200-1204, 1996

37. Li,J, Forhead,AJ, Dauncey,MJ, Gilmour,RS, Fowden,AL: Control of growth hormone receptor and insulin-like growth factor-I expression by cortisol in ovine fetal skeletal muscle. *J Physiol* 541:581-589, 2002

38. Li,J, Gilmour,RS, Saunders,JC, Dauncey,MJ, Fowden,AL: Activation of the adult mode of ovine growth hormone receptor gene expression by cortisol during late fetal development. *FASEB J* 13:545-552, 1999

39. Manikkam,M, Crespi,EJ, Doop,DD, Herkimer,C, Lee,JS, Yu,S, Brown,MB, Foster,DL, Padmanabhan,V: Fetal programming: prenatal testosterone excess leads to fetal growth retardation and postnatal catch-up growth in sheep. *Endocrinology* 145:790-798, 2004

40. Fowden,AL, Forhead,AJ: Endocrine mechanisms of intrauterine programming. *Reproduction* 127:515-526, 2004

41. Jones,RH, Ozanne,SE: Fetal programming of glucose-insulin metabolism. *Mol Cell Endocrinol* 297:4-9, 2009

42. Kolas,T, Nakling,J, Salvesen,KA: Smoking during pregnancy increases the risk of preterm births among parous women. *Acta Obstet Gynecol Scand* 79:644-648, 2000

43. Williams,LA, Evans,SF, Newnham,JP: Prospective cohort study of factors influencing the relative weights of the placenta and the newborn infant. *BMJ* 314:1864-1868, 1997

44. Esposito,ER, Horn,KH, Greene,RM, Pisano,MM: An animal model of cigarette smoke-induced in utero growth retardation. *Toxicology* 246:193-202, 2008

45. Farkas,S, Hussein,J, Ariano,RE, Sitar,DS, Hasan,SU: Prenatal cigarette smoke exposure: Pregnancy outcome and gestational changes in plasma nicotine concentration, hematocrit, and carboxyhemoglobin in a newly standardized rat model. *Toxicol Appl Pharmacol* 2006

46. Mason,RJ, Buist,AS, Fisher,EB, Merchant,JA, Samet,JM, Welsh,CH: Cigarette smoking and health. *Am Rev Respir Dis* 132:1133-1136, 1985

47. Glynn,DA, Cryan,JF, Kent,P, Flynn,RA, Kennedy,MP: Update on smoking cessation therapies. *Adv Ther* 26:369-382, 2009

48. Andres R.L., Day M.C.: Perinatal complications associated with maternal tobacco use. *Semin Neonatol* 5:231-241, 2000

49. Bergmann K.E., Bergmann R.L., Von Kries R., Bohm O., Richter R., Dudenhausen J.W., Wahn U.: Early determinants of childhood overweight and adiposity in a birth cohort study: role of breast-feeding. *Int J Obes Relat Metab Disord* 27:162-172, 2003

50. Okuyemi,KS, Ahluwalia,JS, Harris,KJ: Pharmacotherapy of smoking cessation. *Arch Fam Med* 9:270-281, 2000

51. Rogers,JM: Tobacco and pregnancy. *Reprod Toxicol* 28:152-160, 2009

52. Cnattingius,S, Lambe,M: Trends in smoking and overweight during pregnancy: prevalence, risks of pregnancy complications, and adverse pregnancy outcomes. *Semin Perinatol* 26:286-295, 2002

53. Ananth C.V., Smulian J.C., Vintzileos A.M.: Incidence of placental abruption in relation to cigarette smoking and hypertensive disorders during pregnancy: a meta-analysis of observational studies. *Obstet Gynecol* 93:622-628, 1999

54. Faiz,AS, Ananth,CV: Etiology and risk factors for placenta previa: an overview and meta-analysis of observational studies. *J Matern Fetal Neonatal Med* 13:175-190, 2003

55. Jauniaux,E, Burton,GJ: Morphological and biological effects of maternal exposure to tobacco smoke on the feto-placental unit. *Early Hum Dev* 83:699-706, 2007

56. Nordentoft,M, Lou,HC, Hansen,D, Nim,J, Pryds,O, Rubin,P, Hemmingsen,R: Intrauterine growth retardation and premature delivery: the influence of maternal smoking and psychosocial factors. *Am J Public Health* 86:347-354, 1996

57. Robinson,JS, Moore,VM, Owens,JA, McMillen,IC: Origins of fetal growth restriction. *Eur J Obstet Gynecol Reprod Biol* 92:13-19, 2000

58. Sasco,AJ, Vainio,H: From in utero and childhood exposure to parental smoking to childhood cancer: a possible link and the need for action. *Hum Exp Toxicol* 18:192-201, 1999

59. Cornelius,MD, Day,NL: Developmental consequences of prenatal tobacco exposure. *Curr Opin Neurol* 22:121-125, 2009

60. Pauly,JR, Slotkin,TA: Maternal tobacco smoking, nicotine replacement and neurobehavioural development. *Acta Paediatr* 97:1331-1337, 2008

61. Wideroe,M, Vik,T, Jacobsen,G, Bakketeig,LS: Does maternal smoking during pregnancy cause childhood overweight? *Paediatr Perinat Epidemiol* 17:171-179, 2003

62. Toschke,AM, Koletzko,B, Slikker,W, Jr., Hermann,M, Von,KR: Childhood obesity is associated with maternal smoking in pregnancy. *Eur J Pediatr* 161:445-448, 2002

63. Power,C, Jefferis,BJ: Fetal environment and subsequent obesity: a study of maternal smoking. *Int J Epidemiol* 31:413-419, 2002

64. Montgomery,SM, Ekbom,A: Smoking during pregnancy and diabetes mellitus in a British longitudinal birth cohort. *BMJ* 324:26-27, 2002

65. Oken,E, Levitan,EB, Gillman,MW: Maternal smoking during pregnancy and child overweight: systematic review and meta-analysis. *Int J Obes (Lond)* 32:201-210, 2008

66. Lindley,AA, Becker,S, Gray,RH, Herman,AA: Effect of continuing or stopping smoking during pregnancy on infant birth weight, crown-heel length, head circumference, ponderal index, and brain:body weight ratio. *Am J Epidemiol* 152:219-225, 2000

67. Pickett,KE, Wakschlag,LS, Dai,L, Leventhal,BL: Fluctuations of maternal smoking during pregnancy. *Obstet Gynecol* 101:140-147, 2003

68. Ebert,LM, Fahy,K: Why do women continue to smoke in pregnancy? *Women Birth* 20:161-168, 2007

69. Coleman,T: Recommendations for the use of pharmacological smoking cessation strategies in pregnant women. *CNS Drugs* 21:983-993, 2007

70. Coleman,T: Reducing harm from tobacco smoke exposure during pregnancy. *Birth Defects Res C Embryo Today* 84:73-79, 2008

71. Dempsey,D, Jacob,P, III, Benowitz,NL: Accelerated metabolism of nicotine and cotinine in pregnant smokers. *J Pharmacol Exp Ther* 301:594-598, 2002

72. Ontario Medical Association. Rethinking stop-smoking medications: myths and facts. 1999.

73. Ginzel,KH, Maritz,GS, Marks,DF, Neuberger,M, Pauly,JR, Polito,JR, Schulte-Hermann,R, Slotkin,TA: Critical review: nicotine for the fetus, the infant and the adolescent? *J Health Psychol* 12:215-224, 2007

74. Jordanov,JS: Cotinine concentrations in amniotic fluid and urine of smoking, passive smoking and non-smoking pregnant women at term and in the urine of their neonates on 1st day of life. *Eur J Pediatr* 149:734-737, 1990

75. Lambers,DS, Clark,KE: The maternal and fetal physiologic effects of nicotine. *Semin Perinatol* 20:115-126, 1996

76. Morales-Suarez-Varela,MM, Bille,C, Christensen,K, Olsen,J: Smoking habits, nicotine use, and congenital malformations. *Obstet Gynecol* 107:51-57, 2006

77. Strandberg-Larsen,K, Tinggaard,M, Nybo Andersen,AM, Olsen,J, Gronbaek,M: Use of nicotine replacement therapy during pregnancy and stillbirth: a cohort study. *BJOG* 115:1405-1410, 2008

78. Dempsey,DA, Benowitz,NL: Risks and benefits of nicotine to aid smoking cessation in pregnancy. *Drug Saf* 24:277-322, 2001

79. Wisborg,K, Henriksen,TB, Jespersen,LB, Secher,NJ: Nicotine patches for pregnant smokers: a randomized controlled study. *Obstet Gynecol* 96:967-971, 2000

80. Dwyer,JB, McQuown,SC, Leslie,FM: The dynamic effects of nicotine on the developing brain. *Pharmacol Ther* 122:125-139, 2009

81. Neff,RA, Simmens,SJ, Evans,C, Mendelowitz,D: Prenatal nicotine exposure alters central cardiorespiratory responses to hypoxia in rats: implications for sudden infant death syndrome. *J Neurosci* 24:9261-9268, 2004

82. Slotkin,TA, Lappi,SE, McCook,EC, Lorber,BA, Seidler,FJ: Loss of neonatal hypoxia tolerance after prenatal nicotine exposure: implications for sudden infant death syndrome. *Brain Res Bull* 38:69-75, 1995

83. Buttigieg,J, Brown,S, Holloway,AC, Nurse,CA: Chronic nicotine blunts hypoxic sensitivity in perinatal rat adrenal chromaffin cells via upregulation of KATP channels: role of alpha7 nicotinic acetylcholine receptor and hypoxia-inducible factor-2alpha. *J Neurosci* 29:7137-7147, 2009

84. Somm,E, Schwitzgebel,VM, Vauthay,DM, Aubert,ML, Huppi,PS: Prenatal nicotine exposure and the programming of metabolic and cardiovascular disorders. *Mol Cell Endocrinol* 304:69-77, 2009

85. Holloway,AC, Kellenberger,LD, Petrik,JJ: Fetal and neonatal exposure to nicotine disrupts ovarian function and fertility in adult female rats. *Endocrine* 30:213-216, 2006

86. Eskenazi,B, Bergmann,JJ: Passive and active maternal smoking during pregnancy, as measured by serum cotinine, and postnatal smoke exposure. I. Effects on physical growth at age 5 years. *Am J Epidemiol* 142:S10-S18, 1995

87. Luck,W, Nau,H: Nicotine and cotinine concentrations in serum and urine of infants exposed via passive smoking or milk from smoking mothers. *J Pediatr* 107:816-820, 1985

88. Gao,YJ, Holloway,AC, Zeng,ZH, Lim,GE, Petrik,JJ, Foster,WG, Lee,RM: Prenatal exposure to nicotine causes postnatal obesity and altered perivascular adipose tissue function. *Obes Res* 13:687-692, 2005

89. Holloway,AC, Lim,GE, Petrik,JJ, Foster,WG, Morrison,KM, Gerstein,HC: Fetal and neonatal exposure to nicotine in Wistar rats results in increased beta cell apoptosis at birth and postnatal endocrine and metabolic changes associated with type 2 diabetes. *Diabetologia* 48:2661-2666, 2005

90. Gao,YJ, Holloway,AC, Su,LY, Takemori,K, Lu,C, Lee,RM: Effects of fetal and neonatal exposure to nicotine on blood pressure and perivascular adipose tissue function in adult life. *Eur J Pharmacol* 590:264-268, 2008

91. Chen,WJ, Kelly,RB: Effect of prenatal or perinatal nicotine exposure on neonatal thyroid status and offspring growth in rats. *Life Sci* 76:1249-1258, 2005

92. Williams,CM, Kanagasabai,T: Maternal adipose tissue response to nicotine administration in the pregnant rat: effects on fetal body fat and cellularity. *Br J Nutr* 51:7-13, 1984

93. Somm,E, Schwitzgebel,VM, Vauthay,DM, Camm,EJ, Chen,CY, Giacobino,JP, Sizonenko,SV, Aubert,ML, Huppi,PS: Prenatal nicotine exposure alters early pancreatic islet and adipose tissue development with consequences on the control of body weight and glucose metabolism later in life. *Endocrinology* 149:6289-6299, 2008

94. Hill,DJ, Duvillie,B: Pancreatic development and adult diabetes. *Pediatr Res* 48:269-274, 2000

95. Bouwens,L, Rooman,I: Regulation of pancreatic beta-cell mass. *Physiol Rev* 85:1255-1270, 2005

96. Petrik,J, Arany,E, McDonald,TJ, Hill,DJ: Apoptosis in the pancreatic islet cells of the neonatal rat is associated with a reduced expression of insulin-like growth factor II that may act as a survival factor. *Endocrinology* 139:2994-3004, 1998

97. Heit,JJ, Karnik,SK, Kim,SK: Intrinsic regulators of pancreatic beta-cell proliferation. *Annu Rev Cell Dev Biol* 22:311-338, 2006

98. Festa,A, Williams,K, D'Agostino,R, Jr., Wagenknecht,LE, Haffner,SM: The natural course of beta-cell function in nondiabetic and diabetic individuals: the Insulin Resistance Atherosclerosis Study. *Diabetes* 55:1114-1120, 2006

99. Rhodes,CJ: Type 2 diabetes-a matter of beta-cell life and death? *Science* 307:380-384, 2005

100. Sakuraba,H, Mizukami,H, Yagihashi,N, Wada,R, Hanyu,C, Yagihashi,S: Reduced beta-cell mass and expression of oxidative stress-related DNA damage in the islet of Japanese Type II diabetic patients. *Diabetologia* 45:85-96, 2002

101. Yoon,KH, Ko,SH, Cho,JH, Lee,JM, Ahn,YB, Song,KH, Yoo,SJ, Kang,MI, Cha,BY, Lee,KW, Son,HY, Kang,SK, Kim,HS, Lee,IK, Bonner-Weir,S: Selective beta-cell loss and alpha-cell expansion in patients with type 2 diabetes mellitus in Korea. *J Clin Endocrinol Metab* 88:2300-2308, 2003

102. Hill,DJ: Development of the endocrine pancreas. *Rev Endocr Metab Disord* 6:229-238, 2005

103. Meier,JJ, Butler,AE, Saisho,Y, Monchamp,T, Galasso,R, Bhushan,A, Rizza,RA, Butler,PC: Beta-cell replication is the primary mechanism subserving the postnatal expansion of beta-cell mass in humans. *Diabetes* 57:1584-1594, 2008

104. Remacle,C, Dumortier,O, Bol,V, Goosse,K, Romanus,P, Theys,N, Bouckenooghe,T, Reusens,B: Intrauterine programming of the endocrine pancreas. *Diabetes Obes Metab* 9 Suppl 2:196-209, 2007

105. Simmons,RA: Role of metabolic programming in the pathogenesis of beta-cell failure in postnatal life. *Rev Endocr Metab Disord* 8:95-104, 2007

106. Holemans,K, Aerts,L, Van Assche,FA: Lifetime consequences of abnormal fetal pancreatic development. *J Physiol* 547:11-20, 2003

107. Snoeck,A, Remacle,C, Reusens,B, Hoet,JJ: Effect of a low protein diet during pregnancy on the fetal rat endocrine pancreas. *Biol Neonate* 57:107-118, 1990

108. Petrik,J, Reusens,B, Arany,E, Remacle,C, Coelho,C, Hoet,JJ, Hill,DJ: A low protein diet alters the balance of islet cell replication and apoptosis in the fetal and neonatal rat and is associated with a reduced pancreatic expression of insulin-like growth factor-II. *Endocrinology* 140:4861-4873, 1999

109. Dumortier,O, Blondeau,B, Duvillie,B, Reusens,B, Breant,B, Remacle,C: Different mechanisms operating during different critical time-windows reduce rat fetal beta cell mass due to a maternal low-protein or low-energy diet. *Diabetologia* 50:2495-2503, 2007

110. Garofano,A, Czernichow,P, Breant,B: In utero undernutrition impairs rat beta-cell development. *Diabetologia* 40:1231-1234, 1997

111. Stoffers,DA, Desai,BM, DeLeon,DD, Simmons,RA: Neonatal exendin-4 prevents the development of diabetes in the intrauterine growth retarded rat. *Diabetes* 52:734-740, 2003

112. Karlin,A, Akabas,MH: Toward a structural basis for the function of nicotinic acetylcholine receptors and their cousins. *Neuron* 15:1231-1244, 1995

113. Dajas-Bailador,F, Wonnacott,S: Nicotinic acetylcholine receptors and the regulation of neuronal signalling. *Trends Pharmacol Sci* 25:317-324, 2004

114. Maus,AD, Pereira,EF, Karachunski,PI, Horton,RM, Navaneetham,D, Macklin,K, Cortes,WS, Albuquerque,EX, Conti-Fine,BM: Human and rodent bronchial epithelial cells express functional nicotinic acetylcholine receptors. *Mol Pharmacol* 54:779-788, 1998

115. Conti-Fine,BM, Navaneetham,D, Lei,S, Maus,AD: Neuronal nicotinic receptors in non-neuronal cells: new mediators of tobacco toxicity? *Eur J Pharmacol* 393:279-294, 2000

116. Yoshikawa,H, Hellstrom-Lindahl,E, Grill,V: Evidence for functional nicotinic receptors on pancreatic beta cells. *Metabolism* 54:247-254, 2005

117. Kaneto,H, Kawamori,D, Matsuoka,TA, Kajimoto,Y, Yamasaki,Y: Oxidative stress and pancreatic beta-cell dysfunction. *Am J Ther* 12:529-533, 2005

118. Luo,ZC, Fraser,WD, Julien,P, Deal,CL, Audibert,F, Smith,GN, Xiong,X, Walker,M: Tracing the origins of "fetal origins" of adult diseases: programming by oxidative stress? *Med Hypotheses* 66:38-44, 2006

119. Wallace,DC: A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. *Annu Rev Genet* 39:359-407, 2005

120. Simmons,RA: Developmental origins of diabetes: the role of oxidative stress. *Free Radic Biol Med* 40:917-922, 2006

121. Ryter,SW, Kim,HP, Hoetzel,A, Park,JW, Nakahira,K, Wang,X, Choi,AM: Mechanisms of cell death in oxidative stress. *Antioxid Redox Signal* 9:49-89, 2007

122. Maechler,P, Wollheim,CB: Mitochondrial function in normal and diabetic beta-cells. *Nature* 414:807-812, 2001

123. Noakes,PS, Thomas,R, Lane,C, Mori,TA, Barden,AE, Devadason,SG, Prescott,SL: Maternal smoking is associated with increased infant oxidative stress at 3 months of age. *Thorax* Epub - ahead of print.: 2007

124. Ermis,B, Ors,R, Yildirim,A, Tastekin,A, Kardas,F, Akcay,F: Influence of smoking on maternal and neonatal serum malondialdehyde, superoxide dismutase, and glutathione peroxidase levels. *Ann Clin Lab Sci* 34:405-409, 2004

125. Zhao,Z, Reece,EA: Nicotine-induced embryonic malformations mediated by apoptosis from increasing intracellular calcium and oxidative stress. *Birth Defects Res B Dev Reprod Toxicol* 74:383-391, 2005

126. Crowley-Weber,CL, Dvorakova,K, Crowley,C, Bernstein,H, Bernstein,C, Garewal,H, Payne,CM: Nicotine increases oxidative stress, activates NF-kappaB and GRP78, induces apoptosis and sensitizes cells to genotoxic/xenobiotic stresses by a multiple stress inducer, deoxycholate: relevance to colon carcinogenesis. *Chem Biol Interact* 145:53-66, 2003

127. Wetscher,GJ, Bagchi,M, Bagchi,D, Perdikis,G, Hinder,PR, Glaser,K, Hinder,RA: Free radical production in nicotine treated pancreatic tissue. *Free Radic Biol Med* 18:877-882, 1995

128. Husain,K, Scott,BR, Reddy,SK, Somani,SM: Chronic ethanol and nicotine interaction on rat tissue antioxidant defense system. *Alcohol* 25:89-97, 2001

129. Yildiz,D: Nicotine, its metabolism and an overview of its biological effects. *Toxicon* 43:619-632, 2004

130. Lenzen,S, Drinkgern,J, Tiedge,M: Low antioxidant enzyme gene expression in pancreatic islets compared with various other mouse tissues. *Free Radic Biol Med* 20:463-466, 1996

131. Tiedge,M, Lortz,S, Drinkgern,J, Lenzen,S: Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells. *Diabetes* 46:1733-1742, 1997

132. Donath,MY, Ehses,JA, Maedler,K, Schumann,DM, Ellingsgaard,H, Eppler,E, Reinecke,M: Mechanisms of beta-cell death in type 2 diabetes. *Diabetes* 54 Suppl 2:S108-S113, 2005

133. Kajimoto,Y, Kaneto,H: Role of oxidative stress in pancreatic beta-cell dysfunction. *Ann N Y Acad Sci* 1011:168-176, 2004

134. Robertson,RP: Oxidative stress and impaired insulin secretion in type 2 diabetes. *Curr Opin Pharmacol* 6:615-619, 2006

135. Robertson,RP, Harmon,JS: Diabetes, glucose toxicity, and oxidative stress: A case of double jeopardy for the pancreatic islet beta cell. *Free Radic Biol Med* 41:177-184, 2006

136. Green,DR, Kroemer,G: The pathophysiology of mitochondrial cell death. *Science* 305:626-629, 2004

137. Gulbins,E, Dreschers,S, Bock,J: Role of mitochondria in apoptosis. *Exp Physiol* 88:85-90, 2003

138. Gupta,S: Molecular signaling in death receptor and mitochondrial pathways of apoptosis (Review). *Int J Oncol* 22:15-20, 2003

139. Zimmermann,KC, Bonzon,C, Green,DR: The machinery of programmed cell death. *Pharmacol Ther* 92:57-70, 2001

140. Jiang,X, Wang,X: Cytochrome C-mediated apoptosis. *Annu Rev Biochem* 73:87-106, 2004

141. Timmer,T, de Vries,EG, de,JS: Fas receptor-mediated apoptosis: a clinical application? *J Pathol* 196:125-134, 2002

142. Szabadkai,G, Duchen,MR: Mitochondria mediated cell death in diabetes. *Apoptosis* 2009

143. Kim,WH, Lee,JW, Suh,YH, Hong,SH, Choi,JS, Lim,JH, Song,JH, Gao,B, Jung,MH: Exposure to chronic high glucose induces beta-cell apoptosis through decreased interaction of glucokinase with mitochondria: downregulation of glucokinase in pancreatic beta-cells. *Diabetes* 54:2602-2611, 2005

144. Maestre,I, Jordan,J, Calvo,S, Reig,JA, Cena,V, Soria,B, Prentki,M, Roche,E: Mitochondrial dysfunction is involved in apoptosis induced by serum withdrawal and fatty acids in the beta-cell line INS-1. *Endocrinology* 144:335-345, 2003

145. Maedler,K, Spinas,GA, Lehmann,R, Sergeev,P, Weber,M, Fontana,A, Kaiser,N, Donath,MY: Glucose induces beta-cell apoptosis via upregulation of the Fas receptor in human islets. *Diabetes* 50:1683-1690, 2001

146. Maechler,P, de Andrade,PB: Mitochondrial damages and the regulation of insulin secretion. *Biochem Soc Trans* 34:824-827, 2006

147. Lowell,BB, Shulman,GI: Mitochondrial dysfunction and type 2 diabetes. *Science* 307:384-387, 2005

148. MacDonald,PE, Joseph,JW, Rorsman,P: Glucose-sensing mechanisms in pancreatic beta-cells. *Philos Trans R Soc Lond B Biol Sci* 360:2211-2225, 2005

149. Anello M., Lupi R., Spampinato D., Piro S., Masini M., Boggi U., Del Prato S., Rabuazzo A.M., Purrello F., Marchetti P.: Functional and morphological alterations of mitochondria in pancreatic beta cells from type 2 diabetic patients. *Diabetologia* 48:282-289, 2005

150. Simmons,RA, Suponitsky-Kroyter,I, Selak,MA: Progressive accumulation of mitochondrial DNA mutations and decline in mitochondrial function lead to beta-cell failure. *J Biol Chem* 280:28785-28791, 2005

151. Ishihara,H, Wang,H, Drewes,LR, Wollheim,CB: Overexpression of monocarboxylate transporter and lactate dehydrogenase alters insulin secretory responses to pyruvate and lactate in beta cells. *J Clin Invest* 104:1621-1629, 1999

152. Maechler,P, Carobbio,S, Rubi,B: In beta-cells, mitochondria integrate and generate metabolic signals controlling insulin secretion. *Int J Biochem Cell Biol* 38:696-709, 2006

153. Ihara,Y, Yamada,Y, Toyokuni,S, Miyawaki,K, Ban,N, Adachi,T, Kuroe,A, Iwakura,T, Kubota,A, Hiai,H, Seino,Y: Antioxidant alpha-tocopherol ameliorates glycemic control of GK rats, a model of type 2 diabetes. *FEBS Lett* 473:24-26, 2000

154. Song,KH, Lee,WJ, Koh,JM, Kim,HS, Youn,JY, Park,HS, Koh,EH, Kim,MS, Youn,JH, Lee,KU, Park,JY: alpha-Lipoic acid prevents diabetes mellitus in diabetes-prone obese rats. *Biochem Biophys Res Commun* 326:197-202, 2005

155. Slyvka,Y, Inman,SR, Malgor,R, Jackson,EJ, Yee,J, Oshogwemoh,O, Adame,J, Nowak,FV: Protective effects of antioxidant-fortified diet on renal function and metabolic profile in obese Zucker rat. *Endocrine* 35:89-100, 2009

156. Sena,CM, Nunes,E, Gomes,A, Santos,MS, Proenca,T, Martins,MI, Seica,RM: Supplementation of coenzyme Q10 and alpha-tocopherol lowers glycated hemoglobin level and lipid peroxidation in pancreas of diabetic rats. *Nutr Res* 28:113-121, 2008

157. Kaneto,H, Kajimoto,Y, Miyagawa,J, Matsuoka,T, Fujitani,Y, Umayahara,Y, Hanafusa,T, Matsuzawa,Y, Yamasaki,Y, Hori,M: Beneficial effects of antioxidants in diabetes: possible protection of pancreatic beta-cells against glucose toxicity. *Diabetes* 48:2398-2406, 1999

158. Gorogawa,S, Kajimoto,Y, Umayahara,Y, Kaneto,H, Watada,H, Kuroda,A, Kawamori,D, Yasuda,T, Matsuhisa,M, Yamasaki,Y, Hori,M: Probucol preserves pancreatic beta-cell function through reduction of oxidative stress in type 2 diabetes. *Diabetes Res Clin Pract* 57:1-10, 2002

159. Hsu,FL, Liu,IM, Kuo,DH, Chen,WC, Su,HC, Cheng,JT: Antihyperglycemic effect of puerarin in streptozotocin-induced diabetic rats. *J Nat Prod* 66:788-792, 2003

160. Murugan,P, Pari,L: Antioxidant effect of tetrahydrocurcumin in streptozotocin-nicotinamide induced diabetic rats. *Life Sci* 79:1720-1728, 2006

161. Xiong,FL, Sun,XH, Gan,L, Yang,XL, Xu,HB: Puerarin protects rat pancreatic islets from damage by hydrogen peroxide. *Eur J Pharmacol* 529:1-7, 2006

162. Tarnopolsky,MA: The mitochondrial cocktail: rationale for combined nutraceutical therapy in mitochondrial cytopathies. *Adv Drug Deliv Rev* 60:1561-1567, 2008

163. Crane,FL: Biochemical functions of coenzyme Q10. *J Am Coll Nutr* 20:591-598, 2001

164. Papucci,L, Schiavone,N, Witort,E, Donnini,M, Lapucci,A, Tempestini,A, Formigli,L, Zecchi-Orlandini,S, Orlandini,G, Carella,G, Brancato,R, Capaccioli,S: Coenzyme q10 prevents apoptosis by inhibiting mitochondrial depolarization independently of its free radical scavenging property. *J Biol Chem* 278:28220-28228, 2003

165. Lass,A, Sohal,RS: Electron transport-linked ubiquinone-dependent recycling of alpha-tocopherol inhibits autooxidation of mitochondrial membranes. *Arch Biochem Biophys* 352:229-236, 1998

166. de Arriba G., de Hornedo J.P., Rubio S.R., Fernandez M.C., Martinez S.B., Camarero M.M., Cid T.P.: Vitamin E protects against the mitochondrial damage caused by cyclosporin A in LLC-PK1 cells. *Toxicol Appl Pharmacol* 2009

167. Sethumadhavan,S, Chinnakannu,P: L-carnitine and alpha-lipoic acid improve age-associated decline in mitochondrial respiratory chain activity of rat heart muscle. *J Gerontol A Biol Sci Med Sci* 61:650-659, 2006

168. Tharakan,B, Holder-Haynes,JG, Hunter,FA, Childs,EW: Alpha lipoic acid attenuates microvascular endothelial cell hyperpermeability by inhibiting the intrinsic apoptotic signaling. *Am J Surg* 195:174-178, 2008

169. Naderi,J, Somayajulu-Nitu,M, Mukerji,A, Sharda,P, Sikorska,M, Borowy-Borowski,H, Antonsson,B, Pandey,S: Water-soluble formulation of Coenzyme Q10 inhibits Bax-induced destabilization of mitochondria in mammalian cells. *Apoptosis* 11:1359-1369, 2006

170. Moon,Y, Lee,KH, Park,JH, Geum,D, Kim,K: Mitochondrial membrane depolarization and the selective death of dopaminergic neurons by rotenone: protective effect of coenzyme Q10. *J Neurochem* 93:1199-1208, 2005

171. Moreira,PI, Santos,MS, Sena,C, Nunes,E, Seica,R, Oliveira,CR: CoQ10 therapy attenuates amyloid beta-peptide toxicity in brain mitochondria isolated from aged diabetic rats. *Exp Neurol* 196:112-119, 2005

172. Bruin,JE, Kellenberger,LD, Gerstein,HC, Morrison,KM, Holloway,AC: Fetal and neonatal nicotine exposure and postnatal glucose homeostasis: identifying critical windows of exposure. *J Endocrinol* 194:171-178, 2007

173. Bruin,JE, Petre,MA, Lehman,MA, Raha,S, Gerstein,HC, Morrison,KM, Holloway,AC: Maternal nicotine exposure increases oxidative stress in the offspring. *Free Radic Biol Med* 44:1919-1925, 2008

174. Bruin,JE, Gerstein,HC, Morrison,KM, Holloway,AC: Increased pancreatic beta cell apoptosis following fetal and neonatal exposure to nicotine is mediated via the mitochondria. *Toxicol Sci* 103:362-370, 2008

175. Bruin,JE, Petre,MA, Raha,S, Morrison,KM, Gerstein,HC, Holloway,AC: Fetal and neonatal nicotine exposure in Wistar rats causes progressive pancreatic mitochondrial damage and beta cell dysfunction. *PLoS One* 3:e3371, 2008

176. Bruin,JE, Woynillowicz,AK, Hettinga,BP, Tarnopolsky,MA, Morrison,KM, Gerstein,HC, Holloway,AC: Maternal dietary antioxidant supplementation prevents beta cell loss and mitochondrial defects following fetal and neonatal nicotine exposure. *Diabetes* (Submitted): 2009

177. Slikker,W, Jr., Xu,ZA, Levin,ED, Slotkin,TA: Mode of action: disruption of brain cell replication, second messenger, and neurotransmitter systems during development leading to cognitive dysfunction--developmental neurotoxicity of nicotine. *Crit Rev Toxicol* 35:703-711, 2005

178. Chen,H, Parker,SL, Matta,SG, Sharp,BM: Gestational nicotine exposure reduces nicotinic cholinergic receptor (nAChR) expression in dopaminergic brain regions of adolescent rats. *Eur J Neurosci* 22:380-388, 2005

179. Fu,XW, Lindstrom,J, Spindel,ER: Nicotine activates and up-regulates nicotinic acetylcholine receptors in bronchial epithelial cells. *Am J Respir Cell Mol Biol* 41:93-99, 2009

180. Dwoskin,LP, Crooks,PA: Competitive neuronal nicotinic receptor antagonists: a new direction for drug discovery. *J Pharmacol Exp Ther* 298:395-402, 2001

181. Gotti,C, Clementi,F: Neuronal nicotinic receptors: from structure to pathology. *Prog Neurobiol* 74:363-396, 2004

182. Sharma,G, Vijayaraghavan,S: Nicotinic receptor signaling in nonexcitable cells. *J Neurobiol* 53:524-534, 2002

183. Wiederkehr,A, Wollheim,CB: Impact of mitochondrial calcium on the coupling of metabolism to insulin secretion in the pancreatic beta-cell. *Cell Calcium* 44:64-76, 2008

184. Habener,JF, Kemp,DM, Thomas,MK: Minireview: transcriptional regulation in pancreatic development. *Endocrinology* 146:1025-1034, 2005

185. Chan,CB, De,LD, Joseph,JW, McQuaid,TS, Ha,XF, Xu,F, Tsushima,RG, Pennefather,PS, Salapatek,AM, Wheeler,MB: Increased uncoupling protein-2 levels in beta-cells are associated with impaired glucose-stimulated insulin secretion: mechanism of action. *Diabetes* 50:1302-1310, 2001

186. Brown,JE, Thomas,S, Digby,JE, Dunmore,SJ: Glucose induces and leptin decreases expression of uncoupling protein-2 mRNA in human islets. *FEBS Lett* 513:189-192, 2002

187. Krauss,S, Zhang,CY, Scorrano,L, Dalgaard,LT, St-Pierre,J, Grey,ST, Lowell,BB: Superoxide-mediated activation of uncoupling protein 2 causes pancreatic beta cell dysfunction. *J Clin Invest* 112:1831-1842, 2003

188. Silva,JP, Kohler,M, Graff,C, Oldfors,A, Magnuson,MA, Berggren,PO, Larsson,NG: Impaired insulin secretion and beta-cell loss in tissue-specific knockout mice with mitochondrial diabetes. *Nat Genet* 26:336-340, 2000

189. Brandle,M, Lehmann,R, Maly,FE, Schmid,C, Spinas,GA: Diminished insulin secretory response to glucose but normal insulin and glucagon secretory responses to arginine in a family with maternally inherited diabetes and deafness caused by mitochondrial tRNA(LEU(UUR)) gene mutation. *Diabetes Care* 24:1253-1258, 2001

190. Maassen,JA, 'T Hart,LM, Van,EE, Heine,RJ, Nijpels,G, Jahangir Tafrechi,RS, Raap,AK, Janssen,GM, Lemkes,HH: Mitochondrial diabetes: molecular mechanisms and clinical presentation. *Diabetes* 53 Suppl 1:S103-S109, 2004

191. Bruin,JE, Petrik,JJ, Raha,S, Hyslop,JR, Tarnopolsky,MA, Gerstein,HC, Holloway,AC: Rosiglitazone improves pancreatic mitochondrial function in an animal model of dysglycemia: Role of the insulin-like growth factor axis. *J Endocrinol* Submitted: 2009

192. Serradas,P, Giroix,MH, Saulnier,C, Gangnerau,MN, Borg,LA, Welsh,M, Portha,B, Welsh,N: Mitochondrial deoxyribonucleic acid content is specifically decreased in adult, but not fetal, pancreatic islets of the Goto-Kakizaki rat, a genetic model of noninsulin-dependent diabetes. *Endocrinology* 136:5623-5631, 1995

193. Ihara,Y, Toyokuni,S, Uchida,K, Odaka,H, Tanaka,T, Ikeda,H, Hiai,H, Seino,Y, Yamada,Y: Hyperglycemia causes oxidative stress in pancreatic beta-cells of GK rats, a model of type 2 diabetes. *Diabetes* 48:927-932, 1999

194. Del Guerra S., Lupi R., Marselli L., Masini M., Bugliani M., Sbrana S., Torri S., Pollera M., Boggi U., Mosca F., Del Prato S., Marchetti P.: Functional and molecular defects of pancreatic islets in human type 2 diabetes. *Diabetes* 54:727-735, 2005

195. Kulkarni,RN, Holzenberger,M, Shih,DQ, Ozcan,U, Stoffel,M, Magnuson,MA, Kahn,CR: beta-cell-specific deletion of the Igf1 receptor leads to hyperinsulinemia and glucose intolerance but does not alter beta-cell mass. *Nat Genet* 31:111-115, 2002

196. Kulkarni,RN, Bruning,JC, Winnay,JN, Postic,C, Magnuson,MA, Kahn,CR: Tissue-specific knockout of the insulin receptor in pancreatic beta cells creates an insulin secretory defect similar to that in type 2 diabetes. *Cell* 96:329-339, 1999

197. Ueki,K, Okada,T, Hu,J, Liew,CW, Assmann,A, Dahlgren,GM, Peters,JL, Shackman,JG, Zhang,M, Artner,I, Satin,LS, Stein,R, Holzenberger,M, Kennedy,RT, Kahn,CR, Kulkarni,RN: Total insulin and IGF-I resistance in pancreatic beta cells causes overt diabetes. *Nat Genet* 38:583-588, 2006

198. Muller,D, Jones,PM, Persaud,SJ: Autocrine anti-apoptotic and proliferative effects of insulin in pancreatic beta-cells. *FEBS Lett* 580:6977-6980, 2006

199. Robertson,K, Lu,Y, De,JK, Li,B, Su,Q, Lund,PK, Liu,JL: A general and islet cell-enriched overexpression of IGF-I results in normal islet cell growth, hypoglycemia, and significant resistance to experimental diabetes. *Am J Physiol Endocrinol Metab* 294:E928-E938, 2008

200. Devedjian,JC, George,M, Casellas,A, Pujol,A, Visa,J, Pelegrin,M, Gros,L, Bosch,F: Transgenic mice overexpressing insulin-like growth factor-II in beta cells develop type 2 diabetes. *J Clin Invest* 105:731-740, 2000

201. Sesti,G, Sciacqua,A, Cardellini,M, Marini,MA, Maio,R, Vatrano,M, Succurro,E, Lauro,R, Federici,M, Perticone,F: Plasma concentration of IGF-I is independently associated with insulin sensitivity in subjects with different degrees of glucose tolerance. *Diabetes Care* 28:120-125, 2005

202. Rajpathak,SN, Gunter,MJ, Wylie-Rosett,J, Ho,GY, Kaplan,RC, Muzumdar,R, Rohan,TE, Strickler,HD: The role of insulin-like growth factor-I and its binding proteins in glucose homeostasis and type 2 diabetes. *Diabetes Metab Res Rev* 25:3-12, 2009

203. Mizukami,H, Wada,R, Koyama,M, Takeo,T, Suga,S, Wakui,M, Yagihashi,S: Augmented beta cell loss and mitochondrial abnormalities in sucrose-fed GK rats. *Virchows Arch* 452:383-392, 2008

204. Calderari,S, Gangnerau,MN, Thibault,M, Meile,MJ, Kassis,N, Alvarez,C, Portha,B, Serradas,P: Defective IGF2 and IGF1R protein production in embryonic pancreas precedes beta cell mass anomaly in the Goto-Kakizaki rat model of type 2 diabetes. *Diabetologia* 50:1463-1471, 2007

205. Labiris R.N., Bruin J.E., Sundararajan C, Besanger T, Valliant J, Gerstein,HC, Holloway A.C.: Tissue-specific regulation of insulin uptake and insulin receptor expression in a rat model of dysglycemia using a novel molecular imaging probe. *(Manuscript in preparation)* 2009

206. Kelley,DE, He,J, Menshikova,EV, Ritov,VB: Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. *Diabetes* 51:2944-2950, 2002

207. Befroy,DE, Petersen,KF, Dufour,S, Mason,GF, de Graaf,RA, Rothman,DL, Shulman,GI: Impaired mitochondrial substrate oxidation in muscle of insulin-resistant offspring of type 2 diabetic patients. *Diabetes* 56:1376-1381, 2007

208. Petersen,KF, Befroy,D, Dufour,S, Dziura,J, Ariyan,C, Rothman,DL, DiPietro,L, Cline,GW, Shulman,GI: Mitochondrial dysfunction in the elderly: possible role in insulin resistance. *Science* 300:1140-1142, 2003

209. Choo,HJ, Kim,JH, Kwon,OB, Lee,CS, Mun,JY, Han,SS, Yoon,YS, Yoon,G, Choi,KM, Ko,YG: Mitochondria are impaired in the adipocytes of type 2 diabetic mice. *Diabetologia* 49:784-791, 2006

210. Peterside,IE, Selak,MA, Simmons,RA: Impaired oxidative phosphorylation in hepatic mitochondria in growth-retarded rats. *Am J Physiol Endocrinol Metab* 285:E1258-E1266, 2003

211. Wang,J, Kim,JM, Donovan,DM, Becker,KG, Li,MD: Significant modulation of mitochondrial electron transport system by nicotine in various rat brain regions. *Mitochondrion* 2009

212. Onal,A, Uysal,A, Ulker,S, Delen,Y, Yurtseven,ME, Evinc,A: Alterations of brain tissue in fetal rats exposed to nicotine in utero: possible involvement of nitric oxide and catecholamines. *Neurotoxicol Teratol* 26:103-112, 2004

213. Matta,SG, Balfour,DJ, Benowitz,NL, Boyd,RT, Buccafusco,JJ, Caggiula,AR, Craig,CR, Collins,AC, Damaj,MI, Donny,EC, Gardiner,PS, Grady,SR, Heberlein,U, Leonard,SS, Levin,ED, Lukas,RJ, Markou,A, Marks,MJ, McCallum,SE, Parameswaran,N, Perkins,KA, Picciotto,MR, Quik,M, Rose,JE, Rothenfluh,A, Schafer,WR, Stolerman,IP, Tyndale,RF, Wehner,JM, Zirger,JM: Guidelines on nicotine dose selection for in vivo research. *Psychopharmacology (Berl)* 190:269-319, 2007

214. Hussein,J, Farkas,S, MacKinnon,Y, Ariano,RE, Sitar,DS, Hasan,SU: Nicotine dose-concentration relationship and pregnancy outcomes in rat: biologic plausibility and implications for future research. *Toxicol Appl Pharmacol* 218:1-10, 2007

215. Holloway,AC, Petrik,JJ, Bruin,JE, Gerstein,HC: Rosiglitazone prevents diabetes by increasing beta-cell mass in an animal model of type 2 diabetes characterized by reduced beta-cell mass at birth. *Diabetes Obes Metab* 10:763-771, 2008

216. Woynillowicz A.K., Raha S., Holloway A.C.: The effects of smoking cessation pharmacotherapies on insulin secretion in a rat beta cell line. (Abstract). *McMaster University Annual Health Sciences Graduate Research Day*, 2009

217. Petrik J.J., Gerstein H.C., Cesta C.E., Kellenberger L.D., Alfaidy N., Holloway A.C.: Effects of rosiglitazone on ovarian function and fertility in animals with reduced fertility following fetal and neonatal exposure to nicotine. *Endocrine* 2009

218. Anzar M, Buhr M, Mirshokraei P, Holloway A.C.: Fetal and neonatal exposure to nicotine adversely affects testicular and epididymal function in rats. (Abstract). *Society for the Study of Reproduction* Omaha, NE. July, 2006

219. Lagunov A, Sadeu J.C., Bruin J.E., Woynillowicz A.K., Foster W.G., Holloway A.C.: Effect of fetal and lactational exposure to nicotine on spermatogenesis in Wistar rats (Abstract). *Canadian Fertility and Andrology Society 55th Annual Meeting.* Montreal, QC., 2009

220. Buttigieg,J, Brown,S, Zhang,M, Lowe,M, Holloway,AC, Nurse,CA: Chronic nicotine in utero selectively suppresses hypoxic sensitivity in neonatal rat adrenal chromaffin cells. *FASEB J* 22:1317-1326, 2008

221. Skorge,TD, Eagan,TM, Eide,GE, Gulsvik,A, Bakke,PS: The adult incidence of asthma and respiratory symptoms by passive smoking in uterus or in childhood. *Am J Respir Crit Care Med* 172:61-66, 2005

222. Lannero,E, Wickman,M, Pershagen,G, Nordvall,L: Maternal smoking during pregnancy increases the risk of recurrent wheezing during the first years of life (BAMSE). *Respir Res* 7:3, 2006

223. Petre MA, Labiris R.N., Inman M.D., Holloway A.C.: Fetal and neonatal exposure to nicotine disrupts postnatal lung development. (Abstract). *Annual Meeting of the American Thoracic Society* Toronto, ON. May, 2008

7.6 DISCUSSION FIGURES

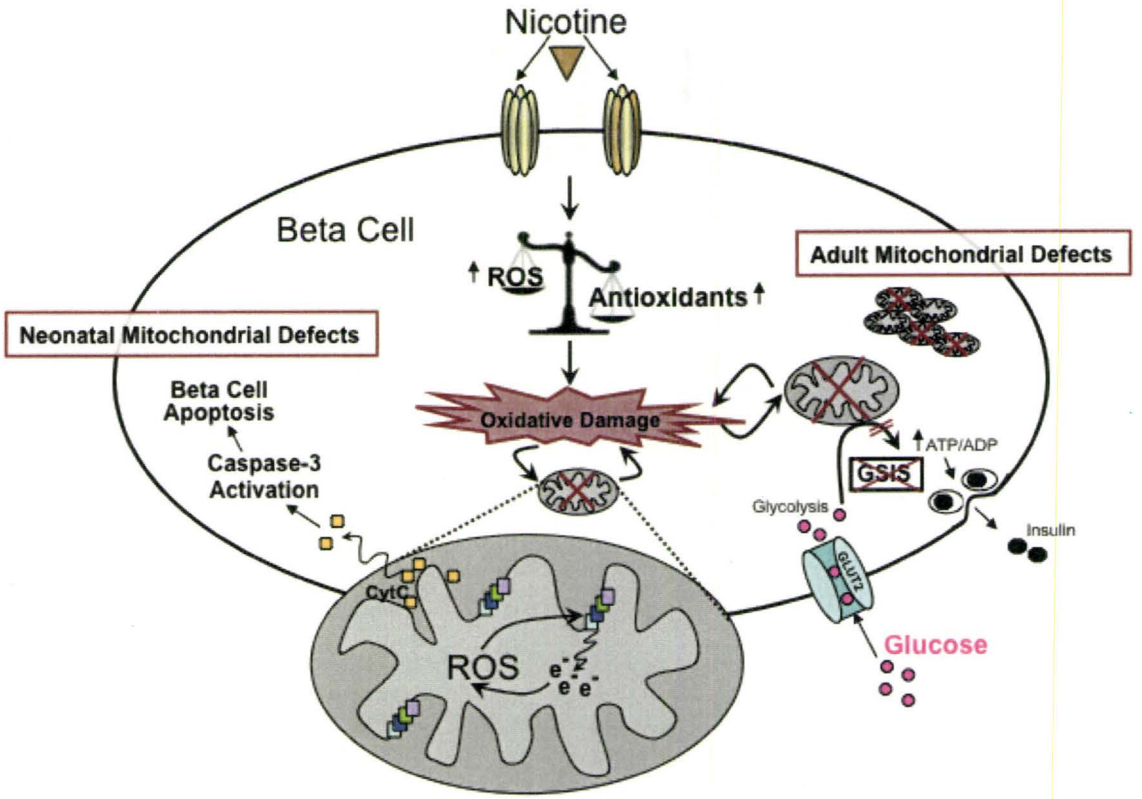


Figure 7.1: Overview of proposed mechanism for nicotine-induced beta cell apoptosis and dysfunction.
Nicotine binds to the nicotinic acetylcholine receptor on the developing beta cell, causing an increase in the production of intracellular reactive oxygen species (ROS), which overwhelms the antioxidant capacity of the cell and leads to oxidative stress. The beta cell mitochondria are particularly susceptible to ROS-induced oxidative damage, and also continue to be a source of ROS production (via electron leakage from dysfunctional electron transport chain enzymes) after cessation of nicotine exposure. In the neonatal beta cells, mitochondria swelling causes release of proteins (e.g. cyt *c*; cytochrome *c*) from the inner membrane space into the cell cytosol, thus triggering beta cell apoptosis and early loss of beta cell mass. As the offspring age, nicotine-induced mitochondrial defects lead to impaired coupling of a glucose stimulus to insulin synthesis and secretion by adult beta cells. Therefore, loss of beta cell mass and function as a result of nicotine-induced oxidative stress and mitochondrial dysfunction likely contribute to the altered glucose homeostasis in this animal model.

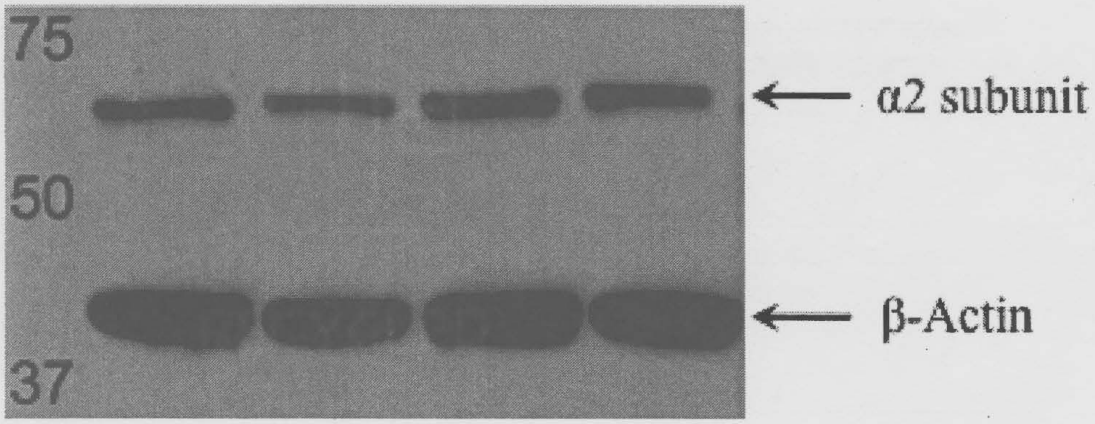


Figure 7.2: nAChR alpha 2 subunit protein expression.
The nAChR alpha 2 subunit (60kDa) was detected in saline-exposed whole pancreatic tissue homogenates at postnatal day 21 by Western blotting and normalized to beta actin as a loading control (45 kDa). (Data from Megan Lehman's 4th year thesis).

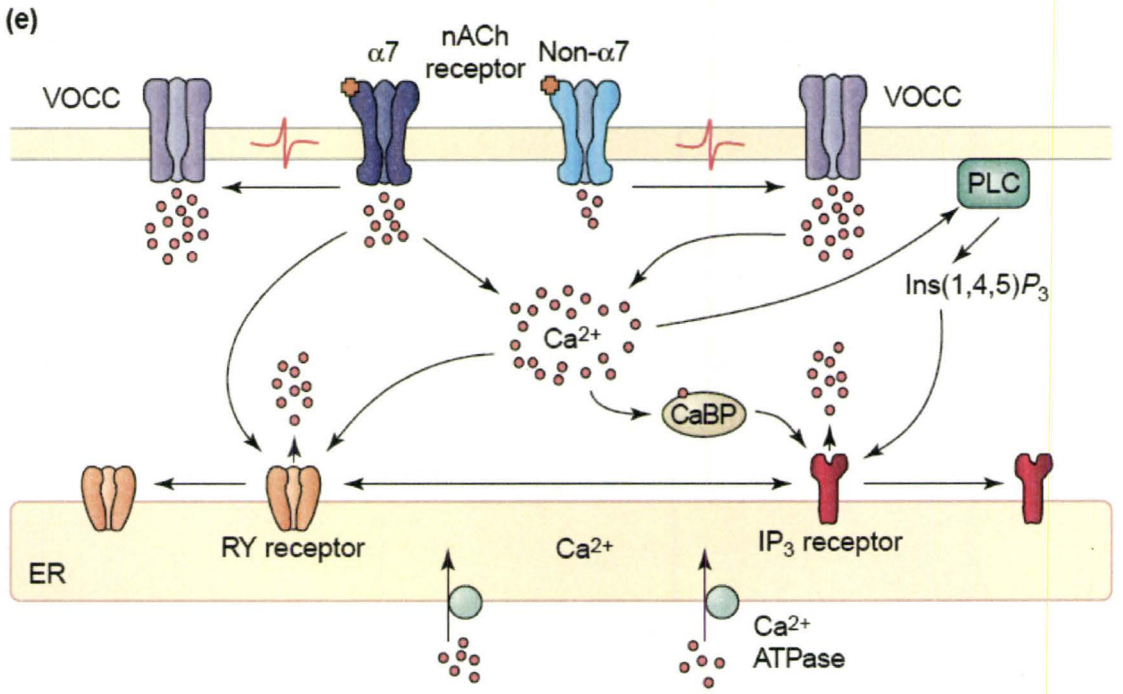


Figure 7.3: Nicotinic acetylcholine receptors (nAChR) and calcium signaling (113). Binding of an agonist to the nAChR causes an increase in intracellular Ca^{2+} via: A) increased ion flux through the active nAChR, B) nAChR-mediated activation of voltage-operated calcium channels (VOCCs), and C) Ca^{2+}-induced Ca^{2+} release from intracellular stores (mediated by ryanodine (RY) receptors and Ins(1,4,5)P_3 (IP_3) receptors).

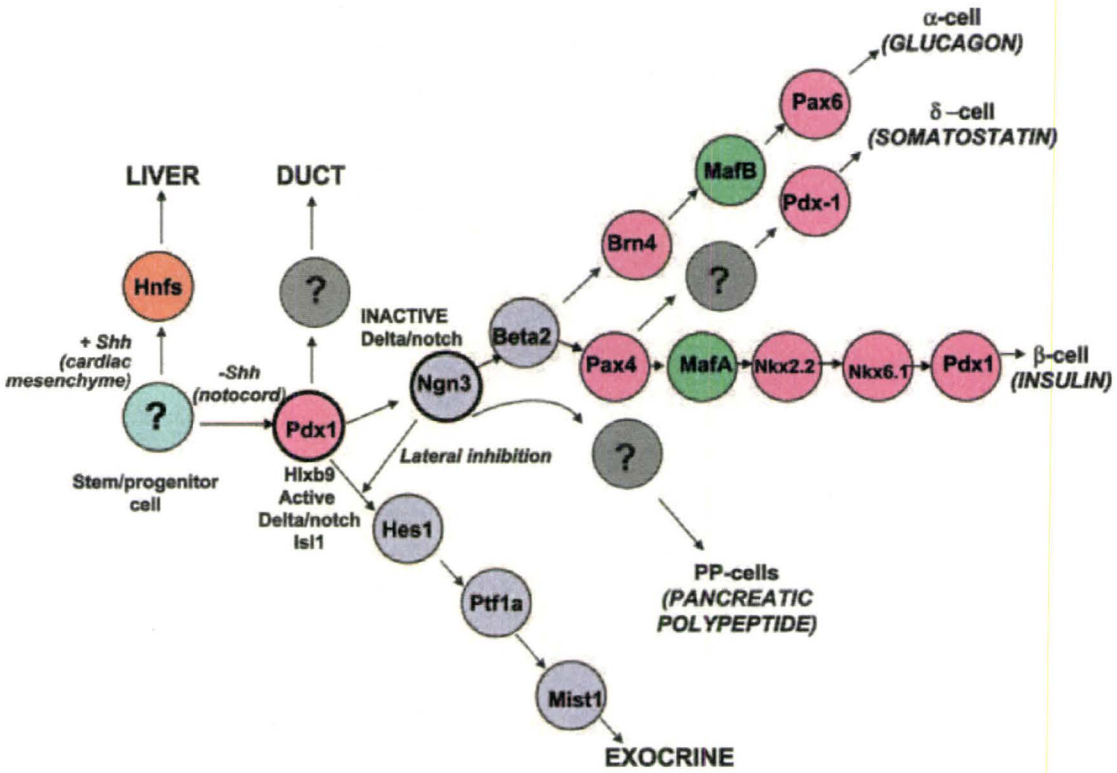


Figure 7.4: Beta cell neogenesis (184).
A simplified schematic of the transcription factors expressed in the different pancreatic cell lineages during development. Beta cells are defined by their unique expression of Pax4, MafA, Nkx2.2, and Nkx6.1 during differentiation.

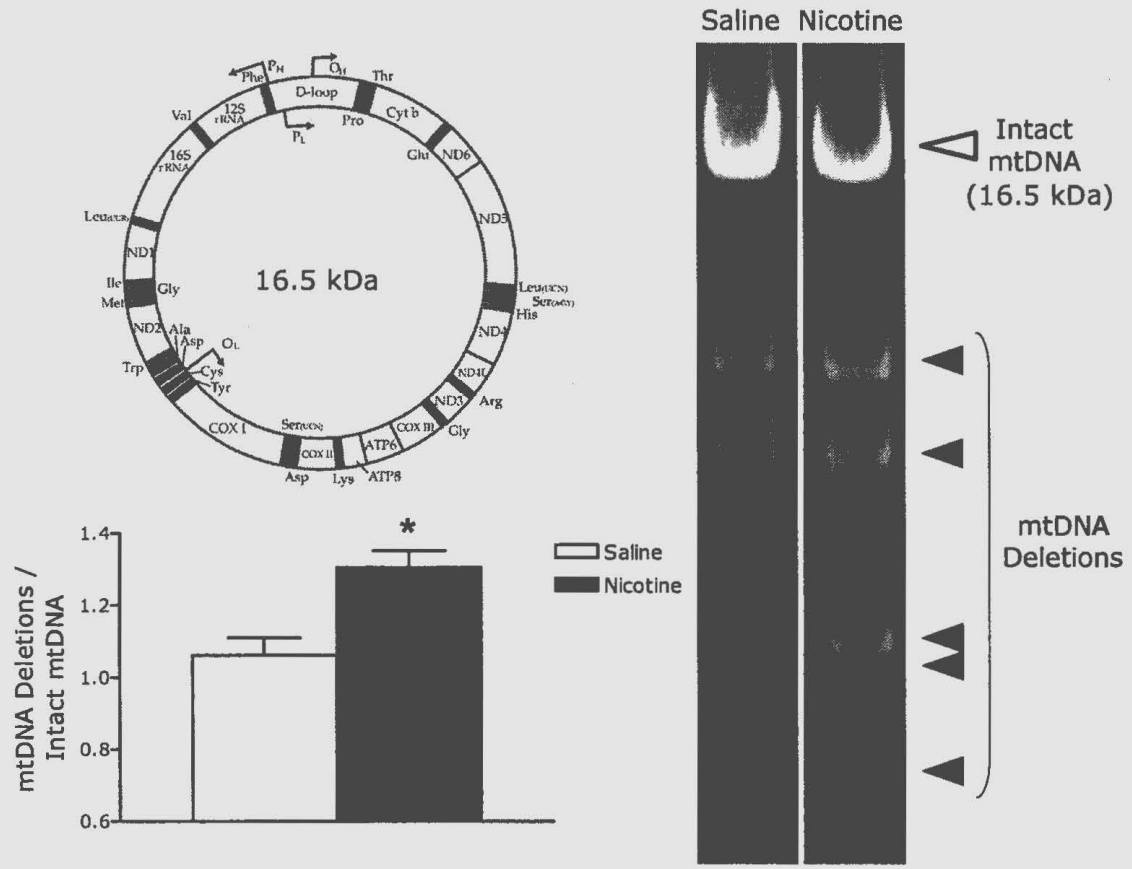


Figure 7.5: Pancreatic mitochondrial DNA (mtDNA) deletions.
A) Diagram of the intact 16.5 kDa mtDNA genome. B) Quantification of mtDNA deletions in the pancreas at 26 weeks of age following saline or nicotine exposure during fetal and neonatal development. An asterisk indicates $p<0.05$. Data are presented as mean ± SEM. C) Representative agarose gels with separated PCR products: intact mtDNA (16.5 kDa) and mtDNA containing deletions (less than 16.5 kDa). Figure was adapted from (119).

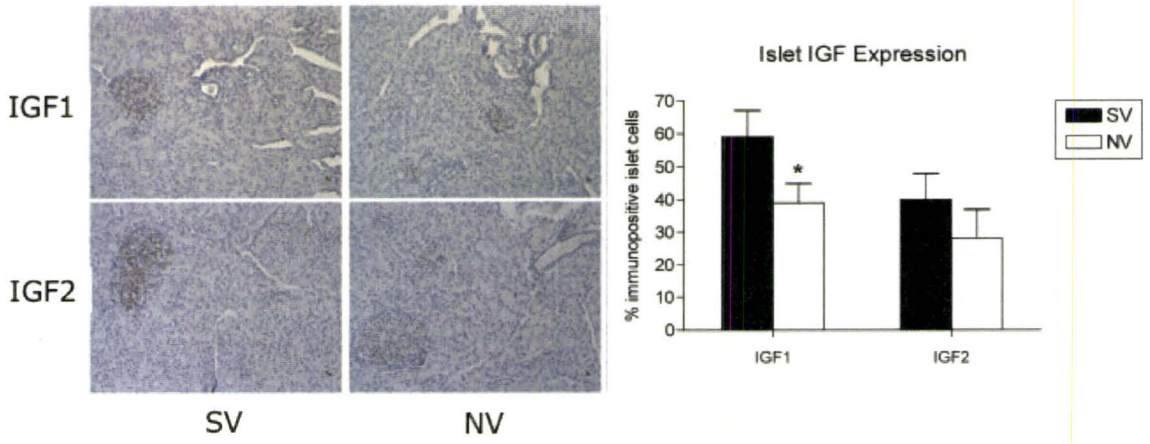


Figure 7.6: Islet insulin-like growth factor (IGF) protein expression.
IGF1 and IGF2 immunostaining in pancreas tissue at 26 weeks of age following saline (SV) or nicotine (NV) exposure during fetal and neonatal development. An asterisk indicates $p<0.05$. Data are expressed as the percentage of immunopositive islet cells and presented as mean ± SEM. Figure was adapted from (119).

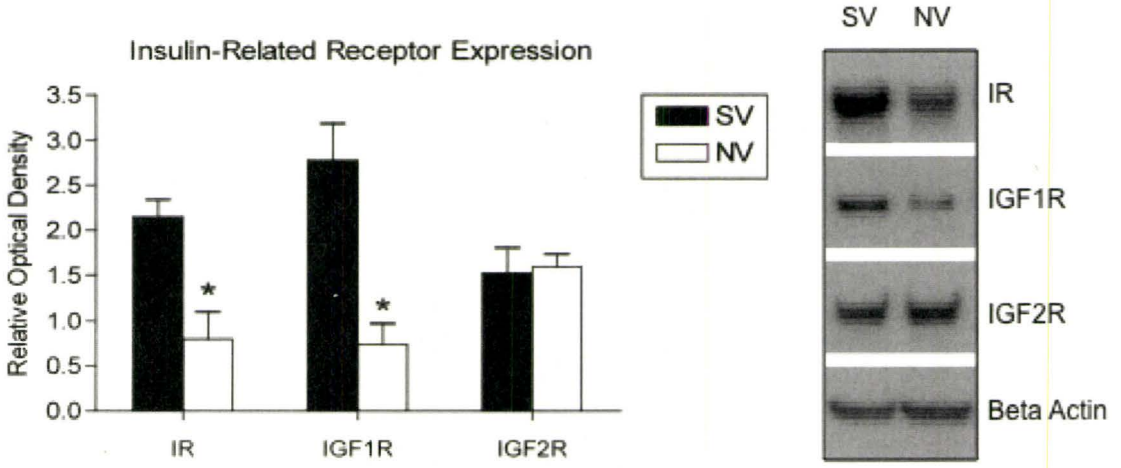


Figure 7.7: Insulin-related receptor protein expression.
Insulin receptor (IR), IGF1R and IGF2R protein expression by western blotting in whole pancreas homogenates at 26 weeks of age following saline (SV) or nicotine (NV) exposure during fetal and neonatal development. An asterisk indicates $p<0.05$. Data are expressed as the relative optical density (normalized to the beta actin loading control) and presented as mean ± SEM. Figure was adapted from (119).

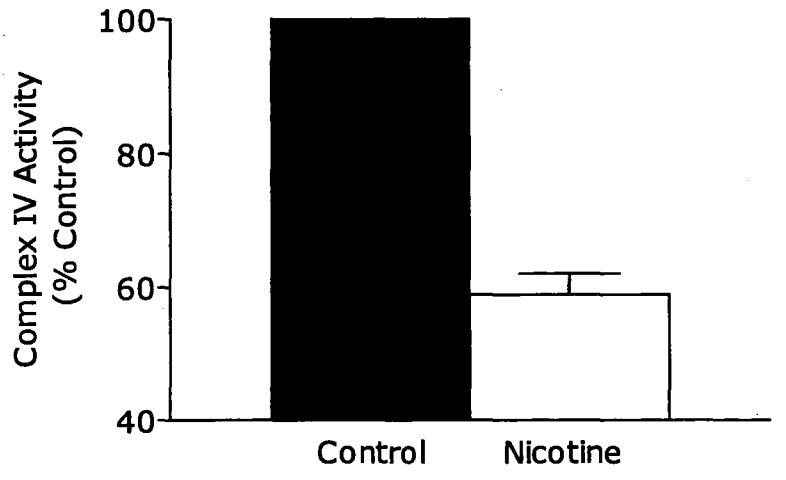


Figure 7.8: Direct effect of nicotine on mitochondrial function in βTC6 cells. Complex IV enzyme activity is expressed as a percentage of the vehicle control following a 1hr nicotine treatment (25 ng/mL) in beta cells *in vitro*. Data are presented as mean ± SEM. (Data provided by Jillian Hyslop).