DOES FOLIC ACID SUPPLEMENTATION PREVENT NICOTINE-INDUCED BETA CELL DYSFUNCTION?

By

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TITLE: Effect of *in vitro* nicotine and folic acid exposure to pancreatic beta cells

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Abstract

Previous studies suggest that nicotine impairs pancreatic function, which may explain the increased risk of T2DM in smokers. We have previously shown that nicotine exposure results in decreased beta cell function, an effect which appears to be mediated via increased beta cell oxidative stress. The goal of this study is to determine whether folic acid, an antioxidant, can prevent nicotine-induced beta cell dysfunction in the beta cell.

INS 1E cells, a rat pancreatic beta cell line, were treated with nicotine or vehicle ± 10µM folic acid for 48 hours. Nicotine treatment decreased both basal and glucose stimulated insulin secretion, but had no effect on insulin content, mitochondrial function or markers of apoptosis. Expression of oxidative stress/damage markers (HSP70 and 4-HNE), antioxidant enzymes (Cu/ZnSOD, MnSOD and CAT), insulin gene transcription factor PDX1 and K_{ATP} channel subunit $kir_{6.2}$ were determined by western blot analysis. Expression of HSP70, 4-HNE and MnSOD were significantly increased with nicotine treatment (p=0.002, 0.05 and 0.03 respectively). Cu/ZnSOD and CAT expression remained unchanged with nicotine treatment. The addition of folic acid significantly reduced HSP70 expression, 4-HNE expression, CAT expression, but did not alter the expression of MnSOD. There was a significant (p<0.0001) increase in expression of PDX1 following treatment with nicotine and folic acid, coinciding with a significant increase in insulin content in this treatment group (p=0.027).Nicotine treatment significantly increased $kir_{6.2}$ expression (p=0.019) which showed a trend toward reduced expression following treatment with folic acid (p=0.067).

Nicotine treatment significantly increases markers of oxidative stress and oxidative damage in pancreatic beta cells; an effect which was reversed by folic acid administration. Nicotine and folic acid treatment increased insulin content, likely mediated through an increase

in the insulin gene transcription factor, PDX1. Furthermore, nicotine treatment increased expression of $kir_{6.2,}$ suggesting a defect in the insulin secretory mechanism. This effect was reversed with folic acid treatment. Although many studies suggest that Canadians are meeting or exceeding recommended folate levels, this is not true in smokers. Our data suggest that additional folate supplementation in smokers may prevent nicotine-induced damage to the pancreas and thus reduce the risk of type 2 diabetes.

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List of Abbreviations

µM	Micromolar
^{125}I-insulin	Iodinated Insulin
4-HNE	4-Hydroxy-2-nonenal
Acetyl CoA	Acetyl-conenzyme A
ADP	Adenosine Diphosphate
ANOVA	Analysis of Variance
Apaf-1	apoptotic protease activating factor 1
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
BCA	Bicinchoninic Acid
Ca^{2+}	Calcium
$CaCl_2$	Calcium Chloride
CAT	Catalase
Cu/ZnSOD	Copper zinc superoxide dismutase
DISC	Death-inducing-signaling -complex
DPBS	Dulbecco's Phosphate Buffered Saline
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunosorbant Assay
ER	Endoplasmic Reticulum
ETC	Electron Transport Chain
$FADH_2$	Flavin Adenine Dinucleotide
FBS	Fetal Bovine Serum
FR	Folate Receptor
GED	General Educational Development

GLUT2	Glucose Transporter 2
GPx	Glutathione Peroxidase
GSIS	Glucose Stimulated Insulin Secretion
H_2O	Water
H_2O_2	Hydrogen Peroxide
H_2PO_4	Dihydrogen Phosphate
HCl	Hydrogen Chloride
HEPES	4-(2-hydroxtlethyl)-1-piperazineethanesulphonic acid
HSP 70	Heat Shock Protein 70
IR	Insulin Receptor
IUGR	Intrauterine Growth Restriction
K^+	Potassium
K_2HPO_4	Phosphoric Acid
K_{ATP}	Adenosine Tri-Phosphate sensitive potassium channel
KCl	Potassium Chloride
kDA	Kilodalton
KRBH	Krebs Ringer Bicarbonate HEPES
MDA	Malondialdehyde
$MgCl_2$	Magnesium Chloride
mM	Millimolar
MnSOD	Manganese superoxide dismutase
nAChR	Nicotinic Acetylcholine Receptor
NaCl	Sodium Chloride
NADH	Nicotinamide Adenine Dinucleotide, reduced form
$NaHCO_3$	Sodium Bicarbonate
NAOH	Sodium Hydroxide
nM	Nanomolar

$O_2^{\bullet -}$	Superoxide
$OH^{\bullet -}$	Hydroxyl Free Radial
PAGE	Polyacrylamide Gel Electrophoresis
PDX-1	Pancreatic and duodenal homeobox 1
RFC	Reduced Folate Carrier
RIPA	Radio Immuno Precipitation Assay
ROS	Reactive Oxygen Species
SAH	S-Adenosylhomocysteine
SAM	S-Adenosylmethionine
SEM	Standard Error of Means
SDS	Sodium Dodecyl Sulfate
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline with 10% (*v/v)* Tween 20
WHO	World Health Organizatio

1.0 Introduction

1.1 Type 2 Diabetes

1.1.1 Prevalence

According to the World Health Organization approximately 285 million people worldwide have diabetes; approximately 90% of patients with diabetes have non-insulin dependent diabetes mellitus otherwise known as type 2 diabetes (WHO 2012). The number of people with diabetes is expected to balloon to 7.7% of the population or 439 million adults by 2030 (Shaw et al 2010). In Canada, about 1 in 4 people have either diabetes or pre-diabetes (approximately 9 million), and the number of people with diabetes is rapidly rising due to an aging population, rising obesity rates and sedentary lifestyles (Canadian Diabetes Association). The other types of diabetes are Type 1 or juvenile onset diabetes and gestational diabetes which will not be discussed here.

1.1.2 Pathology

Type 2 diabetes is characterized by a fasting circulating blood glucose level in excess of 7mmol/l (The expert committee on the diagnosis and classification of Diabetes Mellitus, 2003), or with a blood glucose level >11.1 mmol/l after an oral glucose tolerance test (OGTT). In the pre-diabetes stage, fasting blood glucose levels are 6.1 – 6.9mmol/l with a 2 hour OGTT levels between 7.8 and 11.1mmol/l (Canadian Diabetes Association). This increase in blood glucose is caused by dysfunction of the insulin producing beta cells (inability to produce/secrete insulin in response to a glucose signal) in the endocrine pancreas and/or peripheral insulin resistance (i.e. decreased glucose uptake in skeletal muscle). To maintain glucose homeostasis beta cells

produce and secrete insulin in response to increasing blood glucose levels (i.e. after a meal). Type 2 diabetes is predominantly associated with obesity and inactivity (Prentki and Nolan 2006). Long term consequences of diabetes include cardiovascular complications, retinopathy, kidney failure, and limb amputations (Amos 1997).

1.1.3 Etiology

There are multiple causes of type 2 diabetes, which may involve a combination of beta cell dysfunction and insulin resistance. Dysfunction of beta cells and insulin resistance can occur through both genetic and environmental factors, such as obesity (Kahn, Hull and Utzschneider, 2006), smoking (Ding and Hu, 2007, Wannamethee, Shaper and Perry 2001), single nucleotide polymorphisms (SNP's) (Vassy and Meigs, 2012), and/or fetal insults during pregnancy (Godfrey and Barker 2001, Bertram and Hanson 2001).

The majority of type 2 diabetes cases are associated with a combination of obesity and inactivity. Adipose tissue is able to modulate metabolism by releasing non-esterified fatty acids, glycerol, hormones, namely leptin and adiponectin as well as inflammatory cytokines. When an individual is obese, production of these products (with the exception of adiponectin) is increased. Increased levels of circulating fatty acids are associated with hypertension and may contribute to the pathophysiology of type 2 diabetes, particularly insulin resistance (Boden and Shulman 2002, Kahn et al 2006). Furthermore, chronically elevated fatty acid levels are toxic to the pancreas (i.e lipotoxicity). Elevated lipid levels inhibit glucose stimulated insulin secretion and in addition, inhibits insulin gene expression in the presence of elevated glucose, leading to an increase in beta cell death (Poitout and Robertson 2002, Boden and Shulman 2002, Robertson et al 2004).

In addition to the adverse effects of lipids on beta cell function, chronically elevated glucose levels which are often associated with obesity also have detrimental effects on the pancreatic beta cell (i.e. glucotoxicity). Furthermore, chronically elevated glucose levels lead to some of the secondary complications that occur with diabetes (vascular, retinal and renal tissue abnormalities) (Robertson et al 2007). A chronically high glucose environment leads to significant reduction in insulin secretion (Wallace et al. 2012), an effect likely mediated through oxidative stress (Prentki and Nolan 2006). Lifestyle modifications (weight loss, exercise) can improve the adverse beta cell effects associated with elevated lipids and glucose.

The primary role of the insulin secreted by the pancreatic beta cell is to maintain glucose homeostasis. Glucose homeostasis is achieved by insulin acting on peripheral tissues namely muscle and adipose cells, to stimulate glucose uptake into these tissues while simultaneously reducing hepatic glucose production which occurs through gluconeogenesis. To exert its effects, insulin binds to its receptor (insulin receptor; IR) which is a member of the tyrosine kinase family. Autophosphorylation of IR as a result of insulin binding causes a signaling cascade. One of the most important signals of insulin receptor binding is translocation of the glucose transporter GLUT4 to the cell membrane. Glucose uptake in peripheral tissues occurs through GLUT4, which are highly expressed in skeletal muscle and adipose tissues (Rains and Jain 2011, Saltiel and Pessin 2002 and Choi and Kim 2010).

Insulin resistance occurs when the amount of insulin produced is insufficient to suppress glucose production in the liver and stimulate glucose disposal in skeletal muscle (Strumvoll et al, 2005) Beta cells respond to peripheral insulin resistance by increasing basal and postprandial insulin secretion to compensate for the insulin resistant state (Olefsky and Saltiel, 1996). Once

insulin resistance occurs, glucose levels start to rise giving way to hyperglycemia and its associated complications.

1.1.4 Beta Cell Function

In healthy individuals, beta cells secrete insulin to maintain glucose homeostasis; in normoglycemia plasma glucose concentrations range from 4-8mmol/l non-fasting, 3.9 - <5.5 mmol/l fasting. The ability of the beta cell to secrete insulin at levels which maintain normoglycemia is the result of 2 factors; 1) the responsiveness of the beta cell to a glucose stimulus and 2) the number of beta cells present in the pancreas (i.e. beta cell mass (Kahn et al, 2006).

In a normally functioning beta cell, insulin is released in response to a glucose stimulus. Glucose enters the beta cell via glucose transporters. In humans, this occurs through the glucose transporter GLUT1, in rodents, GLUT2 (De Vos et al, 1995). After entry in to the beta cell, glucose is phosphorylated by glucokinase into glucose-6-phosphate. Via glycolysis, glucose-6-phosphate is converted into pyruvate. Pyruvate enters the mitochondria, is metabolized by the tricarboxylic (TCA) cycle to form NADH and $FADH_2$. Electron transfer from the TCA cycle, by NADH and $FADH_2$, to the respiratory chain promotes the formation of ATP. The production of ATP requires 2 important steps, 1) oxidation of NADH (or $FADH_2$) and 2) phosphorylation of ADP to ATP; these 2 processes are known as oxidative phosphorylation. The NADH and/or $FADH_2$ generated by glycolysis and the TCA cycle are oxidized to NAD^+ or FAD while protons are pumped into the inner mitochondrial membrane through complexes, I, III and IV. Electrons from NADH and $FADH_2$ are pumped through the complexes to O_2, ultimately forming H_2O.

The proton gradients produced by the hydrogen ions are the driving force of ATP synthase to produce ATP from ADP (Kim et al, 2008). In this fashion, oxidative metabolism links the glucose stimulus to subsequent insulin release (Machler and Wollheim, 2001). The formation of ATP leads to an increase in the ATP/ADP ratio, which leads to closure of the ATP sensitive potassium channels (K_{ATP}). K_{ATP} channel closure results in a slow membrane depolarization leading to calcium (Ca^{2+}) influx, rapid membrane depolarization and secretion of insulin granules.

Beta cell mass is a balance between an increase in beta cell number (replication and neogenesis), and increase in beta cell size (hypertrophy) and beta cell death (apoptosis) and a decrease in beta cell size (atrophy) (Butler et al, 2003). An increase in beta cell mass would lead to a potential increase in insulin production and secretion. It appears that beta cell mass increases over a lifetime; in a longitudinal study of Lewis rats, beta cell mass increased into adulthood initially by an increase in cell number, and then by hypertrophy (Bonner-Weir, 2000). It appears that the best stimulus for beta cell mass regulation is glucose. If glucose uptake into peripheral tissues decreases due to inadequate plasma insulin or insulin resistance, then the mild hyperglycemia signals the beta cells that more insulin is needed (Rhodes, 2005). Beta cells then compensate by increasing in number and/or size (increase of beta cell mass) to overcome the increase in glucose. This mild hyperglycemia has beneficial effects, however, chronic or severe hyperglycemia can have detrimental effects (Prentki and Nolan 2006).

1.1.5 Beta Cell Dysfunction in T2DM

In people with type 2 diabetes, beta cell dysfunction is clearly present. Indeed, evidence suggests that beta cell dysfunction is present well in advance of the diagnosis of frank type 2 diabetes (Kahn, 2003). Weir and Bonner-Weir (2004) have proposed 5 stages of beta cell dysfunction during the progression of type 2 diabetes: 1) beta cell compensation, 2) stable adaptation, 3) unstable early decompensation, 4) stable decompensation and 5) severe decompensation. Stage 1 is characterized by an increase in insulin secretion in response to higher circulating blood glucose levels. The increase in insulin secretion can either be a result of increased insulin secretion per cell or from an increase in beta cell number (i.e. increased beta cell mass). During stage 1, blood glucose levels are maintained within the normal range (<6mmol/l fasting). In stage 2, known as 'pre-diabetes', the insulin secreted from the beta cell is no longer able to maintain glucose within the normal range; however, individuals are able to maintain constantly higher glucose levels (6.1-6.9mmol/l fasting glucose) without progressing to diabetes for years. During this stage, there is an increase in oxidative stress, antioxidant and apoptotic markers, as found in rats which have undergone a partial pancreatectomy (Laybutt et al, 2002). Oxidative glucose metabolism leads to the production of reactive oxygen species (ROS), which while necessary for cellular signaling, can be damaging in large quantities. Hyperglycemia is thought to produce large amount of ROS, which overcome the antioxidant defense mechanism, which is low in beta cells (Strumvoll et al, 2005). As previously stated, stage 2 can be maintained for long periods of time, however, at some point the beta cells will be unable to maintain the increased insulin secretion in response to high glucose; this leads to a rapid decline in beta cell mass, due to apoptosis, in conjunction with insulin resistance. This rapid decline in beta cell mass results in even higher glucose levels (i.e. glucotoxicity). This change is stage 3, which is an unstable period and progresses fairly rapidly to stage 4. Stage 4 is

a more stable stage than stage 3. Individuals in stage 4 are still secreting insulin, but at a lower rate due to decreased beta cell mass, which can be as much as 50% reduced. This decrease in beta cell mass is evident by a marked reduction in insulin release in response to a glucose stimulus in diabetic people compared to healthy controls (Kahn, 2000). This stage can last many years, without ever progressing to stage 5, which is complete beta cell failure resulting in dependence on exogenous insulin.

1.1.6 Mechanisms of beta cell dysfunction

Pancreatic beta cell dysfunction results in blood glucose homeostasis that is out of the normal range; this can occur if the beta cells are not responding to the glucose signal (resulting in a high blood glucose level), or conversely, there could be over activity of the beta cell (resulting in a low blood glucose level). The mechanisms leading to beta cell dysfunction leading to hyperglycemia will be discussed here.

The first step for beta cells to secrete insulin is to have glucose transported into the beta cell via the glucose transporter GLUT-2. Potentially, if there are fewer glucose transporters then less glucose will be brought into the beta cell, and less glucose will be metabolized and therefore less insulin will be released. Indeed, it has been shown that in diabetic animal models there is a reduction in the expression of GLUT2 (Thorens et al 1990, Unger 1991, Johnson et al 1990). Conversely, dysfunctional beta cells could have reduced glucose uptake even in the presence of similar glucose transporter expression.

In patients with type 2 diabetes, pancreatic beta cells cannot produce enough insulin to maintain glucose homeostasis. In order for the beta cell to release insulin, there needs to be an

increase in energy production within the beta cell which occurs via the mitochondria. The mitochondria metabolize glucose via glucose oxidation to produce ATP. The production of ATP increases the ratio of ATP/ADP. The increased ATP leads to inhibition of K_{ATP} channels, membrane depolarization, calcium influx and insulin secretion. Therefore, mitochondrial function is linked to insulin secretion via the production of ATP. Mitochondrial dysfunction, has been linked to the development of diabetes (Kim, Wei and Sowers, 2008). Part of this dysfunction has been attributed to reduced cytochrome c oxidase (complex IV) activity (Woynillowicz et al. 2012 and Bruin et al. 2008c) coinciding with increased mitochondrial apoptosis (Bruin et al 2008a).

Hyperglycemia can increase cellular reactive oxygen species (ROS) which negatively impacts cellular functions. But mitochondrial are themselves a source of ROS resulting from imperfectly coupled electron transport. During normal metabolism, superoxide anions are produced but are eliminated by antioxidant defense mechanisms, such as mitochondrial superoxide dismutase (MnSOD). In a hyperglycemic state, it is thought that hyperglycemia increases the electron transfer donors (NADH and $FADH_2$) which in turn increases electron flux through the mitochondrial complexes (Brownlee, 2001). This leads to an increase in the ATP/ADP ratio and hyperpolarization of the inner mitochondrial membrane. The hyperpolarization of the membrane leads to partial inhibition of complex III, which leads to an accumulation of electrons, and generation of superoxide in greater quantities (Rolo and Palmeira 2006).

K_{ATP} channels are located in the plasma membrane of the beta cell and play a key role in linking beta cell metabolism to electrical activity of the membrane, and therefore insulin release. K_{ATP} channels are hetero-octameric complexes containing four pore-forming units ($Kir_{6.x}$)

surrounded by four regulatory units (SUR_X) (Ashcroft, 2006). The K_{ATP} channel in pancreatic beta cells are composed of $Kir_{6.2}$ and SUR1 isomers (MacDonald et al, 2005). In humans, both subunits are located on chromosome 11 relatively close together ($Kir_{6.2}$ is just 4,500 base pairs 3' of the SUR1 gene) (Aguilar-Bryan et al, 1998). ATP, produced by the mitochondria, closes the channel by binding to the kir6.2 subunit at an ATP-binding site located just below the plasma membrane. It is still unclear how ATP binding results in closure the pore (Ashcroft, 2006).

Genetic mutations of the genes encoding $Kir_{6.2}$ and SUR1 (KCNJ11 and ABCC8 respectively) have been shown to cause insulin secretion disorders, such as hyperinsulinemia and type 2 diabetes (Gloyn et al, 2003). These genetic mutations result in gain of function or loss of function of the channel. Loss of function mutations result in hyperinsulinemia, where there is an oversecretion of insulin despite low glucose levels (Flanagan et al, 2009). Loss of function mutations can occur in either ABCC8 or KCNJ11. Conversely, gain of function mutations of either subunit cause diabetes by reducing the responsiveness of the channel to increasing ATP concentrations. This results in an increased potassium efflux from the cell, hyperpolarization of the cell membrane thereby reducing insulin secretion (Flanagan et al, 2007). Although mutations can occur in both subunits, mutations in the KCNJ11 gene are the most common cause of neonatal diabetes and a common polymorphism in KCNJ11 (E23K) is associated with type 2 diabetes in large scale studies (Hattersley and Ashcroft 2005, Flanagan et al 2007). The mutations of KCNJ11 reduce the ability of ATP to inhibit the K_{ATP} channel while simultaneously enhancing channel activity (Ashcroft 2006).

In cases of chronic hyperglycemia, the resulting oxidative stress can lead to apoptosis of the beta cells resulting in a reduction of beta cell mass (Bonner-Weir 2000). Apoptosis is the process of programmed cell death, and typically occurs through two distinct pathways; the

extrinsic ('death-receptor') or intrinsic (mitochondrial mediated) pathway. Both pathways need proteins that are involved in triggering and affecting the apoptotic process; these include the caspases and members of the Bcl-2 family. Caspases are cysteine proteases, each having distinct substrate specificities. The caspases can be divided into groups, the initiator caspases (which start the avalanche of caspase activity) and the effector caspases (which cleave and inactivate vital cell proteins). The Bcl-2 proteins contain pro (Bax, Bak, Bid) and anti-apoptotic (Bcl2, Bcl-xl) members (Cory and Adams 2002, Stasser, O'Conner and Dixit 2000).

The extrinsic pathway involves a death signal binding to a death receptor (Fas/Fas ligand). Ligand binding to the receptor on the cell membrane causes the assembly of a number of proteins in a death-inducing-signaling-complex (DISC). DISC then activates an initiator caspase, caspase-8 and/or caspase-10. What then follows is a Caspase activation cascade, ultimately leading to caspase-3, an effector caspase, activation, (Chandra et al., 2001). Caspase-3 is the central caspase responsible for proteolytic cascade leading to cell death (Ly, Grubb and Lawen 2003)

The intrinsic pathway involves the mitochondria without involving cell surface receptors. The anti-apoptotic members (Bcl-2 and Bcl-xl) work to maintain mitochondrial membrane integrity until they are inactivated by Bid. When there is an apoptotic signal, such as stress, pro-apoptotic members of the Bcl2 family undergo a conformational change (Bax and Bak form homo-oligomers) which allows them to associate with and integrate into the mitochondrial outer membrane causing release of cytochrome c in to the cytosol (Cory and Adams 2002, Gross, McDonnell and Korsmeyer 1999). Released cytochrome c binds to apoptotic protease activating factor 1 (Apaf-1) known as the 'apoptosome'. Apaf-1 which activates once cytochrome c binding

occurs, then binds procaspase-9. Mature caspase-9 is released and then activates the more distal caspase-3 and caspase-7 (Chandra et al., 2001) leading to cell death.

1.2 *Smoking*

1.2.1 Prevalence

The World Health Organization (WHO) states that the global prevalence of cigarette smoking is 22% of people aged 15 and older (WHO 2012). In the United States, approximately 21% of men and 17% of women smoke. The percentage of individuals who smoke declines with age (9.5% of people aged 65 and older compared with 20% of people aged 18-24), but also declines with educational level (45.2% of adults with a general educational development (GED) smoke compared with 6.3% of adults with a postgraduate degree) (Centre for Disease Control, 2012). In Canada, about 43% of the population were current or former smokers in 2011. While this number has decreased from 51% of the population in 1999, the number of smokers has remained the same, while the population has increased (Health Canada, 2012). Of these smokers, approximately 18% are women who reported smoking during pregnancy.

1.2.2 Smoking and type 2 diabetes

Epidemiological studies have shown that heavy cigarette smokers are at an increased risk for the development of type 2 diabetes (Xie et al, 2009, Willi et al 2007 and Haire-Joshu et al 1999). Smoking, and more importantly nicotine itself, has been shown to decrease insulin sensitivity due to impaired glucose uptake in peripheral organs independent of other factors which influence insulin sensitivity (Eliasson et al. 1994, Axelsson et al. 2001). This decrease in insulin sensitivity (a decrease by approximately 10 – 40%) leads to impaired metabolic control in

diabetic patients since more insulin is needed to achieve metabolic control in smokers than non-smokers (Xie et al 2009, Chiolero et al 2008, Eliasson 2003 and Attvall et al 1993) Furthermore, in an animal model of nicotine exposure, nicotine exposed animals were dysglycemic and had an increase in apoptosis of beta cells (Holloway et al., 2005, Bruin et al 2007, 2008a,b and c), this effect is likely through an increase in oxidative stress. Woynillowicz et al (2012) saw an increase in MnSOD expression in an *in vitro* model of nicotine exposure on pancreatic beta cells.

Nicotine is a lipid soluble compound that is metabolized in the liver by cytochrome P450 enzymes CYP2A6 and CYB2B6 and has a half-life of 1-2 hours (Tweed et al, 2012). The primary metabolite of nicotine is cotinine (approximately 70-80% of nicotine is converted to cotinine), which has a half-life of 15-20 hours and serum concentrations are generally 10 times higher in the serum making it a better indicator of nicotine exposure (Lambers and Clark 1996, Benowitz 1996). Nicotine is the main alkaloid found in tobacco and exerts its effects via direct stimulation of neuronal nicotinic acetylcholine receptors (nAChRs). Neuronal acetylcholine receptors are composed of α and β subunits, which can be arranged in a diverse way with α_2 to α_9 and β_2 to β_4 subunits found in brain tissues. The majority of nicotine's effects involve the $\alpha_4\beta_2$, $\alpha_2\beta_2$, and α_7 subunits (Narahashi et al 2000, Benowitz 1996). Nicotine increases synaptic neurotransmission of neurotransmitters such as dopamine, which results in the rewarding and reinforcing effects of nicotine and therefore plays a key role in its highly addictive nature (Tweed et al, 2012).

1.2.3 Nicotine and Oxidative Stress

Cigarette smoke contains both oxidants and pro-oxidants, including nicotine, which are capable of producing reactive oxygen species (ROS) and therefore enhancing oxidative stress (Kosecik et al 2005). Oxidative stress is an imbalance between endogenous and exogenous antioxidants and reactive oxygen species or reactive nitrogen species (RNS). Under some conditions, an increase in oxidants cannot be prevented by antioxidants and, consequently, oxidative stress ensues. Oxidative stress has been implicated in over 100 conditions, including type 2 diabetes. (Kosecik et al 2005, Sies 1997). Oxidants and free radicals stemming from cigarette smoke are capable of directly and indirectly inducing oxidative stress in the body (Aycicek et al 2011). A single puff of cigarette smoke contains 10^{15} free radicals (Alberg 2002).

In addition to cigarette smoke causing oxidative stress, nicotine alone also leads to oxidative stress and damage in tissues (Yildiz et al., 1998, Guan et al., 2003, Suleyman et al., 2002, Zhao and Reece 2005). Chronic exposure to pro-oxidants promotes oxidative injury in tissues and organs leading to disease. Oxidative stress is minimized in cells by exogenous antioxidants (vitamins E and C) and by endogenous antioxidants (super-oxide dismutase and glutathione peroxidase) (Tollefson et al 2010).

In pancreatic beta cells, the levels of antioxidant enzymes are considerably lower than other tissues (Robertson, 2004). Lenzen et al (1996) sought to determine the levels of antioxidant enzymes in various mouse tissues. They found that pancreatic levels of the cytosolic superoxide dismutase and the mitochondrial superoxide dismutase (Cu/ZnSOD and MnSOD) were 38% and 30% that of liver respectively. Levels of glutathione peroxidase (GPx) and catalase (CAT) were even lower, with GPx levels only 15% that of liver and CAT levels were undetectable. These

lower levels of defensive mechanisms can potentially pose problems for the pancreatic beta cell, if exposed to oxidative stress (Lenzen, 2008).

Due to the pro-oxidant effects of nicotine, it is plausible to suggest that treatment with an anti-oxidant might prevent nicotine-induced oxidative stress and damage. Indeed, studies have looked at the effects of antioxidants in nicotine treated tissue and found beneficial effects (Helen et al. 2003, Kalpana and Menon 2004). In a recent study by Bruin et al (2012), a diet enriched in anti-oxidants (Vitamin E, coenzyme Q10 and α-lipoic acid) was given to nicotine-exposed rats throughout pregnancy and lactation. The anti-oxidant intervention was able to decrease beta cell apoptosis and therefore preserve beta cell mass of the offspring. However, the pups exposed to anti-oxidants were significantly smaller. This could, in part, be due to the addition of vitamin E in the diet. There is some discrepancy in the literature regarding vitamin E as to its effectiveness as an anti-oxidant (in higher doses vitamin E tends to act as a pro-oxidant) (Pearson et al, 2006). Due to the promising effect of the antioxidant cocktail on preserving beta cell mass in nicotine exposed animals, finding a different antioxidant that will preserve beta cell function without the adverse effects on growth, could be a viable option to negate the adverse effects of nicotine exposure on beta cell function. One such anti-oxidant is folic acid, a vitamin which is essential during pregnancy. Furthermore, folic acid had been shown to decrease markers of oxidative stress and damage (Lazalde-Ramos et al, 2012).

1.3 *Folic Acid*

1.3.1 Folic Acid

Folate or folic acid is an essential B vitamin that is found in green vegetables, fruits and whole grains. (Mannino et al 2003) It is has been known for some time the importance of

supplementing the diet with folic acid during pregnancy, as dietary folate intake alone may not meet the folate requirements in pregnancy. The need for folate increases dramatically during times of rapid tissue growth, such as pregnancy, where the uterus enlarges and there is growth of the placenta and fetus. Folate deficiency during pregnancy has been associated with an increased risk of neural tube defects (NTD), placental abruption and preeclampsia (Tamura and Piccano, 2006).

Folic acid is involved in nucleic acid synthesis and therefore is required for cell division. Folic acid, in the form of 5-methyltetrahydrofolate, donates a methyl group via the enzyme methionine synthase to form methionine from homocysteine. Methionine then acts a substrate to form s-adenosylmethionine (SAM). SAM is then used to transfer carbon units to purine and pyrimidine bases (Achon et al, 2007). Furthermore, folate is required to metabolize the amino acids methionine, serine, glycine and histidine, which are required in the formation of proteins. Folate deficiency can impair cellular growth and repair in the fetus and/or placenta, increasing the risk for low birth weight and pre-term delivery. (Czeizel et al 1996, Scholl et al 1996, Scholl and Johnson 2006, Duthie and Hawdon 1998 and Jauniaux et al 2007, Wei et al 2003).

1.3.2 Antioxidant properties

Folic acid is not only a co-factor for nucleic acid biosynthesis, but an anti-oxidant as well. Folic acid has been shown to be beneficial in the risk reduction of certain cancers (notably, pancreatic and breast), one such mechanism is the ability for folic acid to scavenge free radicals. (Joshi et al 2001, Stroes et al 2000) It has been shown that folic acid has a potent antioxidant

capacity higher even than Trolox, a derivative of vitamin E, used to reduce oxidative stress or damage (Cano et al, 2001).

Strong evidence shows that cigarette smoking is directly responsible for the lower levels of antioxidant micronutrients, such as folic acid (Alberg 2002). Organic nitrites, nitrous oxide, cyanates found in cigarette smoke interact with folate and convert it to an inactive compound, which could account for the decreased folate level in smokers. (Northrop-Clewes and Thurnham 2007)

It has been shown that folic acid supplementation increases total serum antioxidant capacity (Aghamohammadi et al 2011 and Delfino et al 2007) which could be beneficial in people, such as smokers, who have increased markers of oxidative stress and damage than non-smokers. Furthermore, smokers (as well as individuals exposed to second-hand smoke) have decreased levels of folate than non-smokers (Okumura and Tsukamoto 2011). Because of this, folate supplementation could be a good candidate for the prevention of beta cell damage caused by cigarette smoking.

1.3.3 Use in clinical trials

Numerous clinical trials involving folic acid have been completed, or are ongoing. These clinical trials have looked at outcomes such as stroke (Tighe et al, 2011), rheumatoid arthritis (Morgan et al 1994), cardiovascular events (Albert et al, 2008), renal disease (Wrone et al, 2004), preeclampsia prevention (FACT study, currently recruiting) and cancer prevention (Kim 2004). The doses and length of the trials have varied considerably, which could account for some of the conflicting trial results. The most controversial evidence is with regard to cancer

prevention. Due to the role of folate in *de novo* DNA synthesis, cancer pharmaco-therapies include anti-folate drugs (i.e. methotrexate). The basis for this therapy is that interruption of folate metabolism causes ineffective DNA synthesis, which results in inhibition of cancer tumor growth. Despite the requirement of folate for tumor progression, folate supplementation appears to prevent cancer in people without pre-existing pre-malignant lesions. While, in people who do have these lesions, folate supplementation can accelerate their progression (Kim, 2004). Despite these results, beneficial results have been seen, specifically, folic acid supplementation was shown to reduce the chance of stroke by 18% (Tighe et al, 2011).

In addition, it has been shown by animal data that folic acid supplementation decreases both oxidative stress and damage. In a study investigating the pro-oxidant effect of maternal ethanol administration, it was found that when dams were exposed to ethanol in conjunction with dietary folic acid supplementation, pups were protected against oxidative stress caused by the ethanol treatment. This decrease in oxidative stress was seen as a decrease in thiobarbituric acid reactive substances (TBARS, a byproduct of lipid peroxidation) and protein carbonyl groups in the liver and pancreas in the offspring (Cano et al, 2001). Furthermore, in a human study looking at the effect of folic acid on oxidative stress in individuals with hyperlipidemia and hyperhomocysteinaemia, it was found that folic acid supplementation reduced the oxidative damage marker malondialdehyde (MDA), a marker of lipid peroxidation (Racek, et al 2005). These studies have shown folic acid is able to alleviate oxidative stress and damage in a variety of tissues. Due to this, it is plausible that folic acid will also be able to counteract the negative effects of nicotine on pancreatic beta cells and preserve beta cell function.

1.4 *Hypothesis*

I hypothesize that since *in vitro* exposure to nicotine leads to impaired pancreatic beta cell function via increased oxidative stress that supplementation with the antioxidant folic acid will prevent nicotine-induced beta cell damage.

1.5 *Objectives*

1. To determine *in vitro* whether co-supplementation of folic acid can decrease markers of oxidative stress caused by nicotine treatment
2. To determine the mechanism(s) by which folic acid can reduce oxidative stress in pancreatic beta cells
3. To determine whether or not folic acid supplementation can improve beta cell function in nicotine treated beta cells *in vitro*

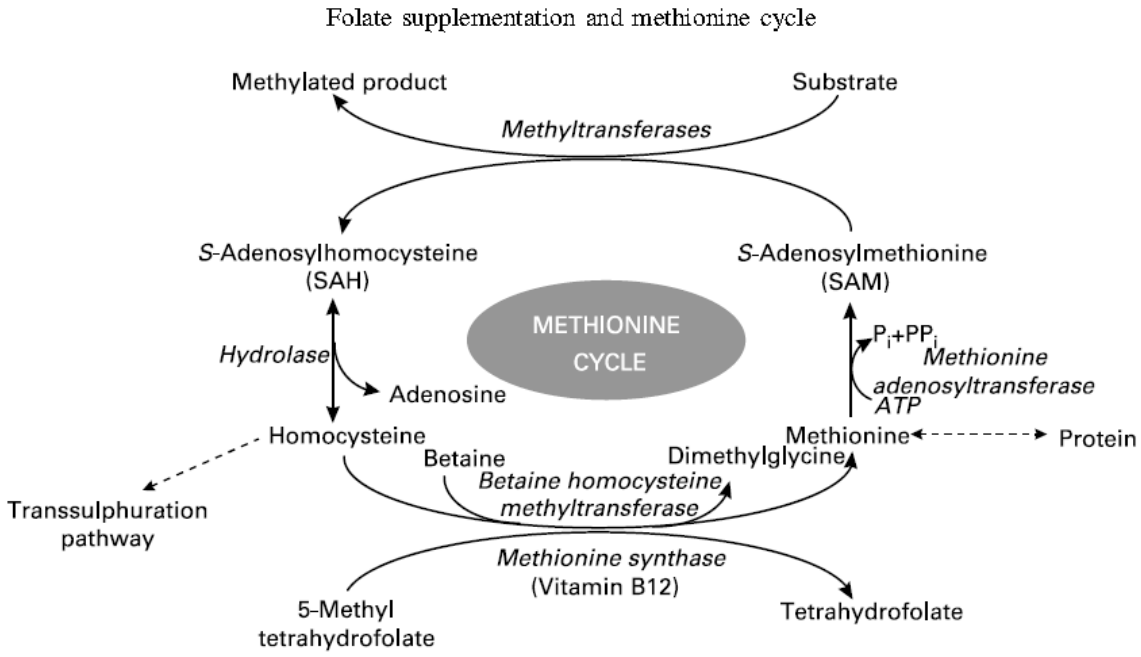

Figure 1.1: Methionine Cycle

This diagram illustrates how folate is involved in methyl donation. 5-methyltetrahydrofolate donates a methyl group with the assistance of methionine transferase for the formation of methionine from homocysteine. Methionine then acts a substrate for the formation of S-Adenosylmethionine (SAM) which is used as a methyl donor or a range of reactions. After the donation of a methyl group, SAM is converted to S-adenosylhomocysteine (SAH), which is then hydrolysed back to homocysteine and adenosine. Figure taken from Achon et al, British Journal of Nutrition, 2007, 98:490-496.

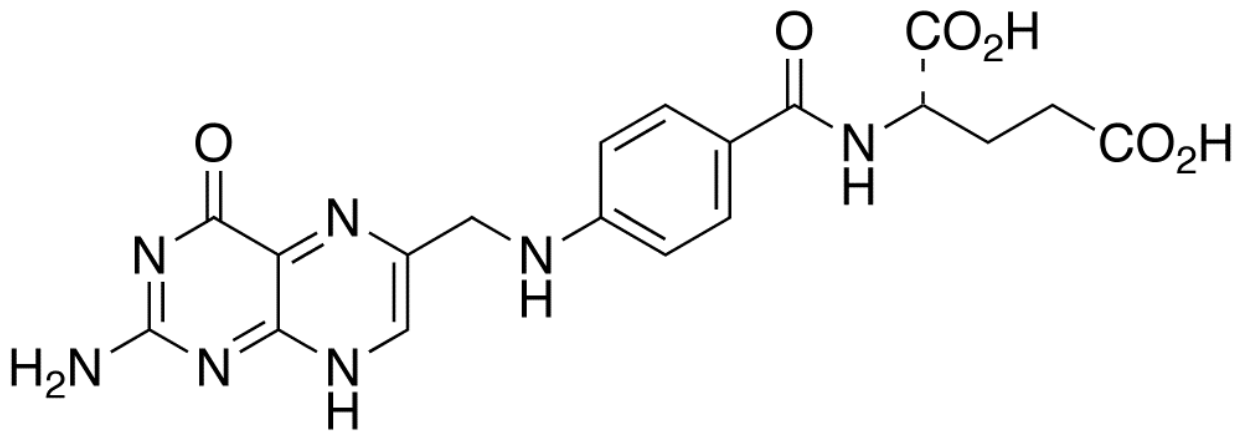

Figure 1.2: Folic acid Structure

Figure taken from Toronto Research Chemicals (TRC), http://www.trc-canada.com/detail.php?CatNum=F680300&CAS=59-30-3&Chemical_Name=Folic Acid&Mol_Formula=C19H19N7O6&Synonym=N-[4-[[(2-Amino-3,4-dihydro-4-oxo-6-pteridinyl)methyl]amino]benzoyl]-L-glutamic Acid; Acifolic; Aspol; Cytofol; Folacid; Folacin; Folbal; Folcidin; Foldine; Folettes; Foliamin; Folicet; Folipac; Folovit; Folsan; Folsaure; Folsav; Folvite; Incafolic; Millafol; Pteroyl-L-monoglutamic Acid; Pteroylglutamic Acid; Pteroylmonoglutamic Acid; Vitamin Bc; Vitamin Be; Vitamin M; NSC 3073;

2.0 Methods

2.1 Effect of nicotine and folic acid treatment on markers of oxidative stress

2.1.1 *Cell Culture Maintenance*

All studies were conducted using INS 1E cells, a rat insulinoma beta cell line generously donated by Dr. Claes Wollheim (Geneva, Switzerland). This cell line is one of the few beta cell lines which secretes insulin in response to insulin within the physiological range (Skelin et al. 2010) Cells were plated in 10 cm non-pyrogenic tissue culture dishes (BD Biosciences, Mississauga, Ontario) and cultured in RPMI 1640 medium (Hyclone, Logan, Utah) supplemented with 10% heat inactivated fetal bovine serum (Hyclone), 1mM Na^+-pyruvate, 50µM β-mercaptoethanol, and 1U/ml penicillin, 1µg/ml streptomycin (Invitrogen, Burlington, ON) and incubated in a humidified atmosphere of 95% O_2 and 5% CO_2. Cells were passaged 1:3 at a confluence of 80% by trypsinization. Briefly, cells were washed with calcium and magnesium free Dulbecco's Phosphate buffered solution (DPBS, Hyclone) and then incubated with 1x trypsin (Invitrogen) with EDTA and NaCl (Invitrogen) for approximately 5 minutes. Trypsin was neutralized with media, and media transferred to 3 new plates and allowed to equilibrate for 24 hours.

2.1.2 *Western Blotting*

INS1E cells were treated for 48 hours with media alone (control), media containing 10 µM folic acid (Sigma Aldrich) or media containing 1µM nicotine alone (Sigma Aldrich, Oakville, ON) or 1µM nicotine 10µM folic acid . This dose of folic acid was chosen to be 4x the amount of folic acid in the media (2.2µM) which is similar to the predicted rise in serum folate levels in women who increase their folate intake (Wald et al., 2001). After treatment, plates were washed

twice with DPBS (Hyclone), then scraped, centrifuged and resuspended in radioimmunoprecipitation assay (RIPA) buffer (15mM Tris-HCl (Bioshop, Burlington, ON), 1% (*v*/v) Triton X-100 (BDH Lab Reagents, Toronto, ON), 0.1% (*w*/v) SDS (Bioshop), 167mM NaCl (Bioshop), 0.5% (*w*/v) sodium deoxycholic acid (Sigma Aldrich)) containing protease inhibitors (Roche, Laval QC). Lysed cells were sonicated at 7Hz for 15 seconds and stored at -80°C until use.

2.1.3 *Folate Receptor and Reduced Folate Carrier Expression*

Folate is able to reach target cells by two methods of transportation; a folate receptor (FR) and a folate carrier known as reduced folate carrier (RFC). To determine if treatment with folic acid can have a beneficial effect on INS 1E cells, I first needed to identify whether a) the receptor and transporter are present in the INS 1E cell line and b) whether nicotine treatment affects the expression level of either FR or RFC.

Protein (20µg) from each treatment group was subjected to SDS-PAGE (at 150V for 45 minutes, Pierce, Rockford IL) then electrotransferred to a PVDF membrane (at 40V for 90 minutes, BioRad Laboratories). Membranes were blocked overnight in 5% (*w/v)* skim milk in TBST (Tris buffered saline (50mM Tris (Bioshop), 150mM NaCl (Bioshop), pH=7.5), 0.5% Tween 20) at 4°C under gentle agitation. Folate receptor primary antibody (1:450, Santa Cruz Biotechnologies, Santa Cruz, CA) and reduced folate carrier (1:200, Santa Cruz Biotechnologies) were incubated for 1 hour at room temperature, followed by 10 washes in TBST. Membranes were then incubated for 1 hour peroxidase conjugated anti-rabbit antibody (1:10,000, Amersham Biosciences, Little Chalfont, Buckinghamshire) on a rocking platform. Membranes were

thoroughly washed in TBST followed by TBS (Tris buffered saline, recipe above). Protein was detected using ECL Plus chemiluminescence (Amersham) and Hyperfilm ECL film (Amersham). Densitometry analysis was performed using Image J 1.45v software. All proteins were quantified relative to beta actin loading control. Briefly, membranes were incubated in stripping buffer (Thermo Scientific, Rockford, IL) for half an hour on a rocking platform. Membranes were quickly washed, and blocked with 1% (*w/v)* BSA (EMD Chemicals Inc., Gibbstown, NJ) in TBST. Following an hour incubation, blocking solution was removed, and β-actin primary antibody was added (1:4000 in 1% BSA (*w/v*) in TBST (Abcam, Cambridge, MA). Membranes were incubated for another hour, washed in TBST (10 times) and rabbit peroxidase conjugated antibody was added for a further hour incubation (1:2000 in 1% BSA *(w/v)* in TBST (Amersham). Following incubation, membranes were washed thoroughly washed in TBST followed by TBS. Protein was detected by ECL Plus Chemiluminescence (Amersham) and Hyperfilm ECL film (Amersham).

2.1.4 *Oxidative Stress Markers*

To determine whether folic acid supplementation could prevent nicotine-induced cellular stress, the expression of heat shock protein 70 (HSP70) was determined. HSP70 acts to protect cells from oxidative stress. Cellular stresses damage proteins which causes protein unfolding. HSP70 temporarily binds to partially denatured proteins allowing them to refold (Beere et al., 2000). We also determined whether or not folic acid could prevent oxidative damage. This was assessed by measuring protein expression of 4-hydroxy-2-nonenal (4-HNE). 4-HNE is produced

by lipid peroxidation in cells and is seen in high quantities in cells under conditions of oxidative stress (Mattson, 2009).

Protein (20µg) from each treatment group was subjected to SDS-PAGE (at 150V for 45 minutes, Pierce, Rockford, IL) then electrotransferred to a PVDF membrane (at 40V for 90 minutes; BioRad Laboratories). Membranes were blocked for either 1 hour with 5% (*w/v)* skim milk in TBST (Tris buffered saline, 0.5% Tween 20) at room temperature (HSP70) or in 5% (*w/v)* BSA in TBST overnight (4-HNE) on a rocking platform and then incubated with HSP70 primary antibody (1:1000, Cell Signaling Technology, Danvers, MA) or 4-HNE primary antibody (1:2000, R&D Systems). After washing with TBST, membranes were incubated for 1 hour with peroxidase conjugated anti-rabbit secondary antibody (HSP70) (1:10,000 Amersham Biosciences, NJ) or peroxidase conjugated anti-mouse secondary anybody (1:10,000 Amersham Biosciences, NJ) on a rocking platform. Membranes were thoroughly washed in TBST followed by TBS washes. Protein was detected using ECL Plus chemiluminescence (Amersham Biosciences, NJ) and Hyperfilm ECL film (Amersham Biosciences, NJ). Densitometry analysis was performed using Image J 1.45v software. All proteins were quantified relative to beta actin loading control. Briefly, membranes were incubated in stripping buffer (Thermo Scientific, Rockford, IL) for half an hour on a rocking platform. Membranes were quickly washed, and blocked with 1% (*w/v)* BSA (EMD Chemicals Inc., Gibbstown, NJ) in TBST. Following an hour incubation, blocking solution was removed, and β-actin primary antibody was added (1:4000 in 1% BSA (*w/v*) in TBST (Abcam, Cambridge, MA). Membranes were incubated for another hour, washed in TBST (10 times) and rabbit peroxidase conjugated antibody was added for a further hour incubation (1:2000 in 1% BSA *(w/v)* in TBST (Amersham). Following incubation,

membranes were washed thoroughly washed in TBST followed by TBS. Protein was detected by ECL Plus Chemiluminescence (Amersham) and Hyperfilm ECL film (Amersham).

2.2 Effect of Nicotine and Folic Acid on Endogenous Antioxidant Enzymes

2.2.1 *Cell Culture Maintenance*

All studies were conducted using INS 1E cells, a rat insulinoma beta cell line generously donated by Dr. Claes Wollheim (Geneva, Switzerland). Cells were maintained as described above.

2.2.2 *Western Blotting*

INS1E cells were treated for 48 hours with media alone (control), media containing 10 µM folic acid (Sigma Aldrich) or media containing 1µM nicotine alone (Sigma Aldrich) or 1µM nicotine with 10µM folic acid . This dose of folic acid was chosen to be 4x the amount of folic acid in the media (2.2µM) which is similar to the predicted rise in serum folate levels in women who increase their folate intake. After treatment, plates were washed twice with DPBS (Hyclone), then scraped, centrifuged and resuspended in radioimmunoprecipitation assay (RIPA) buffer (15mM Tris-HCl, 1% (*v*/v) Triton X-100, 0.1% (*w*/v) SDS, 167mM NaCl, 0.5% (*w*/v) sodium deoxycholic acid) containing protease inhibitors (Roche). Lysed cells were sonicated at 7Hz for 15 seconds.

2.2.3 *Expression of Endogenous Antioxidant Enzymes*

To determine if nicotine treatment up-regulated endogenous antioxidants, western blots were performed to determine expression level of MnSOD, Cu/ZnSOD and Catalase. Protein (20µg for MnSOD and Cu/ZnSOD and 40µg for Catalase) were subjected to SDS-PAGE (at 150V for 45 minutes, Pierce, Rockford IL) then electrotransferred to a PVDF membrane (at 40V for 90 minutes, BioRad Laboratories). Membranes were blocked overnight in 5% (*w/v)* skim milk in TBST (Tris buffered saline, 0.5% Tween 20) (MnSOD) or 5% BSA in TBST (Cu/ZnSOD and Catalase) at 4°C under gentle agitation. Primary antibodies (1:5000 and 1:250 for MnSOD and Cu/ZnSOD respectively, Santa Cruz Biotechnologies and 1:500 for Catalase, Abcam) were incubated for 1 hour at room temperature, followed by several washes in TBST. Membranes were then incubated for 1 hour peroxidase conjugated anti-rabbit antibody (1: 20,000, 1:10,000 and 1:5000 for MnSOD, Cu/ZnSOD and Catalase respectively, Amersham Biosciences NJ) on a rocking platform. Membranes were thoroughly washed in TBST followed by TBS. Protein was detected using ECL Plus chemiluminescence (Amersham, NJ) and Hyperfilm ECL film (Amersham, NJ). Densitometry analysis was performed using Image J 1.45v software. All proteins were quantified relative to beta actin or alpha tubulin loading control. Briefly, membranes were incubated in stripping buffer (Thermo Scientific, Rockford, IL) for half an hour on a rocking platform. Membranes were quickly washed, and blocked with 1% (*w/v)* BSA (EMD Chemicals Inc., Gibbstown, NJ) in TBST. Following an hour incubation, blocking solution was removed, and β-actin primary antibody was added (1:4000 in 1% BSA (*w/v*) in TBST (Abcam, Cambridge, MA). Membranes were incubated for another hour, washed in TBST (10 times) and rabbit peroxidase conjugated antibody was added for a further hour incubation (1:2000 in 1% BSA *(w/v)* in TBST (Amersham). Following incubation, membranes were washed thoroughly

washed in TBST followed by TBS. Protein was detected by ECL Plus Chemiluminescence (Amersham) and Hyperfilm ECL film (Amersham).

2.3 Effect of nicotine and folic acid treatment of beta cell function

2.3.1 *Cell Culture Maintenance*

All studies were conducted using INS 1E cells, a rat insulinoma beta cell line generously donated by Dr. Claes Wollheim (Geneva, Switzerland). Cells were maintained as described above.

2.3.2 *Glucose Stimulated Insulin Secretion*

Cells were seeded to a density of 20,000 cells /well in a 96 well plate (BD Biosciences). After 24 hours to allow cells to adhere; cells were treated for 48 hours with vehicle (control) ± 10µm folic acid or 1µM nicotine ± 10µM folic acid in normal RPMI media. Following treatment, media was removed, plates were washed and glucose free media (Invitrogen) was added. Following a 2 hour incubation period, media was removed and KRBH (Krebs Ringer Buffer with HEPES, 135mM NaCl, 3.6mM KCl, 5mM NaHCO3, 0.5mM CaCl2, 10mM HEPES, 0.1% bovine serum albumin (BSA) at a pH of 7.4) with low (3.3µM) or high (16.7µM) glucose was added and allowed to incubate for 2 hours. This experiment was done in triplicate and assayed in duplicate

2.3.3 *Rat Insulin ELISA*

The amount of insulin secreted in response to a glucose stimulus was assessed in spent media samples from treated INS 1E cells using a commercially available Ultra-Sensitive Rat Insulin ELISA kit (Chrystal Chem, Downers Grove, IL). Standards and samples were prepared according to manufacturer's instructions. Briefly, standards, samples and sample diluent were added to a 96-well plate pre-coated with guinea pig anti-insulin antibody and incubated for 2 hours at 4°C. The bound insulin in the standards and samples are then bound with a horse-radish peroxidase anti-insulin antibody followed by a detection with 3,3',5,5'-tetramethylbenzidine (TMB) solution. Absorbance was determined at 450nm (primary wavelength) and 630nm (background wavelength). The amounts of insulin in the samples were determined by comparing sample values to the standard curve.

2.3.4 *Basal Insulin Secretion*

INS 1E cells were seeded to a density of 250,000 cells per well in 12-well plates (BD Biosciences). After allowing 24 hours to equilibrate, cells were treated with vehicle (control) ± 10 µM folic acid or nicotine ± 10µM folic acid in duplicate for 48 hours. After treatment, media was removed, and cells were washed with 2.8mM glucose in KRBH (Krebs Ringer Bicarbonate buffer with HEPES, 135mM NaCl, 3.6mM KCl, 5mM NaHCO3, 0.5mM CaCl2, 10mM HEPES, 0.1% bovine serum albumin (BSA) at a pH of 7.4,) then incubated for 30 minutes with 2.8mM glucose in KRBH. 500 µL of media was removed and stored at -20°C for assay by RIA.

2.3.5 *Insulin Content*

INS 1E cells were seeded to a density of 250,000 cells per well in 12-well plates (BD Biosciences). After allowing 24 hours to equilibrate, cells were treated as control ± 10µM folic acid or nicotine ± 10µM folic acid in duplicate for 48 hours. After treatment, media was removed, and cells were washed with 2.8mM glucose in KRBH (Krebs Ringer Bicarbonate buffer with HEPES, 135mM NaCl, 3.6mM KCl, 5mM NaHCO3, 0.5mM CaCl2, 10mM HEPES, 0.1% bovine serum albumin (BSA) at a pH of 7.4,) then incubated for 30 minutes with 2.8mM glucose in KRBH. Media was removed, and 1ml of acid ethanol (0.18M HCl in 70% Ethanol) was added to each well to lyse cells and release intracellular insulin. Following an overnight incubation at 4°C, media was removed and stored at -20°C for analysis by RIA.

2.3.6 *Radioimmunoassay (RIA)*

Insulin secretion was measured from spent media samples using a Sensitive Rat Insulin RIA Kit (Millipore, Billerica, MA) according to manufacturer's instructions. Briefly, insulin samples collected from spent media were diluted 1:20 in assay buffer in 12x75mm glass test tubes in order to ensure that insulin concentrations fell within the linear portion of the standard curve. The following were added to all test tubes (with the exception of total counts tubes and non-specific binding tubes) for a final reaction volume of 300µl: 100µl spent media sample in duplicate (diluted 1:20) or 100µl standard insulin (known concentration) in duplicate; 100µl radiolabelled insulin; and 100µl insulin antibody. The total counts tubes only contained 100µl in duplicate of radiolabelled insulin; the non-specific binding tubes contained 200µl in duplicate of assay buffer. All tubes were incubated at 4°C for 20-24 hours. Following incubation, 1.0mL of

precipitating reagent was added to all tubes (except total counts tubes); tubes were incubated at 4°C for an additional 20 minutes. The test tubes were then centrifuged for 40 minutes at 2,500 rpm in 4°C (except total counts tubes). Following centrifugation, the supernatant was immediately decanted and all tubes were added to a gamma counter. All samples were read using the Wizard 1470 Automatic Gamma Counter (Perkin Elmer, Montreal, QC, CA). Samples were counted for 1 minute. The concentration (ng/ml) of rat insulin was automatically calculated by RIACalc (Perkin Elmer, Montreal, QC) from the standard curve.

2.3.7 *Western Blotting*

INS1E cells were treated for 48 hours with media alone (control), media containing 10µM folic acid (Sigma Aldrich) or media containing 1µM nicotine alone (Sigma Aldrich) or 1µM nicotine with 10µM folic acid . This dose of folic acid was chosen to be 4x the amount of folic acid in the media (2.2µM) which is similar to the predicted rise in serum folate levels in women who increase their folate intake. After treatment, plates were washed twice with DPBS (Hyclone), then scraped, centrifuged and resuspended in radioimmunoprecipitation assay (RIPA) buffer (15mM Tris-HCl, 1% (*v*/v) Triton X-100, 0.1% (*w*/v) SDS, 167mM NaCl, 0.5% (*w*/v) sodium deoxycholic acid) containing protease inhibitors (Roche). Lysed cells were sonicated at 7Hz for 15 seconds.

2.3.8 *Insulin Regulation*

PDX-1 (pancreatic and duodenal homeobox 1) is a transcription factor that is necessary for the development of the pancreas and islets but also for the regulation of insulin gene expression (Fujimoto and Polonsky, 2009). Since nicotine treatment in vivo impaired insulin

secretion, it is conceivable that the nicotine treatment is leading to a down regulation of PDX-1 which could in part, account for the decrease in insulin. In addition, the transcription factor Foxa2 is also responsible for insulin expression in mature beta cells and is a regulator of PDX-1 (Lee et al., 2002). For this reason, expression of Foxa2 will also be assessed.

Proteins (30µg for PDX-1 and 40µg for Foxa2) from control and nicotine treatment groups were subjected to SDS-PAGE (at 150V for 45 minutes, Pierce, Rockford IL) then electrotransferred to a PVDF membrane (at 40V for 90 minutes, BioRad Laboratories). Membranes were blocked for 3 hours at room temperature in 5% (w/v) skim milk in TBST (Tris buffered saline, 0.5% Tween 20) for PDX-1 and 3x 20 minutes in 10% skim milk at room temperature for foxa2 under gentle agitation. PDX-1 primary antibody (1:500, Santa Cruz Biotechnologies) and Foxa2 primary antibody (1:5000, Abcam) were incubated overnight at 4°C under gentle agitation, followed by several washes in TBST. Membranes were then incubated for 1 hour peroxidase conjugated anti-rabbit antibody (1:70,000 for PDX-1 and 1:4000 for Foxa2, Amersham Biosciences NJ) on a rocking platform. Membranes were thoroughly washed in TBST followed by TBS. Protein was detected using ECL Plus chemiluminescence (Amersham, NJ) and Hyperfilm ECL film (Amersham, NJ). Densitometry analysis was performed using Image J 1.45v software. All proteins were quantified relative to alpha tubulin loading control. Briefly, membranes were incubated in stripping buffer (Thermo Scientific, Rockford, IL) for half an hour on a rocking platform. Membranes were quickly washed, and blocked with 1% (*w/v)* BSA (EMD Chemicals Inc., Gibbstown, NJ) in TBST. Following an hour incubation, blocking solution was removed, and α-tubulin primary antibody was added (1:10,000 in 1% BSA (*w/v*) in TBST (Abcam, Cambridge, MA). Membranes were incubated for another hour, washed in TBST (10 times) and rabbit peroxidase conjugated antibody was added for a further hour incubation

(1:4000 in 1% BSA *(w/v)* in TBST (Amersham). Following incubation, membranes were washed thoroughly washed in TBST followed by TBS. Protein was detected by ECL Plus Chemiluminescence (Amersham) and Hyperfilm ECL film (Amersham).

2.3.9 *MTS cell viability*

Cell viability was done to determine whether nicotine treatment lead to a decrease in cell survival which could indicate an increase in beta cell apoptosis.

INS 1E cells were seeded to a density of 10,000 cells per well in 96-well plates and allowed to equilibrate for 24 hours. The cells were treated as control or 1µM, 10µM, 50µM or 100µM nicotine dissolved in regular cell media. An MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay was performed according to manufacturers' instructions at 48 hours post treatment.

2.3.10 *Apoptosis*

To determine if the decreased glucose-stimulated insulin secretion was due to due a decrease in beta cell number, the expression level of Caspase 3 was assessed. Caspase 3 is activated by all three apoptotic pathways (Intrinsic and extrinsic).

Protein (20µg) from each treatment group was subjected to SDS-PAGE (at 150V for 45 minutes, Pierce, Rockford, IL) then electrotransferred to a PVDF membrane (at 40V for 90 minutes; BioRad Laboratories). Membranes were blocked overnight with 5% (w/v) BSA in TBST (Tris buffered saline, 0.5% Tween 20) on a rocking platform and then incubated with

Caspase 3 primary antibody (1:250, Santa Cruz). After washing with TBST, membranes were incubated for 1 hour with peroxidase conjugated anti-rabbit secondary antibody (1:10,000 Amersham Biosciences, NJ) on a rocking platform. Membranes were thoroughly washed in TBST followed by TBS washes. Protein was detected using ECL Plus chemiluminescence (Amersham Biosciences, NJ) and Hyperfilm ECL film (Amersham Biosciences, NJ). Densitometry analysis was performed using Image J 1.45v software. All proteins were quantified relative to alpha tubulin loading control. Briefly, membranes were incubated in stripping buffer (Thermo Scientific, Rockford, IL) for half an hour on a rocking platform. Membranes were quickly washed, and blocked with 1% (*w/v)* BSA (EMD Chemicals Inc., Gibbstown, NJ) in TBST. Following an hour incubation, blocking solution was removed, and α-tubulin primary antibody was added (1:10,000 in 1% BSA (*w/v*) in TBST (Abcam, Cambridge, MA). Membranes were incubated for another hour, washed in TBST (10 times) and rabbit peroxidase conjugated antibody was added for a further 1 hour incubation (1:4000 in 1% BSA *(w/v)* in TBST (Amersham). Following incubation, membranes were washed thoroughly washed in TBST followed by TBS. Protein was detected by ECL Plus Chemiluminescence (Amersham) and Hyperfilm ECL film (Amersham).

2.3.11 *Glucose Uptake*

To determine if the decrease in insulin secretion is due to decreased glucose uptake in to the beta cell, expression of the glucose transport GLUT2 was measured.

Protein (20µg) from each treatment group was subjected to SDS-PAGE (at 150V for 45 minutes, Pierce, Rockford, IL) then electrotransferred to a PVDF membrane (at 40V for 90

minutes; BioRad Laboratories). Membranes were blocked overnight with 5% (w/v) skim milk in TBST (Tris buffered saline, 0.5% Tween 20) for Kir6.2 or 5% (w/v) BSA in TBST for GLUT2 on a rocking platform and then incubated with primary antibody concentrations of 1:1000 (GLUT2, Millipore), 1:200 (Kir6.2, Almone Labs). After washing with TBST, membranes were incubated for 1 hour with peroxidase conjugated anti-rabbit secondary antibody (1:10,000 and 1:5000 for GLUT2 and Kir6.2 respectively, Amersham Biosciences, NJ) on a rocking platform. Membranes were thoroughly washed in TBST followed by TBS washes. Protein was detected using ECL Plus chemiluminescence (Amersham Biosciences, NJ) and Hyperfilm ECL film (Amersham Biosciences, NJ). Densitometry analysis was performed using Image J 1.45v software. All proteins were quantified relative to alpha tubulin loading control. Briefly, membranes were incubated in stripping buffer (Thermo Scientific, Rockford, IL) for half an hour on a rocking platform. Membranes were quickly washed, and blocked with 1% (*w/v)* BSA (EMD Chemicals Inc., Gibbstown, NJ) in TBST. Following an hour incubation, blocking solution was removed, and α-tubulin primary antibody was added (1:10,000 in 1% BSA (*w/v*) in TBST (Abcam, Cambridge, MA). Membranes were incubated for another hour, washed in TBST (10 times) and rabbit peroxidase conjugated antibody was added for a further hour incubation (1:4000 in 1% BSA *(w/v)* in TBST (Amersham). Following incubation, membranes were washed thoroughly washed in TBST followed by TBS. Protein was detected by ECL Plus Chemiluminescence (Amersham) and Hyperfilm ECL film (Amersham).

2.3.12 *Glucose Consumption*

To determine if the reduction of glucose-stimulated insulin secretion was due to a reduction in glucose uptake into INS 1E cells, glucose consumption was assessed.

INS 1E cells were seeded to a density of 100,000 cells per well in a 24-well cell culture plate (BD Biosciences). Following 24 hours to allow cells to equilibrate, cells were treated for 48 hours as no cell control (contains the maximal amount of glucose in the media), control ± 10µM folic acid or 1µM nicotine ± 10µM folic acid in normal cell culture media (RPMI-1640). Following treatment, media was removed and cells were washed with DPBS followed by a 1 hour incubation with normal (11.1mM) and high (16.7mM) glucose in KRBH. This media was removed and stored at -20°C until assay.

2.3.13 *Glucose Oxidase Assay*

Glucose consumption was measured by glucose oxidase assay (Point Scientific, Canton MI) according to manufactures instructions, briefly; 2.5µl of sample media was added to 250µl glucose oxidase reagent in a 96-well microplate and incubated at 37°C for 10 minutes. Following incubation, absorbance was measured at 492nm. Glucose consumption was determined by comparing the amount of glucose in the no cell control media to the treated cell media and normalizing it to the amount of protein in that well.

2.3.14 *Mitochondrial Isolation*

To assess whether the decrease in insulin secretion was due to mitochondrial dysfunction, I measured mitochondrial electron transport chain activity. To generate mitochondrial fractions, INS 1E cells were treated for 48 hours with media containing vehicle (control) 10µmol/l folic acid or nicotine ± 10µmol/l folic acid, in 4 independent experiments. Five plates per treatment group were used to ensure adequate protein concentrations for analysis. After treatment, cells were washed with Ca^{2+} and Mg^{2+} free DPBS, scraped and collected in DPBS. Cells were pelleted by centrifugation (2,000 rpm for 5 minutes) and the pellet resuspended in HEPES buffer (100mM KCl, 70mM Sucrose, 220 mM mannitol, 1mM EGTA, 5mM HEPES, pH 7.4) supplemented with BSA and EDTA-free protease inhibitors (Roche, Laval, QC). Cells were manually homogenized in a glass Teflon dounce homogenizer and centrifuged at 3000 rpm for 10 minutes to spin out nuclei. The supernatants were then centrifuged at 12,000 rpm for 10 minutes. The resultant pellets were resuspended in HEPES buffer without BSA, spun twice more at 11,000 rpm for 10 minutes then snap frozen in liquid nitrogen and stored at -80°C until analysis.

2.3.15 *Mitochondrial Function*

Activity of the electron transport chain (ETC) was analyzed to determine mitochondrial function. All samples were analyzed in duplicate on a UV spectrophotometer (Varian Inc. Palo Alto, CA). Complex IV activity was determined by measuring the oxidation of cytochrome c by cytochrome c oxidase. Briefly, 15µl reduced cytochrome c (2µM, from equine heart, Sigma Aldrich) was added to 1ml of potassium phosphate (K2HPO4) buffer, pH 7.4, and mixed then

10µl of mitochondrial homogenate was added and allowed to equilibrate for 30 seconds and then measured. Reduced cytochrome c has a strong absorbance at 550nm. Once oxidized to ferricytochrome c by cytochrome c oxidase in the sample there is a decreased absorbance resulting in a negative slope. To determine the amount of mitochondria in the sample, mitochondrial sample were also analyzed for citrate synthase (a marker of mitochondrial mass). Briefly, 20µl of mitochondrial homogenate was added to 850µl Tris buffer (0.1M, pH 8.0), 100µl DNTB (1mg/2.5ml in Tris buffer), and 10µl acetyl CoA in a cuvette and mixed. After an equilibration period 30µlof oxaloacetate was added to start the reaction. Citrate synthase in the sample catalyzes the conversion of acetyl CoA by oxaloacetate to form citrate and regenerate coenzyme A. An increase in absorbance is measured at 412 nm with a positive slope obtained.

2.3.16 *Expression of K_{ATP} channels*

Protein (20µg) from each treatment group was subjected to SDS-PAGE (at 150V for 45 minutes, Pierce, Rockford, IL) then electrotransferred to a PVDF membrane (at 40V for 90 minutes; BioRad Laboratories). The membrane was blocked overnight with 5% (w/v) skim milk in TBST (Tris buffered saline, 0.5% Tween 20) on a rocking platform and then incubated with primary antibody (1:200 in 5% skim milk (*w/v)* in TBST (Almone Labs, Jerusalem, Isreal) for 2 hours. After washing with TBST, membranes were incubated for 1 hour with peroxidase conjugated anti-rabbit secondary antibody (1:5000, Amersham Biosciences, NJ) on a rocking platform. The membrane was thoroughly washed in TBST followed by TBS washes. Protein was detected using ECL Plus chemiluminescence (Amersham Biosciences, NJ) and Hyperfilm ECL film (Amersham Biosciences, NJ). Densitometry analysis was performed using Image J 1.45v

software. All proteins were quantified relative to alpha tubulin loading control. Briefly, membranes were incubated in stripping buffer (Thermo Scientific, Rockford, IL) for half an hour on a rocking platform. Membranes were quickly washed, and blocked with 1% (*w/v)* BSA (EMD Chemicals Inc., Gibbstown, NJ) in TBST. Following an hour incubation, blocking solution was removed, and α-tubulin primary antibody was added (1:10,000 in 1% BSA (*w/v*) in TBST (Abcam, Cambridge, MA). Membranes were incubated for another hour, washed in TBST (10 times) and rabbit peroxidase conjugated antibody was added for a further hour incubation (1:4000 in 1% BSA *(w/v)* in TBST (Amersham). Following incubation, membranes were washed thoroughly washed in TBST followed by TBS. Protein was detected by ECL Plus Chemiluminescence (Amersham) and Hyperfilm ECL film (Amersham).

2.3.17 *Statistical Analysis*

All statistical analyses were performed using GraphPad Prism 5.0. With the exception of expression of FR and RFC (students *t* test), analysis was performed using one way ANOVA. When significance was indicated by ANOVA, Tukey *post hoc* comparison tests were applied. All data were tested for normality as well as equal variance. Outliers were calculated using GraphPad online outlier calculator (http://www.graphpad.com/quickcalcs/grubbsl.cfm). Values that were calculated to be outliers were excluded from analysis. Values are presented as mean ± SEM. A p value of less than 0.05 was considered significant.

3.0 Results

3.1 Effect of Nicotine and folic acid treatment on markers of oxidative stress

3.1.1 *Folate Transporters*

Folate receptor and reduced folate carrier are present in the INS 1E cell line (figures 1.2 and 1.3); nicotine treatment did not significantly alter expression of either protein (p= 0.83 and p= 0.39, respectively, n=3).

3.1.2 *Oxidative stress*

Cells treated with nicotine had significantly higher expression of heat shock protein 70 (HSP70) relative to control cells (p=0.0021, n=3, figure 1.4). In INS 1E cells treated with 1µM nicotine and 10µM folic acid, there was a significant decrease in HSP70 compared to nicotine treated cells (p=0.0047, n=3, figure 1.5)

4-HNE was significantly up-regulated (p= 0.05, n=2) in nicotine treated INS 1E cells (figure 1.6). In INS 1E cells treated with nicotine and 10µM folic acid, there was a significant decrease in 4-HNE expression (p= 0.01, n=2); in addition, folic acid treatment restored 4-HNE levels back to control levels. The addition of 10µM folic acid to control INS IE cells had no effect on 4-HNE levels.

3.2 Effect of Nicotine and folic acid treatment on endogenous antioxidants

3.2.1 *MnSOD*

Expression of MnSOD was significantly increased with treatment of 1µM nicotine (p=0.028, n=3) compared to control (figure 2.1). The addition of 10µM folic acid to the control media did

not alter expression versus control. The addition of 10µM folic acid did not reduce expression levels of MnSOD compared with control, and remained at the same level as the nicotine treatment alone.

3.2.2 *Cu/ZnSOD*

Expression of Cu/ZnSOD did not change with nicotine treatment compared with control (p=0.40, n=3). There was a significant (p=0.047) decrease in expression in the nicotine + 10µm folic acid group versus nicotine treatment only (figure 2.2).

3.2.3 *Catalase*

Expression of Catalase was unchanged with treatment of nicotine compared with control; likewise, the control + 10µm folic acid treatment group remained unchanged. There was a significant decrease with the addition of 10µm folic acid in the nicotine treated group compared to nicotine treatment alone (p=0.0023, n=3), figure 2.3.

3.3 Effect of nicotine and folic acid treatment on beta cell function

3.3.1 *Glucose Stimulated Insulin Secretion*

There was a significant increase in insulin secretion following high glucose stimulation in control INS 1E cells (p=0.0056, n=3, figure 3.1). In cells treated with folic acid, nicotine or nicotine plus folic acid (i.e., control + 10µm folic acid, nicotine, and nicotine + 10µm folic acid), the insulin secretion in response to high glucose was not significantly different than that seen under low (i.e. 3.3mM) glucose conditions.

3.3.2 *Basal Insulin secretion*

There was a significant decrease in basal insulin secretion following nicotine treatment compared with control (p=0.023, n=5, figure 3.2).

3.3.3 *Insulin Content*

There was a significant increase in insulin content in the 1µm nicotine + 10µm folic acid compared to control and control + 10µm folic acid (p=0.027 and p=0.008 respectively, n=5). There was no change in insulin content in the nicotine group compared with any other treatment group, Figure 3.3.

3.3.4 *Insulin Regulation*

There was no change in expression of PDX-1 between control, control + 10µm folic acid and nicotine treatment groups. There was a significant increase of expression when comparing PDX1 expression in cells treated with 1µm nicotine + 10µm folic acid (p<0.0001, n=3, figure 3.4) relative to all other groups.

There was no significant effect of any treatment on fox a2 (p=0.75, n=3, figure 3.5).

3.3.5 *MTS Cell Viability*

Nicotine, at concentrations up to 100µM had no effect on INS 1E cell viability (p= 0.77, figure 3.6).

3.3.6 *Apoptosis*

Expression of Caspase 3 between the 4 treatment groups was not significantly different (p=0.43). See figure 3.8.

3.3.7 *Glucose Uptake*

Expression of GLUT2 between the 4 treatment groups was not significantly different (p=0.34).

3.3.8 *Glucose Consumption*

There was no change in the amount of glucose consumed at normal (11.1mM) or high (16.7mM) glucose concentrations (p= 0.23 and 0.57 respectively , n=5), figure 3.9 A, B and C

3.3.9 *Mitochondrial Function*

Nicotine treatment alone or in combination with folic acid did not significantly alter Complex IV activity (p=0.71, n=4). Moreover, there was no effect of any treatment on citrate synthase activity (p=0.85, n=4) or the ratio of complex IV to citrate synthase activity (p=0.84, n=4, figures 3.10A, B and C)

3.3.10 *Glucose Signaling*

Expression of $Kir_{6.2}$ was significantly increased in INS 1E cells treated with 1µm nicotine compared to control (p=0.019, n=3, figure 3.11). There was a trend toward decreasing expression with the addition of folic acid (p=0.067).

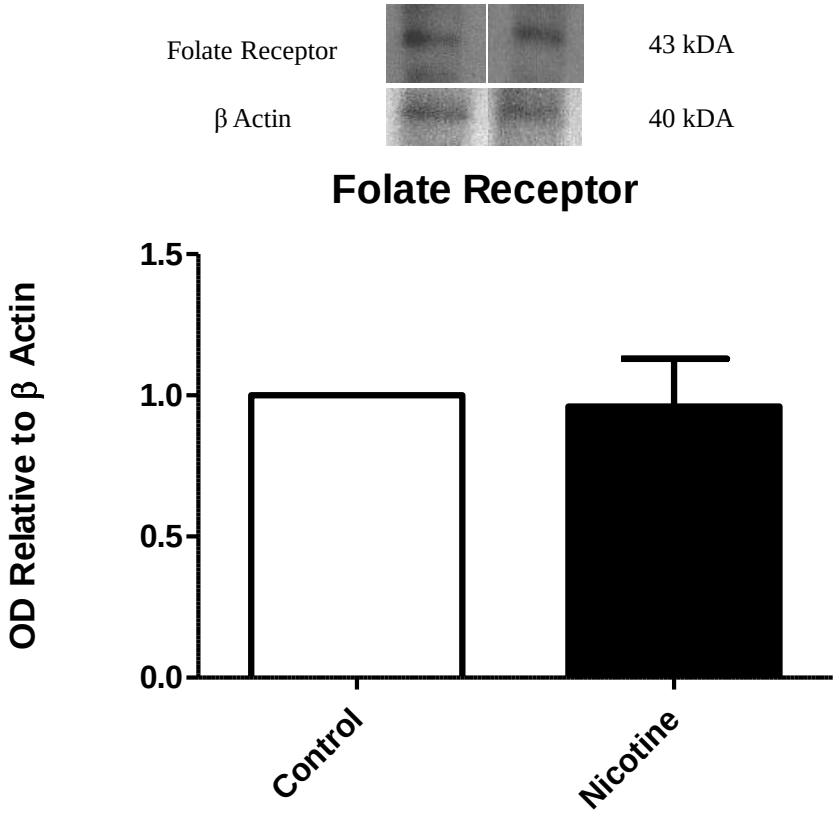

Figure 1.2 Effect of nicotine treatment on folate receptor expression

Folate receptor expression in INS 1E cells 48 hours post treatment with saline or 1µM nicotine. Data are expressed as optical density relative to beta actin loading control and represented as mean ± SEM, n=3. The above panels are a representative band for each treatment group from the western blot. Each graph is a quantification of all samples.

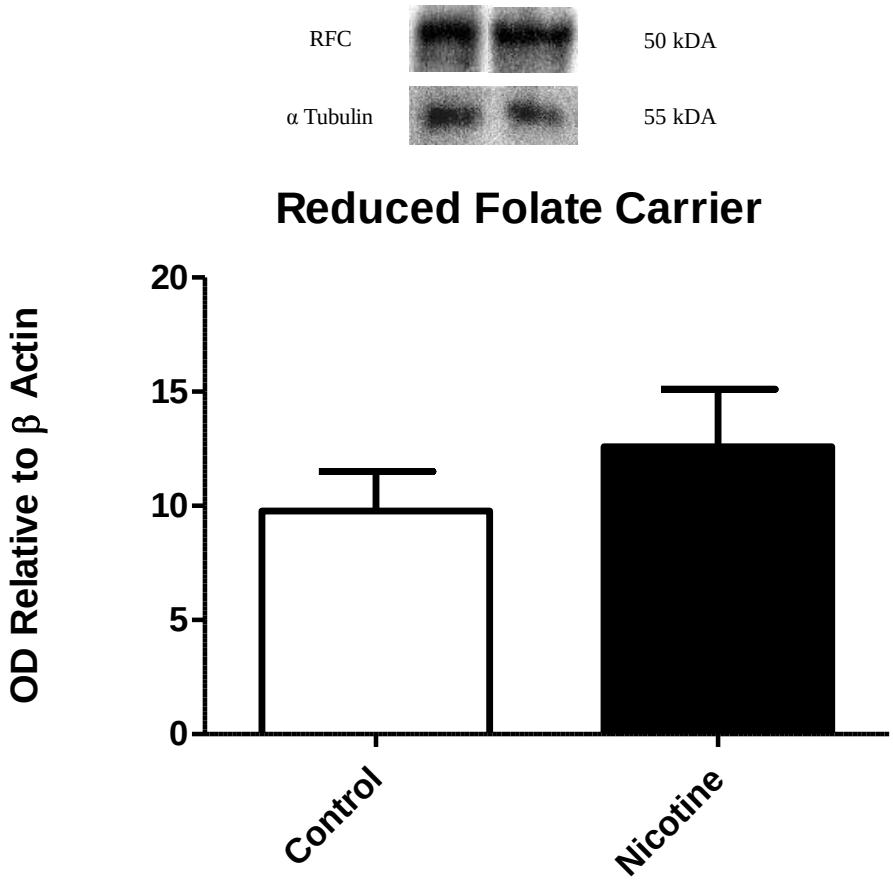

Figure 1.3 Effect of nicotine treatment on reduced folate carrier expression

Reduced folate carrier expression in INS 1E cells 48 hours post treatment with saline or 1µM nicotine. Data are expressed as optical density relative to beta actin loading control as represented as mean ± SEM, n=3. The above panels are a representative band for each treatment group from the western blot. Each graph is a quantification of all samples.

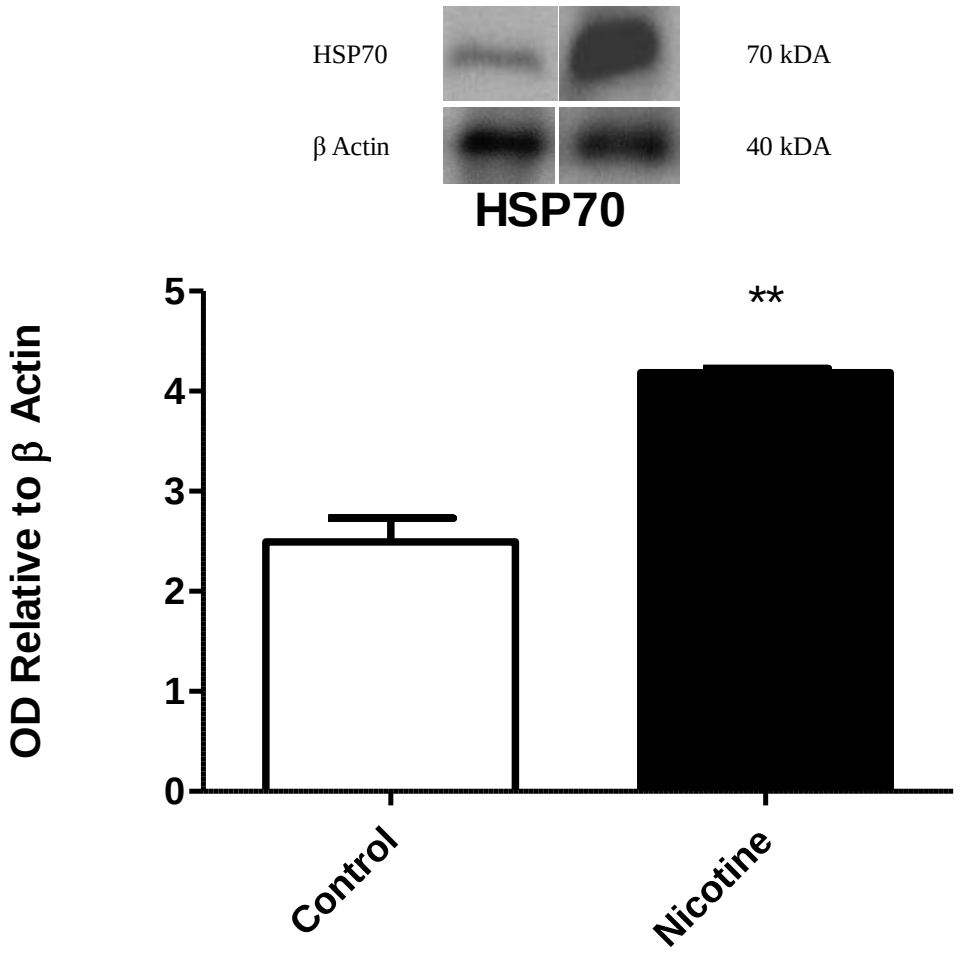

Figure 1.4 Effect of nicotine treatment on HSP70 Expression

HSP70 expression in INS 1E cells 48 hours post treatment with saline or 1µM nicotine. Data are expressed as optical density relative to beta actin loading control and represented as mean ± SEM, n=3. A double asterisk denotes a p value <0.01. The above panels are a representative band for each treatment group from the western blot. Each graph is a quantification of all samples.

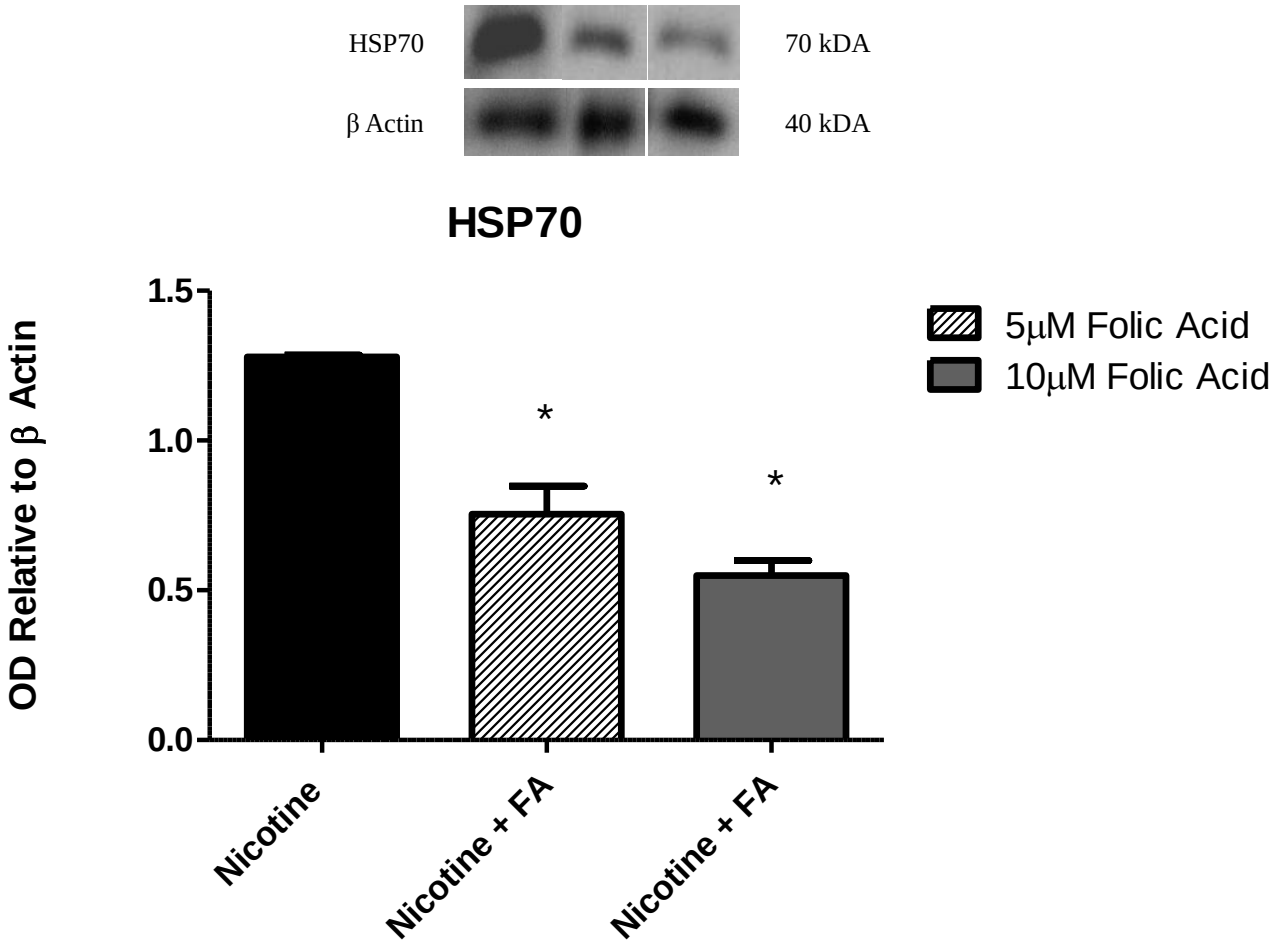

Figure 1.5 Effect of nicotine and folic acid treatment on HSP70 Expression

HSP70 expression in INS IE cells 48 hours post treatment with 1μM nicotine ± 5 or 10μM folic acid. Data are expressed as optical density relative to beta actin loading control and represented as mean ± SEM, n=3. An asterisk denotes a p value <0.05. The above panels are a representative band for each treatment group from the western blot. Each graph is a quantification of all samples.

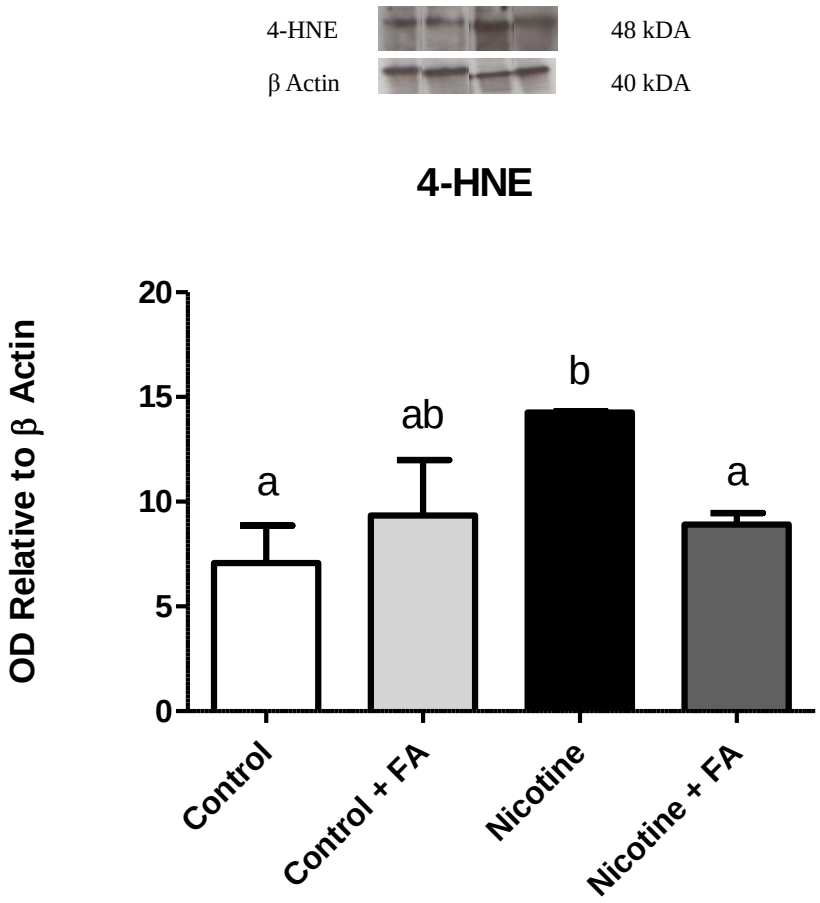

Figure 1.6 Effect of nicotine and folic acid on 4-HNE expression

4-HNE expression in INS 1E cells 48 hours post treatment with saline ± 10µM folic acid or 1µM nicotine ± 10µM folic acid. Data are expressed as optical density relative to beta actin loading control and represented as mean ± SEM, n=3. Bars with different superscripts are significantly ($p<0.05$) different. The above panels are a representative band for each treatment group from the western blot. Each graph is a quantification of all samples.

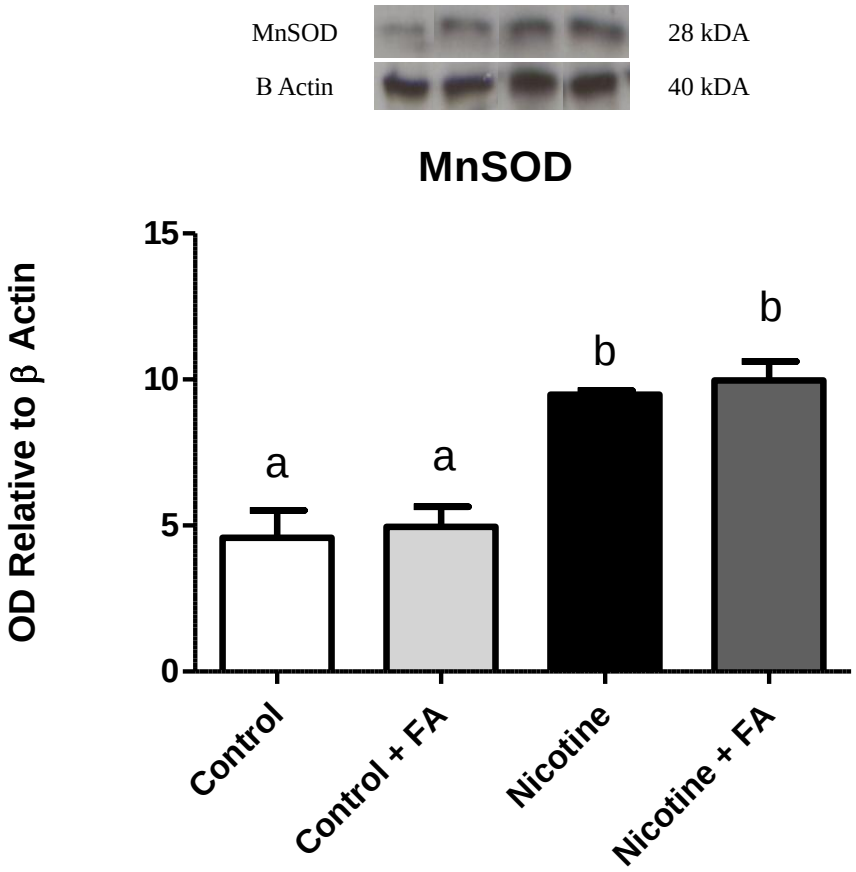

Figure 2.1 Effect of nicotine and folic acid on MnSOD expression

MnSOD expression in INS 1E cells 48 hours post treatment with saline ± 10µM folic acid or 1µM nicotine ± 10µM folic acid. Data are expressed as optical density relative to beta actin loading control and represented as mean ± SEM, n=3. Bars with different superscripts are significantly ($p<0.05$) different. The above panels are a representative band for each treatment group from the western blot. Each graph is a quantification of all samples.

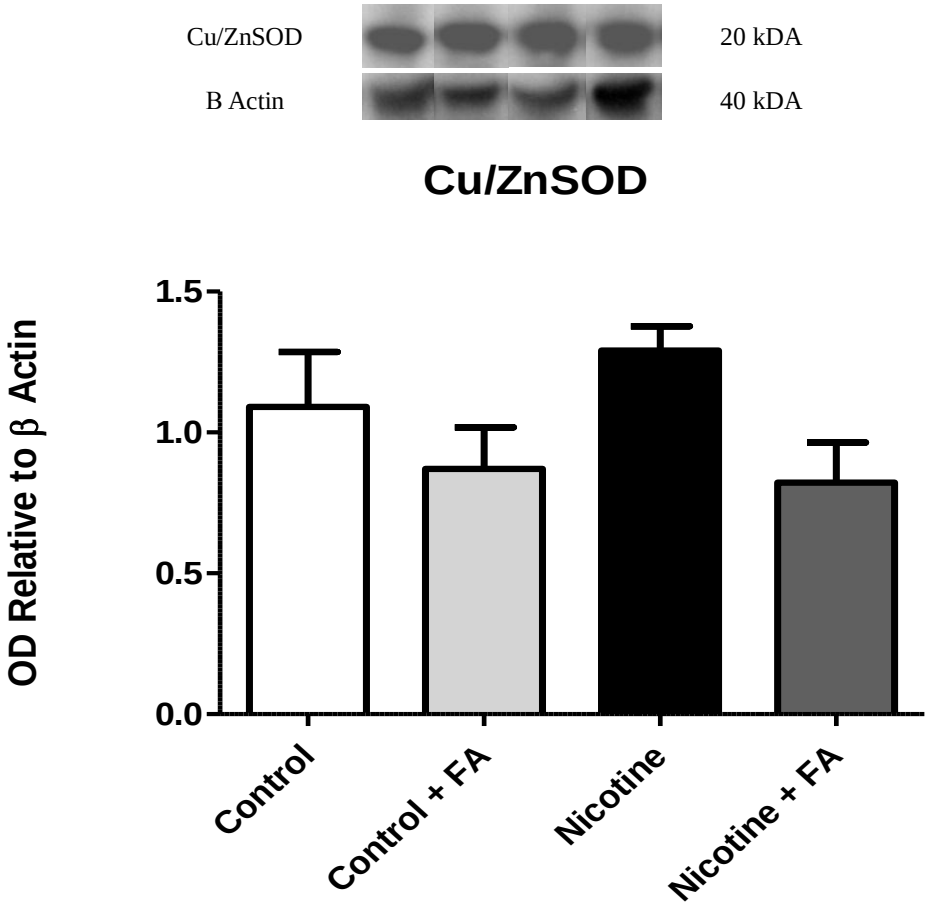

Figure 2.2 Effect of nicotine and folic acid on Cu/ZnSOD expression

Cu/ZnSOD expression in INS 1E cells 48 hours post treatment with saline ± 10µM folic acid or 1µM nicotine ± 10µM folic acid. Data are expressed as optical density relative to beta actin loading control and represented as mean ± SEM, n=3. Bars with different superscripts are significantly ($p<0.05$) different. The above panels are a representative band for each treatment group from the western blot. Each graph is a quantification of all samples.

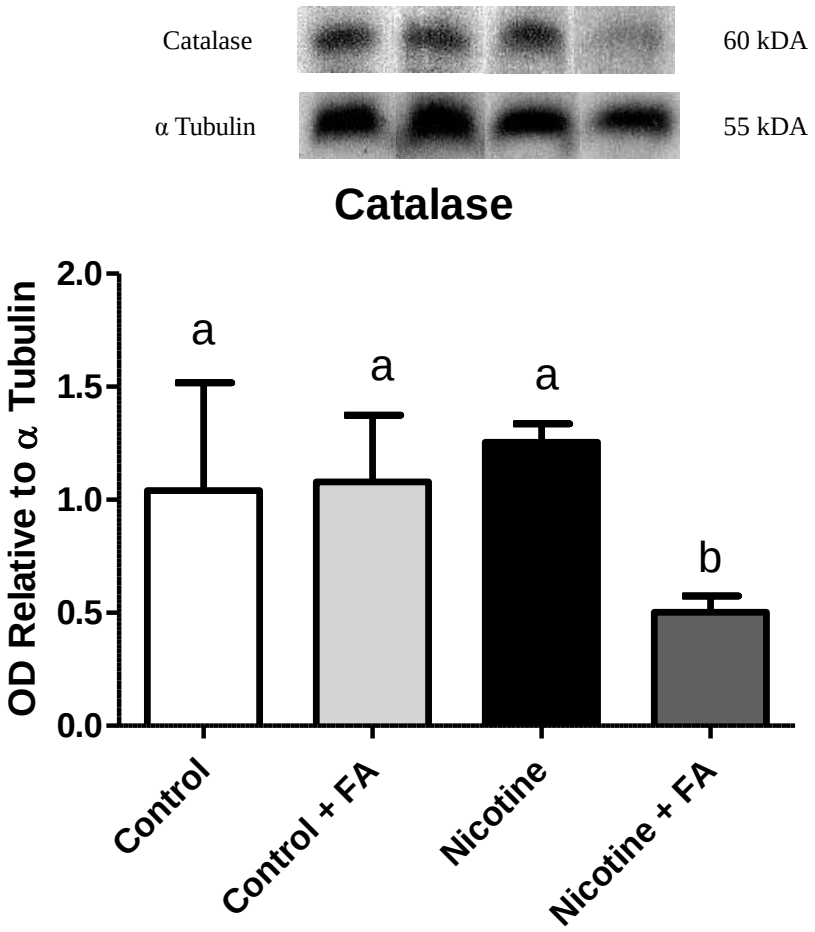

Figure 2.3 Effect of nicotine and folic acid on catalase expression

Catalase expression in INS 1E cells 48 hours post treatment with saline ± 10µM folic acid or 1µM nicotine ± 10µM folic acid. Data are expressed as optical density relative to alpha tubulin loading control and represent as mean ± SEM, n=3. Bars with different superscripts are significantly ($p<0.05$) different. The above panels are a representative band for each treatment group from the western blot. Each graph is a quantification of all samples.

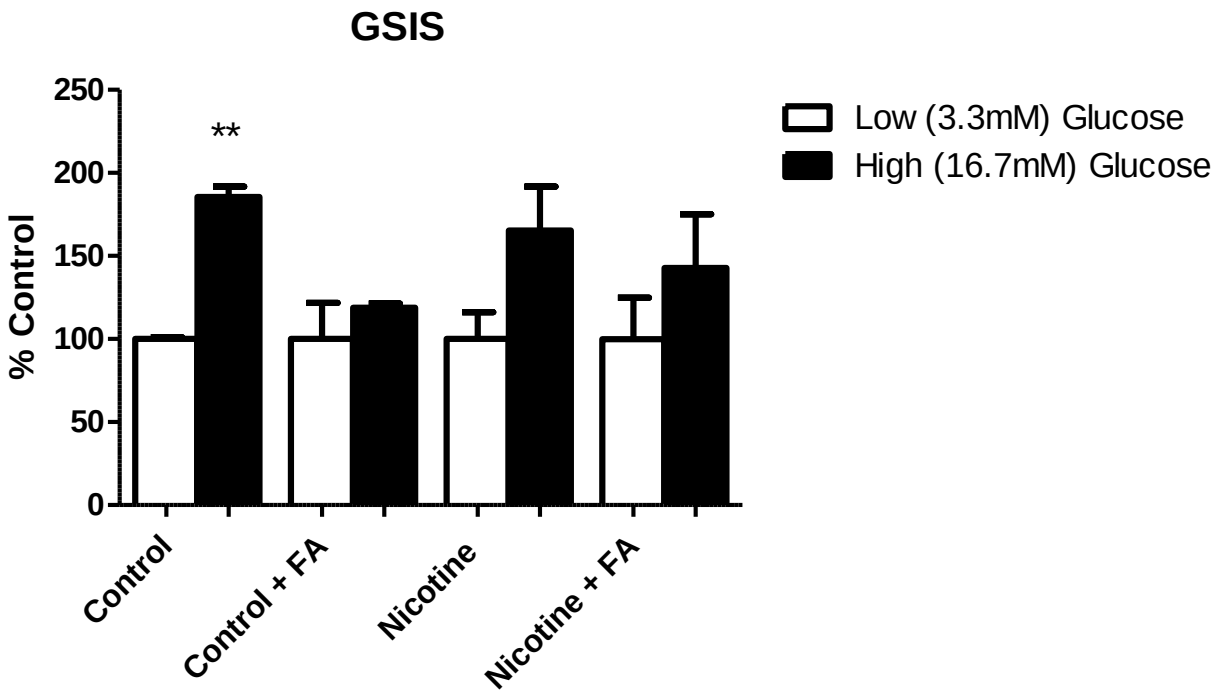

Figure 3.1 Effect of nicotine and folic acid on glucose stimulated insulin secretion

Glucose stimulated insulin secretion in INS 1E cells 48 hours post treatment with saline ± 10µM folic acid or 1µM nicotine ± 10µM folic acid. Data are expressed as a percentage of the low glucose control, n=3. An asterisk denotes a p value <0.05.

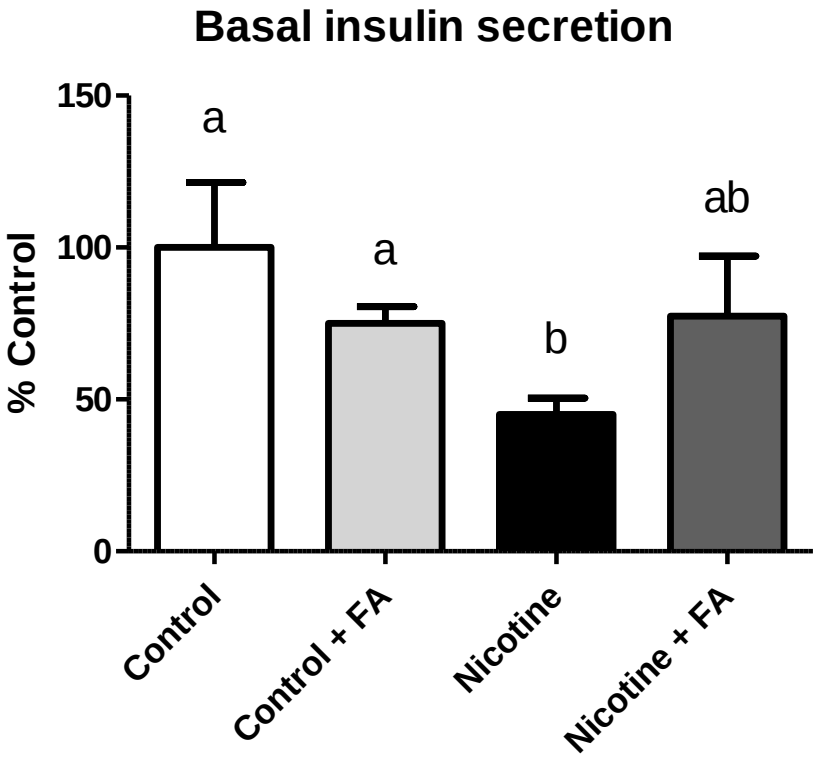

Figure 3.2 Effect of nicotine and folic acid on basal insulin secretion

Basal Insulin secretion inn INS 1E cells 48 hours post treatment with saline or 1µM nicotine ± 5 or 10µM nicotine. Data are expressed as a percentage of the control, n=5. Bars with different superscripts are significantly ($p<0.05$) different.

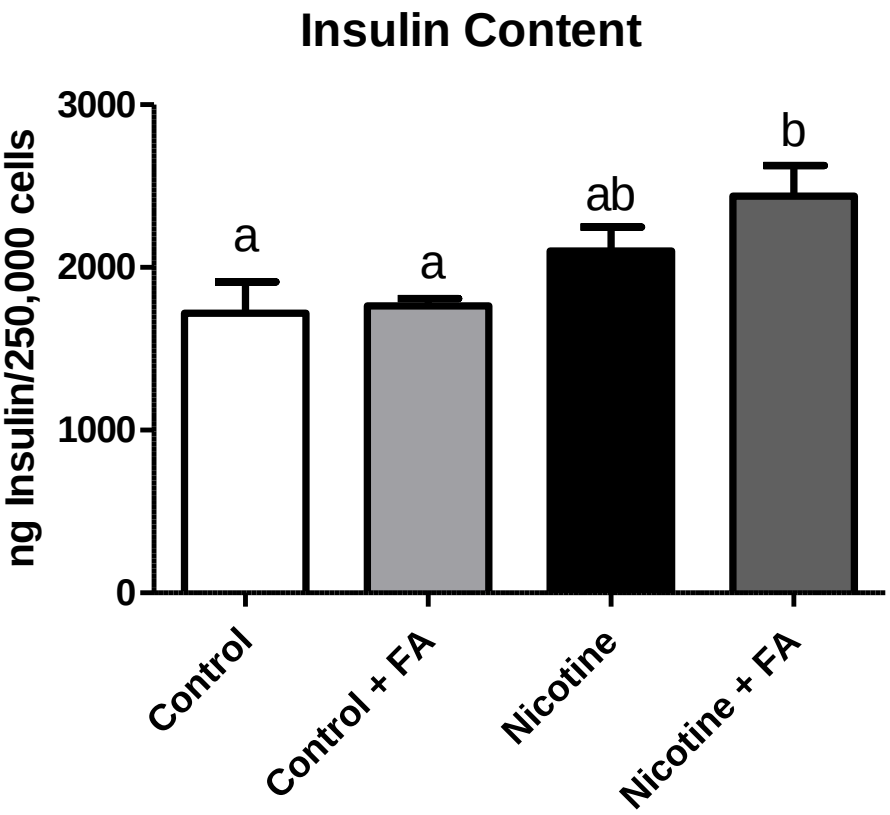

Figure 3.3 Effect of nicotine and folic acid on insulin content

Insulin content in INS 1E cells 48 hours post treatment with saline ± 10µM folic acid or 1µ nicotine ± 10µM folic acid. Data are expressed as ng insulin per 250,000 cells, n=5. Bars with different superscripts are significantly ($p<0.05$) different.

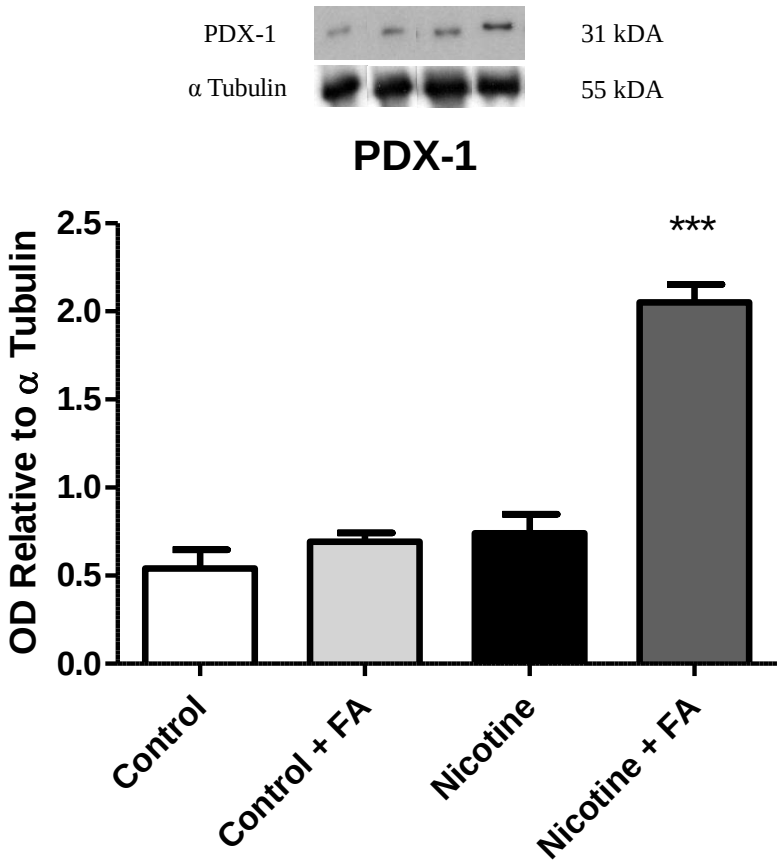

Figure 3.4 Effect of nicotine and folic acid on PDX-1 expression

PDX-1 expression in INS 1E cells 48 hours post treatment with saline ± 10µM folic acid or 1µM nicotine ± 10µM folic acid. Data are expressed as optical density relative to alpha tubulin loading control and represented as mean ± SEM, n=3. A triple asterisk denotes a p value <0.0001. The above panels are a representative band for each treatment group from the western blot. Each graph is a quantification of all samples.

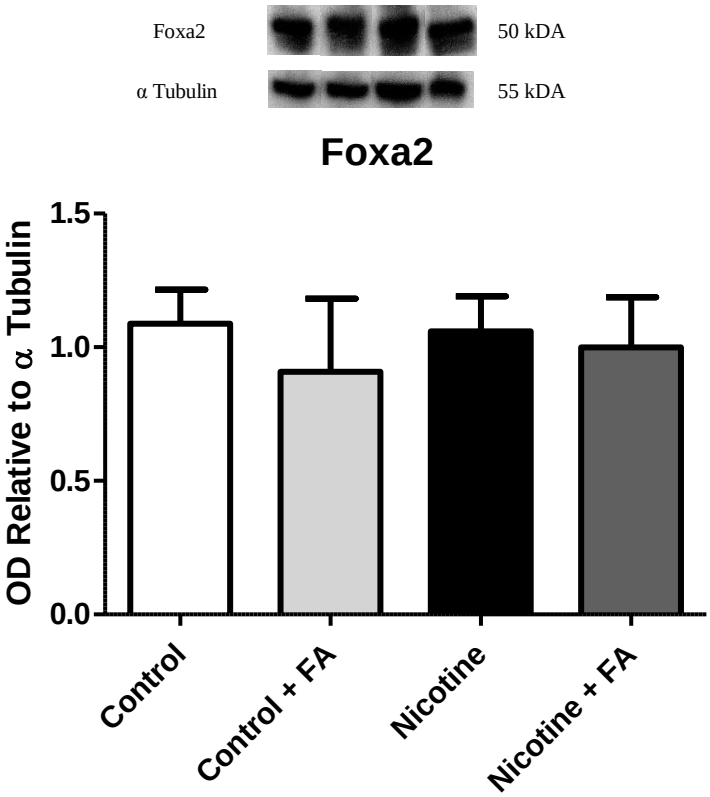

Figure 3.5 Effect of nicotine and folic acid on foxa2 expression

Foxa2 expression in INS 1E cells 48 hours post treatment with saline ± 10µM folic acid or 1µM nicotine ± 10µM folic acid. Data are expressed as optical density relative to alpha tubulin loading control and represented as mean ± SEM, n=3. The above panels are a representative band for each treatment group from the western blot. Each graph is a quantification of all samples.

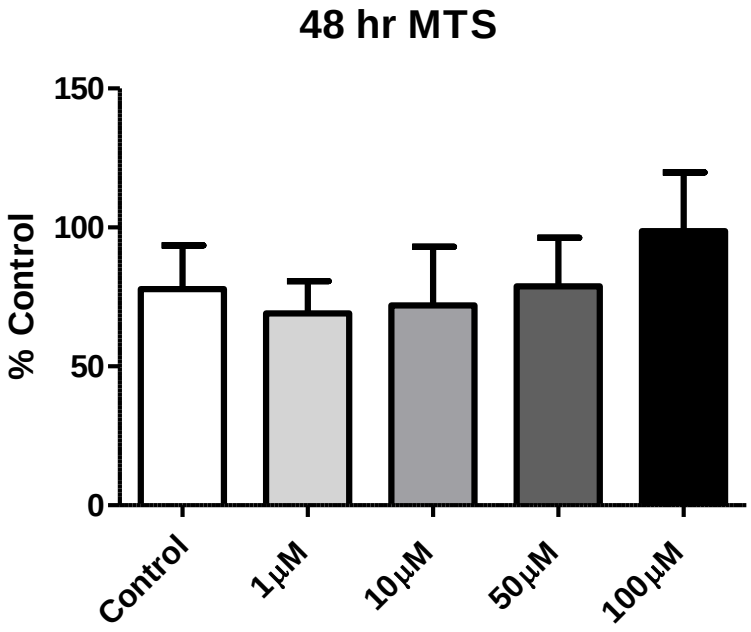

Figure 3.6 Effect of nicotine treatment on cell viability

MTS cell viability 48 hours post treatment with saline or 1, 10, 50 or 100μM nicotine. Data are expressed as a percentage of the control.

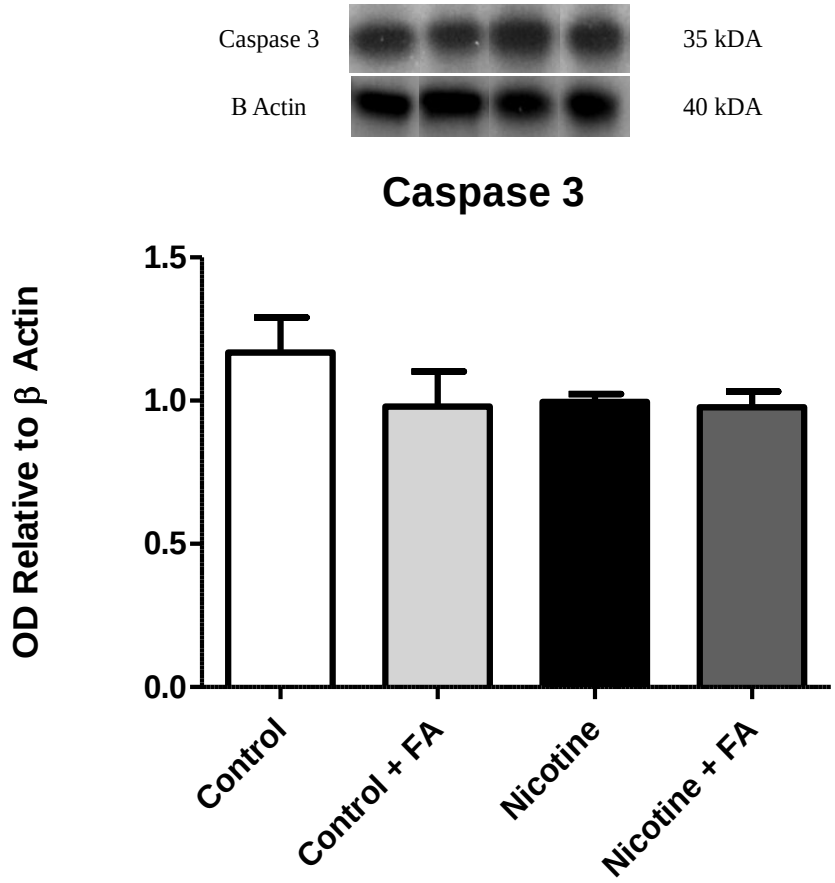

Figure 3.7 Effect of nicotine and folic acid on Caspase 3 expression

Caspase 3 expression in INS 1E cells 48 hours post treatment with saline ± 10µM folic acid or 1µM nicotine ± 10µM folic acid. Data are expressed as optical density relative to beta actin loading control and represented as mean ± SEM, n=3. The above panels are a representative band for each treatment group from the western blot. Each graph is a quantification of all samples.

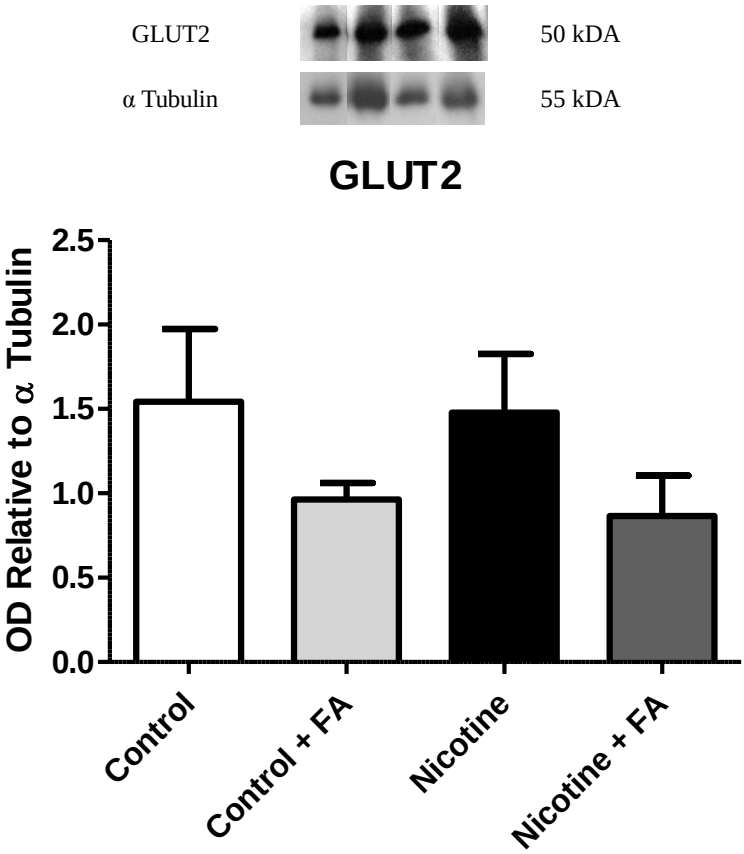

Figure 3.8 Effect of nicotine and folic acid on GLUT2 expression

GLUT2 expression in INS 1E cells 48 hours post treatment with saline ± 10µM folic acid or 1µM nicotine ± 10µM folic acid. Data are expressed as optical density relative to alpha tubulin loading control and represented as mean ± SEM, n=3. The above panels are a representative band for each treatment group from the western blot. Each graph is a quantification of all samples.

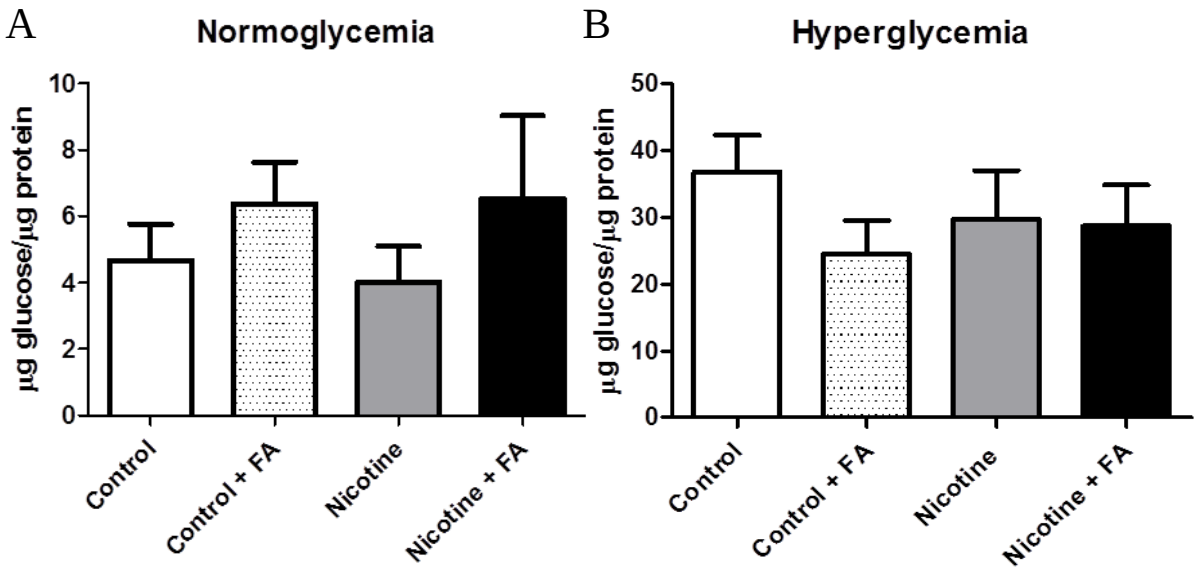

Figure 3.9 Effect of nicotine and folic acid on glucose consumption at normal and high glucose concentrations

Glucose consumption in INS 1E cells 48 hours post treatment with saline ± 10µM folic acid or 1µM nicotine ± 10µM folic acid at A) normal (11.1mM) glucose or B) high (16.7mM) glucose concentrations. Data are presented as µg glucose consumed per µg protein, n=5.

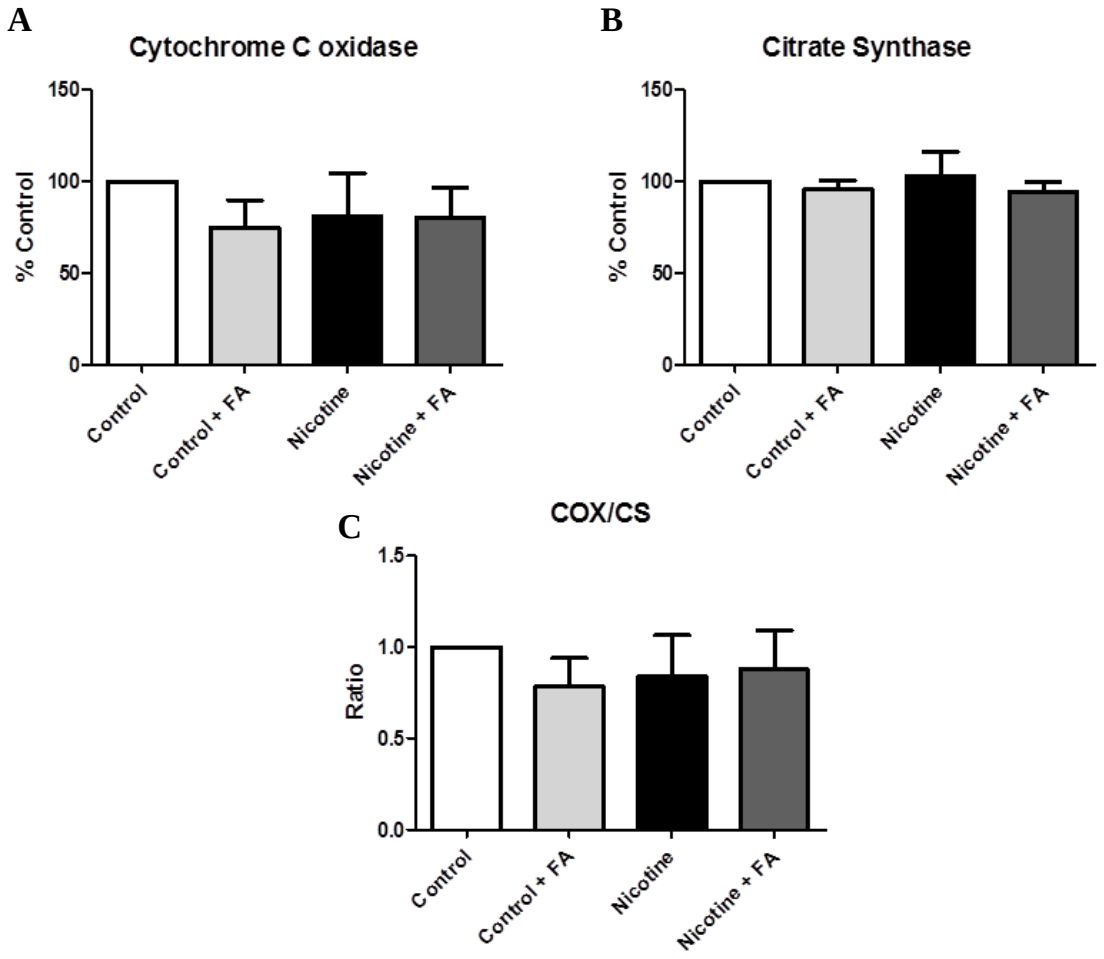

Figure 3.10 Effect of nicotine and folic acid on mitochondrial function

Mitochondrial function in INS 1E cells 48 hours post treatment with saline ± 10µM folic acid or 1µM nicotine ± 10µM folic acid. A) Effect on cytochrome c oxidase (complex IV), B) effect on citrate synthase and C) effect on ratio of cytochrome c oxidase to citrate synthase. Data are expressed as a percentage of the control, n=4.

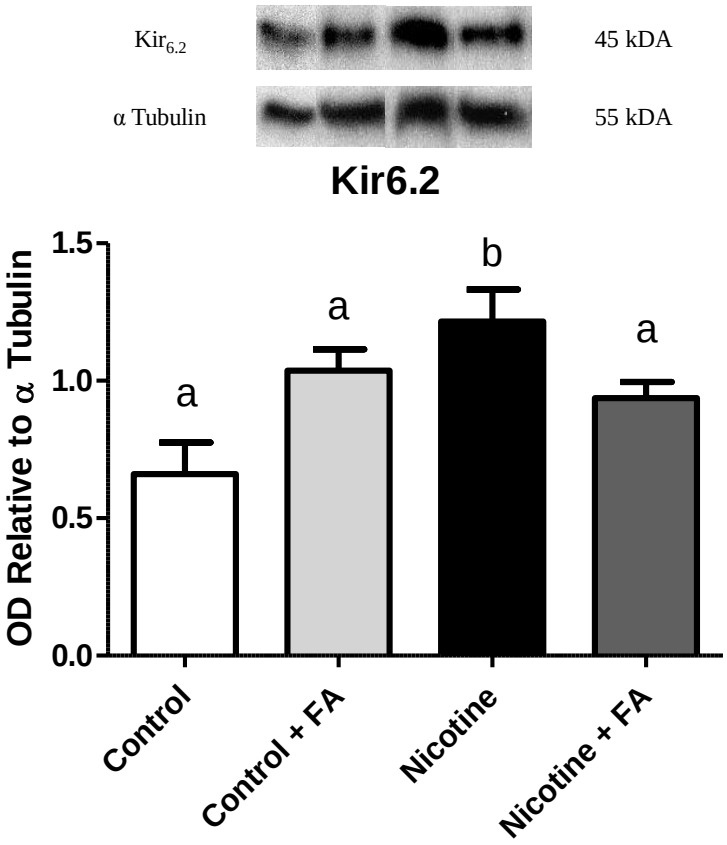

Figure 3.11 Effect of nicotine and folic acid on $kir_{6.2}$ expression

$Kir_{6.2}$ expression in INS 1E cells 48 hours post treatment with saline ± 10µM folic acid or 1µM nicotine ± 10µM folic acid. Data are expressed as optical density relative to alpha tubulin loading control and represented as mean ± SEM, n=3. Bars with different superscripts are significantly ($p<0.05$) different. The above panels are a representative band for each treatment group from the western blot. Each graph is a quantification of all samples.

4.0 Discussion

4.1 Effect of nicotine and folic acid treatments on markers of oxidative stress

4.1.1 *Maternal smoking*

Approximately 10-20% of Canadian women smoke during pregnancy (The Source 2011), although this number varies widely according to ethnic and geographical regions reaching as high as 85% (Mehaffey K, 2010). Smoking during pregnancy causes several adverse obstetrical outcomes including risk of low birthweight, perinatal mortality and sudden infant death syndrome (Castles A, 1999). The nicotine found in tobacco smoke is readily able to cross the placental barrier and fetal concentrations can generally be 15% higher than maternal levels. (Lambers and Clark 1996) In developed countries cigarette smoke is the most modifiable chemical insult encountered during pregnancy (Gruslin A et al. 2009 and Lambers DS 1996). Furthermore, mounting epidemiological evidence demonstrates that fetal exposure to cigarette smoke increases the risk of developing type 2 diabetes during adulthood (Behl M et at. 2012). Animal data suggest that nicotine, the major addictive component of cigarette smoke, maybe responsible, in part, for this increased risk of diabetes. Indeed, animal studies have demonstrated that fetal and neonatal exposure to nicotine results in dysglycemia in rodents (Bruin et al. 2007). It has also been shown that nicotine induces oxidative stress in beta cells of neonates, which subsequently triggers beta cell death and the onset of dysglycemia in early adulthood (Bruin 2008b, 2008c). Previous work from our lab has demonstrated that an antioxidant intervention during pregnancy preserves beta cell mass, however the antioxidant cocktail used in this study (i.e., vitamin E, alpha lipoic acid and COQ10) had adverse effects on fetal growth (Bruin et al. 2012). Therefore the goal of this study was to identify in vitro whether or not a different

antioxidant, namely folic acid, could preserve beta cell function. We selected folic acid as our antioxidant for this study because of: a) its safety in pregnancy; b) its widespread acceptance amongst both care providers and women for its beneficial effects on preventing neural tube defects; c) its ability to improve glucose tolerance in adult rats (Buettner et al. 2010) and d) the fact that folate levels are lower in smokers vs. non-smokers (van Wersch et al 2002, Cogswell et al 2003, Stark et al 2005, Jauniaux et al 2007).

4.1.2 *Smoking and folate status*

It is well established that smokers have decreased levels of serum antioxidants and in particular, the level of folate is decreased in smokers vs non-smokers (22.7 vs 29.4nM) (van Wersch et al. 2002, Cogswell et al. 2003, Stark et al. 2005 and Jauniaux et al. 2007).

Although it has been suggested that difference seen in folate levels in smokers vs non-smokers is due to dietary differences (smokers tend to eat less vegetables than non-smokers), evidence suggests that this is not the case. It has been found that the dietary folate intake is not significantly different between smokers and non-smokers (McDonald et al 2002, Mansoor et al 1997). However, some socio-economic factors could also account for the discrepancy. An epidemiological study from Norway suggested that the mean *supplemental* folic acid intake was higher in women who did not smoke, were married or co-habiting, and had a higher education (Nilsen et al 2010). The discrepancy in levels of folate supplementation between smokers and non-smokers suggests that this group might be at risk for the complications of folate deficiency; and could benefit from additional folate.

Recent studies (Wani et al., 2011) have shown that ethanol exposure can reduce expression of folate transporters in the rat pancreas. This reduction in folate transporters leads to reduced uptake folate within the pancreas. To date, no such studies have been done to determine whether nicotine exposure has an effect on folate transporter expression in the pancreas, and specifically, in the beta cell. If chemical exposures are able to disrupt the expression levels of folate transporters, then this could explain the decreased levels of folate that are seen in smokers. Our studies showed no effect of nicotine treatment on the folate transporters FR and RFC in pancreatic beta cells. But, it is possible that there could still be decreased folate uptake. Indeed, in placental tissue, high glucose (30mM), nicotine (0.1 and 10µM) and other drugs of abuse reduced folate uptake in human cytotrophoblasts *in vitro* (Keating et al, 2009). If this is true for beta cells as well, then the reduced folate uptake could contribute to disrupted one carbon metabolism (i.e. could affect beta cell mass through reduction in beta cell proliferation).

4.1.3 *Effect of nicotine and folic acid on oxidative stress*

Both nicotine and low folate are associated with oxidative stress (Wetscher et al 1995, Huang et al, 2004, Chern et al. 2001, Barr et al. 2007). Nicotine exposure has been shown to increase levels of HSP70 (a marker of oxidative stress), protein carbonyls (a marker of oxidative damage) and antioxidant enzymes in pancreatic tissue *in vitro* and *in vivo (*Ermis et al 2004, Woynillowicz et al 2012, Bruin et al 2008b). Indeed, in a study of folate depletion, low folate decreased Cu/ZnSOD and GPx and increased lipid peroxidation in rat livers (Huang et al 2001). Like low folic acid, nicotine also negatively affects tissues, and most importantly, adversely affects endocrine pancreas tissue (Bruin et al. 2008b, Holloway et al. 2005, Wetscher et a. 1995).

Low folate status is associated with increased homocysteine levels (Gupta et al, 1998) and increasing homocysteine levels have been implicated in a number of disease states including diabetes (Elias and Eng 2005). Folate supplementation has been shown to improve both oxidative stress and increased homocysteine levels in various tissues (Torrens et al. 2006, Delfino et al. 2007, Al-Maskari et al. 2012), making it a viable option as an antioxidant therapy. In particular, folate supplementation has been shown to improve endothelial dysfunction associated with T2DM (Title et al., 2006) and reduce the risk of developing T2DM according to the Pune Maternal Nutrition Study (PMNS) (Yajnik et al., 2008).

The goal of my study was to determine if folic acid supplementation could reduce or prevent nicotine-induced damage to pancreatic beta cells *in vitro.*

With nicotine treatment there was increase in expression of the chaperone protein HSP70, which is a marker of overall cellular stress, including oxidative stress. This finding is consistent with data from the literature showing the stress-inducing nature of nicotine (Woynillowicz et al 2012). The function of HSP70 is to prevent cellular apoptosis by associating with Apaf-1 (part of the apoptotic complex, the apoptosome) (Beere et al. 2000 and Saleh et al. 2000). Furthermore, nicotine treatment also caused an increase in the oxidative damage marker 4-HNE. In this instance oxidative damage is in the form of lipid peroxidation and is the oxidative degradation of lipids, where free radicals remove electrons in cellular membranes which results in cellular damage (Uchida 2003, Blokhina et al. 2003). Destruction to cellular membranes by lipid peroxidation can result in significant tissue damage, since lipid peroxidation is a self-propagating reaction (Mylonas and Kouretas, 1999). 4-HNE damages fatty acids by initiating a free radical attack on the unsaturated bonds of membrane fatty acids. If 4-HNE levels are increased excessively, cellular function can be compromised resulting in death of the cell (Mattson, 2009).

I have shown that with nicotine treatment in INS 1E cells of 1uM for 48 hours, there is a significant increase in the overall cellular stress marker HSP 70 and oxidative damage marker 4-HNE. This data are consistent with the adverse effects of nicotine found by others (Helen et al. 1999, Bruin et al. 2008b, Chattopadhyay and Chattopadhyay 2008) Furthermore, co-treatment INS1 E cells with nicotine and folic acid (10uM) significantly reduced the up-regulation of HSP70 and 4 HNE. This suggests that folic acid treatment is able to counteract the damaging effects of nicotine administration. In both instances, the addition of folic acid reduced the expression of both proteins significantly.

Individuals with low folate status have higher concentrations of homocysteine. Hyperhomocysteinaemia is associated with adverse health outcomes, including impaired insulin secretion by pancreatic beta cells. However, exposure to homocysteine did not alter cellular insulin content, cell viability or apoptosis (Patterson et al. 2006). Furthermore, increasing levels of homocysteine impaired the secretory response to the drug tolbutamide. Tolbutamide interacts with the SUR1 subunit of the K_{ATP} channel, causing channel closure and insulin release. In addition, increasing levels of homocysteine caused an inhibition of insulin secretion to beta cells undergoing direct membrane depolarization (Patterson et al, 2006). Since smoking leads to decreasing folate levels, increased homocysteine associated with low folate could be how smoking/nicotine exposure increases the risk for developing diabetes. Indeed, Maloney et al (2011) have examined glucose homeostasis in the offspring exposed to a low folate maternal diet. The low folate diet had no initial effect of body mass of the offspring compared with control pups, although the litters were smaller in number. At the end of the study males exposed to the low folate diet *in utero* were significantly heavier than controls, were insulin resistant and had elevated fasting glucose levels. Therefore, the offspring born to women who smoke which have

low folate levels could be at risk for the development of type 2 diabetes. It is possible that the adverse effects seen with nicotine treatment *in vitro* would be more pronounced in folate-deficient media, but this would have to be the focus of future studies.

4.2 Effect of nicotine and folic acid treatment on antioxidants

All eukaryotic cells contain antioxidant enzymes which function to offset the negative effects of oxidative stress. One of the major families of antioxidant enzymes are the superoxide dismutases (SOD). The primary function of SOD is the conversion of superoxide to oxygen and hydrogen peroxide (Tollefson et al, 2010).The antioxidant catalase (CAT) functions to convert hydrogen peroxide to water and oxygen. These antioxidants are an important defense mechanism to protect cells from the damaging effects of reactive oxygen species (ROS). This is especially true for the pancreatic beta cell, which is particularly vulnerable to ROS (Xu et al. 1999, Robertson 2004). Compared to other tissue types (i.e. liver, kidney, brain), pancreatic islets have the lowest levels of endogenous antioxidants (i.e. SOD, CAT and GPx) (Lenzen et al., 1996). ROS are necessary molecules for cellular signaling (i.e. conveying extracellular and intracellular signals to the nucleus), however, an overproduction of ROS can be detrimental to cells (Poli et al. 2004, Martin and Barrett 2002).

There have been several studies linking cigarette smoking and/or nicotine exposure to increased oxidative stress (Aycicek et al, 2010, Menon et al, 2011 and Crowley-Weber et al, 2003). Datum from my experiments has shown that treatment with 1µm nicotine causes a significant increase in the mitochondrial SOD, MnSOD compared with vehicle treated cells. There was no increase in the cytosolic SOD, Cu/ZnSOD nor was there an increase in catalase.

With the addition of 10µm folic acid to the nicotine group, there was a significant decrease in expression in both Cu/ZnSOD and catalase compared to the nicotine only treatment group. Interestingly, the addition of folic acid failed to reduce the expression of MnSOD which remained at the same level as the nicotine only group. It is possible that the oxidative stress caused by the nicotine is affecting the mitochondria within the beta cell more than the cell as a whole. Indeed, it has been shown that mitochondria are sensitive to the damaging effects of ROS (Duprez et al, 2012). Crowley-Weber et al. (2003) showed that 0.8µM of nicotine is sufficient to decrease the mitochondrial membrane potential which could lead to mitochondrial dysfunction. In an *in vivo* model of fetal nicotine exposure, nicotine treatment caused mitochondrial dysfunction in the pancreas of the offspring (Bruin et al., 2008c). It is therefore possible that the nicotine-induced reduction in insulin secretion from INS1E cells reported by Woynillowicz et al. (2012) and in this study (Figure 3.1) is a result of mitochondrial stress.

Pi et al. suggest that while an increase in the endogenous anti-oxidant expression is beneficial for preventing oxidative damage and possible cell death (2010), it could be a double-edged sword. The group also suggests that the increase in endogenous anti-oxidant expression decrease the signals needed for adequate glucose stimulated insulin secretion (GSIS), thereby reducing insulin secretion in the INS 1(823/13) cell line (2007). Although the addition of folic acid to the nicotine group did not reduce expression of MnSOD, there was a reduction of both oxidative stress in this treatment group (as seen with reduced expression of HSP70 and 4-HNE). Perhaps the consistent elevation of MnSOD, even though there is a decrease in oxidative stress, could account for persistent decrease In GSIS seen in this group.

4.3Effect of nicotine and folic acid treatment on beta cell function

4.3.1 *Effect on insulin secretion*

Yoshikawa et al (2005) and Bruin et al (2008b), showed that the nicotinic acetylcholine receptors (nAChR's) are present within both the pancreas and the beta cell. Results from my study and Woynillowicz et al. (2012) have demonstrated that nicotine can have a *direct* negative affect on the beta cell to reduce GSIS; an effect which is likely mediated through an increase in oxidative stress.

Results from my work have shown that treatment of INS 1E cells with 1µm nicotine for 48 hours caused a significant decrease in basal insulin secretion and an ablation of GSIS. We wanted to determine if an antioxidant intervention would preserve insulin secretion

With the addition of 10µm folic acid, there was a trend toward increased basal and glucose stimulated insulin secretion in nicotine-treated cells. While the basal insulin secretion in the nicotine plus folic acid treated cells was not significantly different from that seen in cells treated with nicotine alone, it was also not significantly decreased from the control group, suggesting that the addition of 10µm folic acid returned basal insulin secretion in nicotine treated cells to normal levels. It is interesting to note that control cells treated with folic acid also failed to produce an insulin response to high glucose (i.e. ablation of GSIS). A possible explanation could be that in healthy individuals, the administration of antioxidants can have a negative impact on health (i.e. high doses of vitamin E can increase mortality), whereas someone in a disease state would find beneficial effects of antioxidant administration (Anderson et al, 2010, Miller et al. 2005). Indeed, in clinical studies the effects of antioxidant interventions are not always beneficial. For example, folic acid has been shown to be effective for stroke prevention

(Wang et al, 2007), whereas other studies have shown no effect or dual effects (Wrone et al, 2004, Mullin 2011, Ly et al 2011, Kim 2004). It should be noted, however, that the best possible candidates may not have been chosen to participate in the clinical trials, which could lead to the confounding results seen with antioxidant intervention (Collins 2005). Although the adverse effects of excessive ROS have been well documented, ROS at physiological concentrations serve as cellular signals, and are therefore essential. The addition of additional antioxidants where none are needed may also have detrimental effects to the cell, as its signals to function have diminished (Thannickal and Fanburg 2000).

4.3.2 *Effect on Glucose uptake*

Patients with T2D show a marked reduction in the glucose transporter GLUT2 (Del Guerra et al, 2005), which could be partially responsible for the decrease in insulin secretion in diabetes.

In the Zucker diabetic rat model, it has been found that the expression of GLUT 2 is normal in pre-diabetic rats, but is vastly reduced (75%) once blood glucose levels exceed 11mM (Unger 1991). This reduction in GLUT-2 could be partially responsible for the reduced GSIS seen with this model. Similar to this, Thorens et al (1990 and 1992) showed that the reduction in GLUT2 expression was proportional to the severity of hyperglycemia (i.e. the more severe the hyperglycemia the more profound the reduction in GLUT2 expression). Furthermore, they found the loss of GLUT2 is induced by the diabetic environment and can be reversed by transplanting islets from diabetic *db/db* mice to non-diabetic *db/+* mice. To determine if treatment with nicotine reduced GLUT-2 in the INS 1E cells, we determined the expression of GLUT-2 by

western blot and found no change, leading us to the assumption that the reduced GSIS is occurring through another mechanism.

To determine if there is a decreased activity of the glucose transporter we quantified the amount of glucose that was taken up by the cells from the cell culture media. If less glucose is being consumed by nicotine treated cells; then less ATP will produced from the glucose and ultimately, less insulin released. We determined that at both normal (11.1mM) and high (16.7mM) glucose concentrations, there was no change in glucose consumption. Studies have shown that cigarette smoke can decrease peripheral glucose uptake (Attvall et al. 1993), however this is the first study showing the effects of nicotine exposure on beta cell glucose uptake.

4.3.3 *Effect on insulin content*

To determine if the reduced GSIS in nicotine treated cells was due to an overall reduction in the amount of insulin within the cell, we quantified the amount of insulin in treated INS 1E cells. We found that there was no reduction in insulin content with nicotine treatment, suggesting that the reduction in GSIS is not due to less insulin present within the cell (and therefore less insulin to secrete). Interestingly, we found a significant increase in the amount of insulin in the nicotine plus folic acid group, which is consistent with the increased expression of the transcription factor pancreatic and duodenal homeobox 1 (PDX1).

PDX-1 is an important transcription factor required for early pancreatic development. Indeed, studies have shown that PDX1 null mice show pancreatic agenesis (Fujimoto and Polonsky 2009, Kim and Hebrok 2001). In the mature pancreas, PDX1 becomes restricted to mature β cells where it regulates β cell specific genes, namely insulin, where it binds directly to

A/T rich elements of the *Ins* promoter (Melloul 2004 and Chakrabarti and Mirmira 2003). PDX1 is essential is mature β-cell function; missense and frameshift mutations within the *Pdx1* gene result in defective insulin secretion (Fujimoto and Polonsky, 2009 and Brissova et al 2002). *In vivo* studies of both intrauterine growth restriction and nicotine exposure show a reduction in expression of the transcription factor PDX-1 (Xu et al, 2011 and Somm E et al, 2008). Furthermore, progressive epigenetic silencing of *pdx1* leads to type 2 diabetes in adulthood (Park et al, 2008). I found no such reduction in PDX -1 expression with nicotine treatment alone, however, it maybe that in my *in vitro* model the exposure time wasn't long enough to see such changes.

The transcription factor Foxa2 (formerly known as HNF3β), like PDX1, is necessary for proper pancreas formation. In addition, β-cell specific deletion of Foxa2 results in attenuation of the genes encoding PDX1 and K_{ATP} channels resulting in profound hypoglycemia (Sund et al 2001, Chakrabarti and Mirmira 2003), showing that Foxa2 is an important regulator of these two genes. Furthermore, Foxa2 is also required in the mature β cell for insulin secretion (Gao et al, 2007). Due to its function as an activator of PDX1, we determined if, like PDX1, expression of foxa2 was increased in INS 1E cells treated with nicotine and folic acid. We found that although PDX1 was significantly increased, there was no similar increase in Foxa2, suggesting that the increase in PDX1 was occurring through another pathway. To date, this is the first study which shows that folate supplementation may alter PDX-1 expression. Poor maternal nutrition leads to decreased expression of PDX-1 through decreased histone acetylation at the PDX-1 promoter coinciding with increased DNA methylation. These changes result in epigenetic silencing of PDX-1 and the progression toward type 2 diabetes of the offspring (Simmons, 2007). It is possible that folate supplementation (a methyl donor) is increasing H3K4 methylation on the

PDX-1 promoter leading to an increase in expression (as opposed to increased H3Kp methylation which is consistent with gene silencing (Park et al. 2008).

4.3.4 *Effect on apoptosis*

Patients with type 2 diabetes have reduced beta cell mass (Rhodes, 2005). In the initial stages of diabetes, the pancreas is able to initially increase beta cell mass to compensate for the increasing glucose levels (Weir et al 2001, Weir and Bonner-Weir 2004 and Kahn 2001). However, increased beta cell mass is unable to be sustained indefinitely. The end result is reduced beta cell mass, an effect which occurs through beta cell apoptosis (Butler et al, 2003). Animal studies of fetal and neonatal nicotine exposure show a reduction in serum insulin levels coinciding with an increase in apoptosis at birth (Holloway et al, 2005), an effect which was later shown to be mediated via the mitochondrial apoptotic pathway (Bruin et al, 2008a).

I had previously found an increase in expression of 4-HNE, a marker of lipid peroxidation. Lipid peroxidation of cellular membranes can affect cellular function and cause apoptosis (Mattson, 2009). To determine if the reduction in insulin release was due to a reduction in beta cell number (due to apoptosis) expression of Caspase 3 was assessed. Caspase 3 is the last Caspase to be activated in the Caspase signaling cascade. Caspase 3 is the only Caspase to be activated by all apoptotic pathways (intrinsic and extrinsic); because of this we chose to determine the expression level of Caspase 3. I found no increase in Caspase 3. This suggests that the reduction in insulin secretion *in vitro* in the nicotine treated cells is unlikely to be occurring through apoptosis *in vitro*. This is consistent with MTS data (used to show effects on cell viability/proliferation) where nicotine treatment had no effect. Other studies have looked at the

effects of nicotine exposure on the effects of apoptosis. These studies showed an increase in Caspase 3 activation, however, the doses were considerably higher than the dose we used (5mM and 50uM) (Kang et al. 2011, Ramlochansingh et al. 2011). Due to the low concentration of the nicotine in the present study (1µM), it is likely that we would not see an increase in apoptosis at the time point tested (48 hours).

4.3.5 *Effect on mitochondrial function (glucose sensing)*

There is considerable evidence linking mitochondrial dysfunction to the development of type 2 diabetes (Lowell et al 2005). The role of mitochondria in the beta cell is to link glucose uptake to insulin secretion. If the mitochondria are not working properly then there will be a decrease in the amount of ATP produced and therefore a reduction in the amount of insulin released. Studies have shown *in vivo* and *in vitro,* that nicotine has a negative impact on mitochondrial function within the beta cell (Bruin et al 2008, Bruin et al 2010, Woynillowicz et al 2012). Data from my experiments, however, has shown that *in vitro* nicotine exposure to the INS 1E beta cell line does not result in mitochondrial dysfunction, despite the reduction in basal and glucose stimulated insulin secretion. This is in disagreement with Woynilowicz et al (2012); who found that *in vitro* nicotine exposure lead to dysfunction of complex IV in the mitochondria. It has been noted that passage number of a cell line can affect its function (American Type Culture Collection (ATCC) 2007), where the stress of cell culturing can create phenotypic changes in the cells. In addition, the INS 1E cell line tends to be particularly sensitive to temperature and humidity changes (personal observation). This could account for the discrepancy of findings seen in this experiment and in those of Woynilowitcz et al.. It is then

likely that the reduction in insulin release, at least initially, is occurring through another mechanism.

Since there was no effect of nicotine treatment on GLUT2 expression and on mitochondrial function, then therefore we assume that there would be no change in the amount of ATP produced in control and nicotine treated beta cells. Therefore, the impairment of insulin secretion seen with nicotine treatment could be due to a defect in the secretory mechanism of insulin secretion.

4.3.6 *K_{ATP} Channels*

An important part of insulin secretion is the ATP sensitive potassium channel (K_{ATP}). K_{ATP} channels are inhibited by ATP, resulting in closure of the channel and subsequent insulin release. In the absence of glucose, there is a reduction in the ATP/ADP ratio and the channels are open and no insulin is released (Huopio et al 2002). K_{ATP} channels are octomers composed of a pore-forming unit ($Kir_{6.2}$) surrounded by a regulatory unit (SUR1) which associate in a 4:4 ratio (Ashcroft FM, 2007 and Hiliart M, 2008).

Genetic studies have shown that mutations in either subunit of the K_{ATP} channel, $Kir_{6.2}$ or SUR1, causes defects in insulin secretion resulting in hyperinsulinism or diabetes. These defects in insulin secretion are due to gain of function mutations (leading to a reduction in insulin release) or loss of function mutations (leading to an over-stimulation of insulin release) (Ashcroft FM, 2006).

I have shown that nicotine treatment significantly increases the expression of the K_{ATP} subunit $Kir_{6.2}$. In nicotine only treatment group there was a reduction in glucose stimulated and basal insulin secretion. If the increase in expression of $Kir_{6.2}$ is similar to gain of function mutations, then this could be a possible explanation why there is a reduction in insulin secretion with nicotine exposure. The addition of folic acid did reduce this expression, but not to levels seen with the control. This reduction on $kir_{6.2}$ expression coincides with an increase in basal insulin secretion and glucose-stimulated insulin secretion (although not significant). In a review of K_{ATP} channels by Ashcroft 2005, it was stated that a total loss of protein (of both subunits) would result in loss of channel activity and therefore hyperinsulinism. Therefore, it is conceivable that an increase in in K_{ATP} subunits would result in more channel activity leading to hypoinsulinism. Indeed, it has been shown that increased expression of K_{ATP} subunits stabilizes the beta cell near its resting membrane potential of -70mV which raises the concentration of glucose needed to elicit an insulin response (Chen et al, 2011 and Ashcroft 2006). Therefore, nicotine treatment appears to affect the secretory mechanism of beta cells which precedes mitochondrial dysfunction and increased apoptosis seen in *in vivo* models (Bruin et al., 2008c and a, respectfully).

4.4 Future Directions

The next step in determining whether folic acid supplementation would have beneficial effects on pancreatic beta cells would be an animal study. Bruin et al., (2007, 2008a, b and c) showed that fetal nicotine exposure increased pancreatic oxidative stress, apoptosis and mitochondrial dysfunction. The combination of these factors resulted in impaired glucose

homeostasis. These data give support to the fetal origins hypothesis, where offspring are 'programmed' *in utero* for a certain lifestyle, which if not met in adult life can lead to disease.

4.4.1 *Fetal origins of adult disease*

The causes of the rise in the metabolic syndrome seen throughout the world are not entirely clear. A common hypothesis for this is the 'fetal origins', 'fetal programming', 'developmental origins of adult disease' or 'thrifty phenotype' theory. The premise of this theory is that utero-insufficiency combined with modern day lifestyles (inactivity, abundant food) leads to disease (hypertension, diabetes, coronary heart disease, and kidney disease) later in life. (Yajnik and Deshmukh 2008, Simmons 2004). Utero-insufficiency can be in the form of maternal smoking, pre-eclampsia, maternal undernutrition, or toxic exposure among others (Hendrix and Berghella 2008, Ong 2006). Placental insufficiency leads to a reduction in birth weight, which when coupled with catch-up growth in childhood, increases the risk of developing disease. For example, those at the highest risk for the development of T2D were born small and became overweight as children (Simmons, 2004, Hales and Ozanne 2003).

How intra-uterine growth restriction leads to disease later in life is not entirely clear, however, it is likely that the developing fetus will put most nutrients toward the development of vital organs (i.e. brain) at the expense of less vital organs (i.e. pancreas). The effect of this is a reduction in both β-cell mass, and function shortly after birth, with an age-related decline in β-cell mass (in an intrauterine growth restriction (IUGR) rat model there is a 50% reduction

compared to controls at 15weeks of age and at 26 weeks a 65% reduction) (Simmons 2009, Martin-Gronert and Ozanne 2007)

It has been shown that smoking during pregnancy leads to intra-uterine growth restriction (Wang et al., 2009). Both smoking during pregnancy and IUGR alone lead to the development of type 2 diabetes (Dennery, 2012), a growing concern worldwide.

The results of my experiments show that folic acid supplementation may be a viable option to protect the pancreatic beta cell from the adverse effects of nicotine. In addition, I have shown the potential for new mechanisms to explore (effects of PDX1 and $kir_{6.2}$). Indeed, recent human studies have shown the promising effects of folate supplementation in patients with T2DM. Aghamohammadi et al (2011) showed that folate supplementation (5mg/d for eight weeks) in a diabetic population increased antioxidant capacity and decreased MDA (a marker of oxidative damage). Furthermore, Gargari et al (2011) showed that folate supplementation (5mg/d for eight weeks) lowered plasma homocysteine levels, improved glycemic control and insulin resistance in male patients with T2DM.

5.0 Conclusions

The epidemic of type 2 diabetes worldwide has led to a vast amount of research being done to try and determine how and why this is occurring. Our research group has focused on fetal programming of the endocrine pancreas through chemical insults during pregnancy and lactation. One of these chemicals is nicotine, a chemical which can affect the fetal pancreas through cigarette smoking and nicotine replacement therapy use. Approximately 20% of Canadian women smoke during pregnancy, possibly making nicotine exposure a major player in the diabetes epidemic. Due to this, determining an antioxidant intervention that would alleviate nicotine-induced pancreatic damage could reduce the numbers of diabetes in the future.

To try and elucidate how nicotine and folic acid exposure can affect insulin secretion of pancreatic beta cells, we used an *in vitro* model to determine the pathways in which nicotine/folic acid can affect insulin secretion. We found that folic acid is a viable option as an antioxidant to prevent oxidative stress and damage due to its beneficial effects on nicotine exposed beta cells.

In conclusion, it appears that *in vitro* nicotine exposure to beta cells leads to a reduction in insulin release through increased K_{ATP} channel expression and not through a reduction in insulin content. With the addition of folic acid, there is an increase in insulin release likely mediated through an increased insulin availability which may be a result of increased gene expression of insulin mediated via the transcription factor PDX-1.

6.0 References

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