

**THE EFFECT OF UNCOUPLING PROTEIN 2 ON PROINSULIN  
PROCESSING**

A Thesis

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in Partial Fulfilment of the Requirements  
for the Degree of

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in the Department of Biomedical Sciences  
Faculty of Veterinary Medicine  
University of Prince Edward Island

**Narudee Kashemsant**  
**Charlottetown, Prince Edward Island, Canada**

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## ABSTRACT

Diets high in fat have an effect on both of the cardinal features of type 2 diabetes mellitus, insulin resistance and beta cell dysfunction. However, the mechanisms by which high fat diet is detrimental to beta cell function are only partially elucidated. Impaired glucose stimulated insulin secretion and hyperproinsulinaemia are well documented in this disease. In addition, these two characteristics are ATP dependent. Therefore there might be some defect in ATP availability in beta cells in type 2 diabetes. Uncoupling protein 2 (UCP2) is a negative regulator of ATP production and its expression is elevated in diabetic models and by dietary fat. Therefore it is plausible that the defective insulin secretion and proinsulin processing found in diabetic patients might result from increased expression of beta cell UCP2. The overall hypothesis of this thesis is that chronic high fatty acid exposure would induce UCP2, subsequently reducing ATP, to impair glucose stimulated insulin secretion and proinsulin conversion to mature insulin. To test this hypothesis, three studies were carried out using paradigms predicted to increase UCP2 expression. In the first study, UCP2 cDNA was directly transfected into INS-1 cells. In the second study, INS-1 cells were cultured in the presence of fatty acid. The last study was an *in vivo* study in which wildtype and UCP2 knockout mice were fed with either standard rodent chow or high fat diet for five weeks.

The present study showed that UCP2 reduced ATP content without altering ATP synthase, thereby impairing GSIS. UCP2 increased insulin gene transcription but reduced proinsulin processing in the UCP2 overexpression model. Free fatty acid exposure of beta cells also caused impaired insulin processing, but this could not be directly attributed to UCP2 gene expression or correlated with reductions in ATP production. Lastly, high fat diet in wildtype mice was sufficient to impair GSIS and proinsulin processing but did not significantly increase UCP2 expression, or reduce intracellular ATP. From the UCP2 overexpression studies we conclude that induction of UCP2 can influence proinsulin processing, presumably by reducing intracellular ATP concentrations. Whether fatty acids influence proinsulin processing via their effects on UCP2 expression or activity was inconclusive. The five week dietary regime was sufficient to induce changes in glucose-stimulated insulin secretion and proinsulin processing but was insufficient to affect UCP2. This suggests that early changes in insulin secretion are not attributable to UCP2 expression, although effects on activity cannot be ruled out.

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## LIST OF ABBREVIATIONS

Abbreviations	Definition
ACC	acetyl CoA carboxylase
ADP	adenosine diphosphate
AMV-RT	avian myeloblastosis virus reverse transcriptase
Arg	arginine
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
Ca <sup>2+</sup>	calcium ion
cDNA	complementary DNA
CPT-I	carnitine palmitoyl transferase I
DAG	diacylglycerol
DEPC	diethyl pyrocarbonate
DME medium	Dulbecco's modified Eagle's medium
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
ELISA	enzyme-linked immunosorbent assay
FADH <sub>2</sub>	flavin adenine dinucleotide (reduced form)

FBS	fetal bovine serum
FCCP	carbonyl cyanide p- (trifluoromethoxy)
	phenylhydrazone
FFA	free fatty acid
GAPDH	glyceraldehyde 3 phosphate dehydrogenase
GK	glucokinase
glc	glucose
Glut4 (Glut2)	glucose transporter 4 (glucose transporter2)
GSIS	glucose stimulated insulin secretion
HFD	high fat diet
IRS-1	Insulin receptor substrate-1
ISG	immature secretory granule
K <sub>ATP</sub> channel	ATP dependent potassium channel
KC	knockout mouse fed with control diet
kD	kilodalton
KF	knockout mouse fed with high fat diet

Km	the Michaelis-Menten constant
KO	knockout
KRB	Krebs Ringer bicarbonate
LC CoA	long chain acyl CoA
LDCV	large dense core vesicle
LDL	low density lipoprotein
Lys	lysine
min	minute
MODY	maturity onset diabetes of the young
mRNA	messenger RNA
MSG	mature secretory granule
NADH	nicotinamide adenine dinucleotide (reduced form)
NSF	N-ethylmaleimide sensitive factor
PA	palmitate
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDH	pyruvate dehydrogenase
PDX-1	pancreas duodenum homeobox-1
PI-3 kinase	phosphoinositide 3-kinase
PKC	protein kinase C

PPAR	peroxisome proliferator-activated receptor
PPRE	peroxisome proliferator response element
RaM	rapid mode
RER	rough endoplasmic reticulum
RIA	radio-immunoassay
RNA	ribonucleic acid
RNA Pol II	RNA polymerase II
ROS	reactive oxygen species
rRNA	ribosomal RNA
s	second
SDS	sodium dodecyl (lauryl) sulfate
Ser	serine
SERCA	sarco/endoplasmic reticulum calcium ATPase pump
SFA	saturated fatty acid
SNAP-25	synaptosomal-associated protein of 25 kDa

SPC1/3 (SPC2)	subtilisin like prohormone convertase1/3 (subtilisin like prohormone convertase 2)
SRD	standard rodent diet
SREBP 1c	sterol regulatory element binding protein 1c
STZ	streptozotocin
TCA cycle	tricarboxylic acid cycle
TFIIH	transcription factor for RNA pol IIH
TG	triacylglycerol, triglyceride
TGN	trans Golgi network
Thr	threonine
TNF $\alpha$	tumour necrosis factor $\alpha$
UCP	uncoupling protein
VDCC	voltage dependent calcium channel
VLDL	very low density lipoprotein
WAT	white adipose tissue
WC	wild type mouse fed with control diet

WF

wild type mouse fed with high

fat diet

WT

wild type

ω-6 PUFA

ω-6 polyunsaturated fatty acid

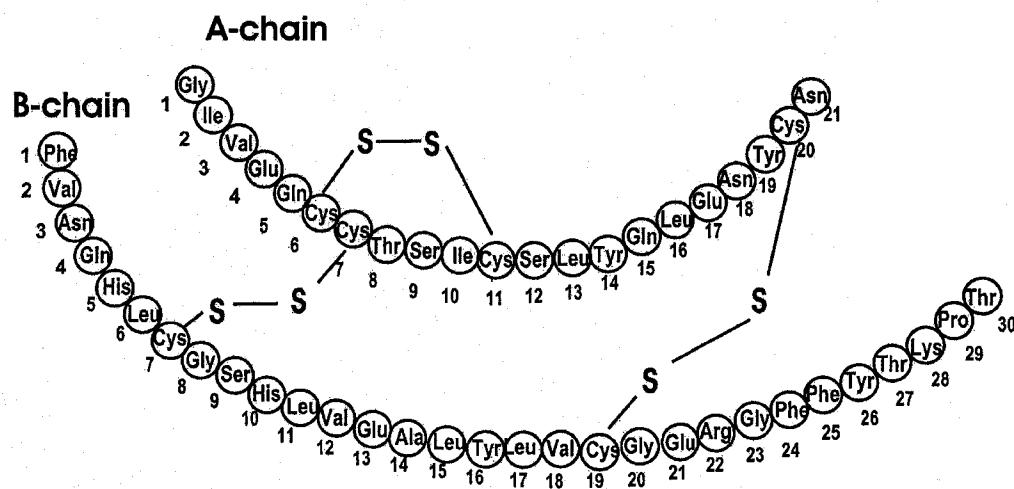
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# CHAPTER 1

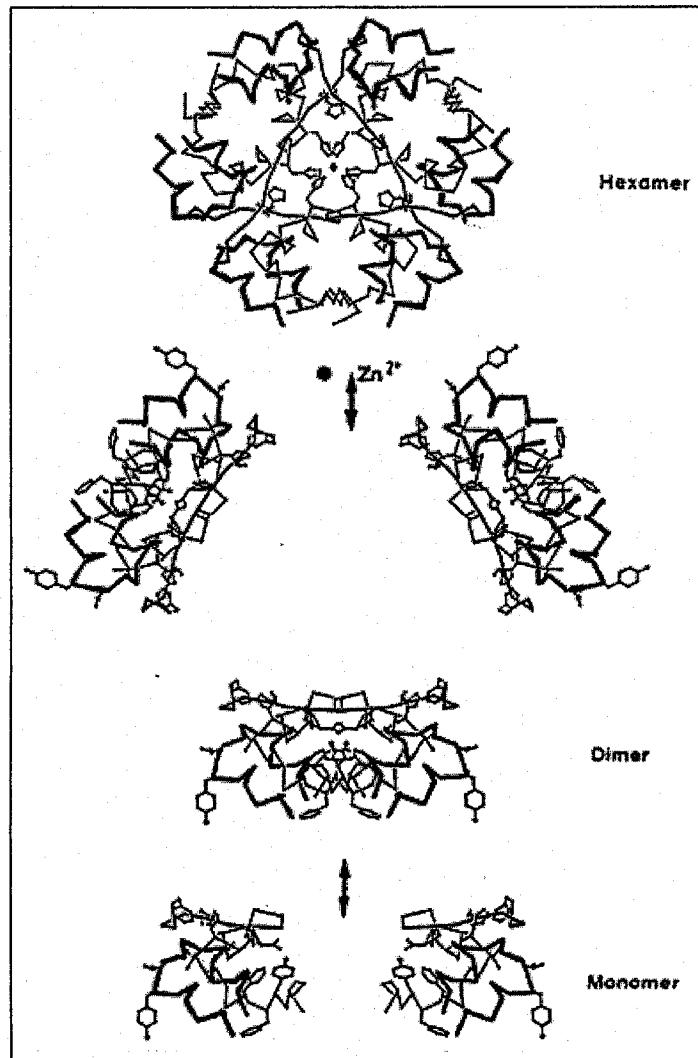
## GENERAL INTRODUCTION

### Insulin structure

Insulin is a small peptide hormone produced by beta cells of the islets of Langerhans. It consists of two chains referred to as the A-chain and B-chain, connected together by two disulfide bonds. Human A-chain contains 21 amino acids, while the B-chain contains 30 amino acids (Figure 1-1). The first disulfide bond connects the seventh amino acid from the A-chain to the seventh amino acid from the B-chain (A-7 to B-7). The second disulfide bond connects the twentieth amino acid of the A-chain to the nineteenth amino acid of the B-chain (A-20 to B-19). There is another disulfide bond internally connecting the sixth amino acid to the eleventh amino acid of the A-chain (A-6 to A-11). In all mammalian insulins the position of all three disulfide bonds is similar, suggesting that this globular protein has been strictly conserved during evolution (1). The N- and C-terminal regions of the A-chain and the hydrophobic residues of the B-chain are also highly conserved (2). Under physiologic conditions, insulin is normally in a monomeric structure. However, at high concentrations (such as are found in the secretory granules of the beta cells), insulin tends to self-associate to form hexamers (Figure 1-2) with three dimers arranged around an axis with two atoms of zinc. This insulin structure is important for storage capacity (3).



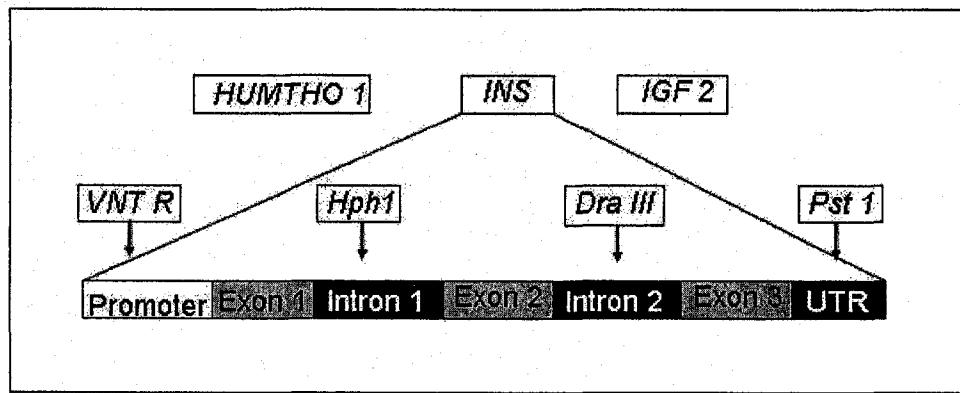
**Figure 1-1:** Human insulin structure (reproduced with permission from (4)).



**Figure 1-2:** Assembly and disassembly of the insulin hexamer. The A-chain of insulin is illustrated as a thickened black line, and the B-chain as a thinner line. Insulin monomers assemble into dimers as the concentration of protein increases, and into hexamers in the presence of divalent cations such as zinc (reproduced with permission from (4)).

## Insulin synthesis

The insulin gene has three exons and two introns (Figure 1-3). Exon 1 is located in the untranslated region of the gene. Exon 2 contains sequence coding for the signal peptide, B-chain and connecting (C)-peptide and exon 3 contains the A-chain sequence (5). The human insulin gene is located on the short arm of chromosome 11 on the band 11p15 (6). The human insulin promoter region regulates the transcriptional response to glucose and other nutrients and limits transcription of the gene to pancreatic beta cells (7). In contrast with humans, two rodent insulin genes are present. Both rat insulin genes are on chromosome 1 (8), while mouse insulin I is on chromosome 19 (9) and insulin II is on chromosome 7 (10). The rodent insulin II is similar to the human insulin gene, having three exons and two introns, while the second intron is missing from rodent insulin gene I (11). Moreover, based on mouse preproinsulin these two proteins differ by two amino acids in the B-chain and three amino acids in C-peptide (12;13).



**Figure 1-3:** Diagrammatic illustration of the insulin gene (INS), which consists of three exons and two introns (adapted from (14)).

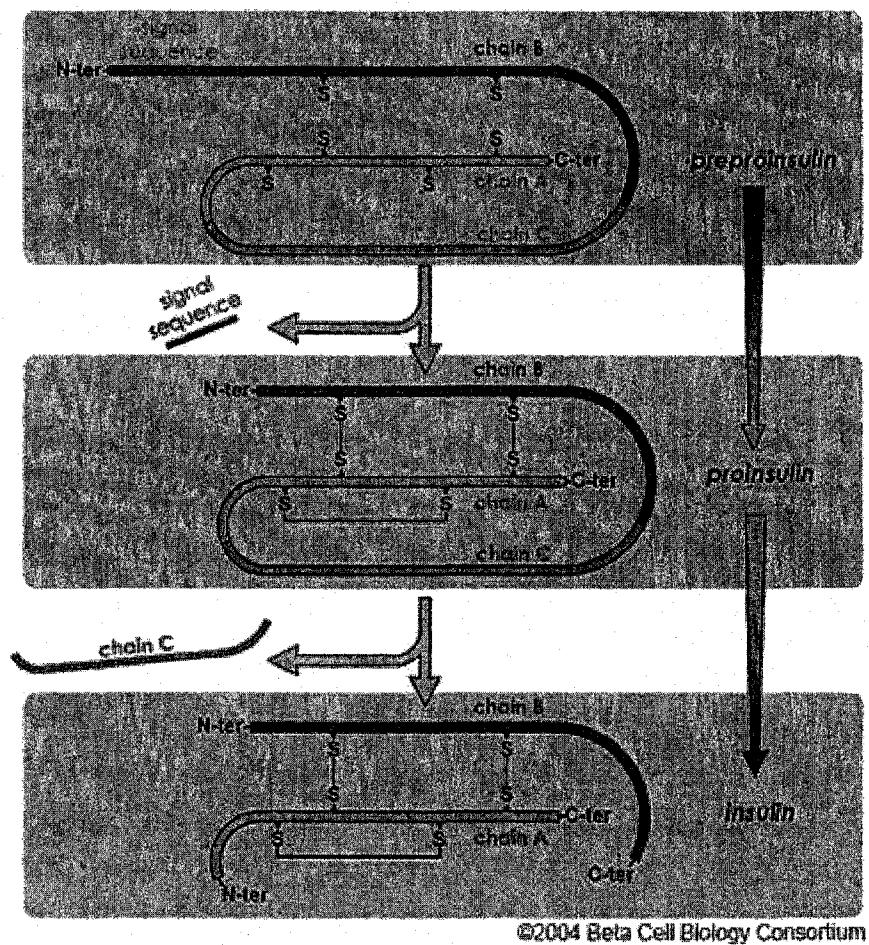
Glucose is the predominant stimulant of insulin biosynthesis. When glucose enters the beta cell to undergo metabolism and consequently induce insulin secretion, it also stimulates insulin synthesis (15). In addition to glucose, insulin itself is also reported as an insulin synthesis regulator (16;17). Insulin activates the insulin gene promoter via phosphatidyl inositol 3-kinase (PI 3-kinase)-Ia and the p70S6 kinase pathway (17). Moreover, it is reported that the effect of glucose on insulin synthesis is dependent on the presence of insulin (18).

The biosynthetic precursor of insulin is preproinsulin (Figure1-4). Human preproinsulin mRNA encodes an 11500 dalton protein. It comprises the entire proinsulin moiety and a 24 amino acid signal peptide. The signal protein helps preproinsulin to be sorted by ribonucleoproteins, called signal recognition particles. Signal recognition particles also contain a binding site for docking protein, which is on the cytosolic side of the rough endoplasmic reticulum (RER). Once the protein docks, preproinsulin is guided to enter the lumen of the rough endoplasmic reticulum. Subsequently, the signal protein is cleaved by signal peptidase, leaving proinsulin (1).

Proinsulin is the immediate biosynthetic precursor of insulin and unlike many peptide hormone precursors that do not have hormonal activity, proinsulin itself is a functional hormone (19). Proinsulin is a 9000 dalton peptide that includes the entire insulin and C-peptide sequences. The C-peptide chain varies in sequence among different species, but usually connects to insulin by Arg-Arg to the C-terminus of the B-chain and by Lys-Arg to the N-terminus of the A-chain (1). Newly synthesized proinsulin undergoes glycosylation before being transported to

the Golgi network. Transport of proinsulin from the RER to the Golgi network is facilitated by a binding protein on the cisternal face. The RER membrane buds to cover the newly synthesized proinsulin, subsequently the vesicle containing the newly synthesized proinsulin will fuse with the bud from the trans-Golgi apparatus. The passage of vesicles from the RER, through cis-, medial- and trans- Golgi apparatus (20) is an energy requiring event (21). During this translocation and in the zinc- and calcium-containing environment of the Golgi, proinsulin reorganizes itself from a monomeric to a hexameric conformation (22).

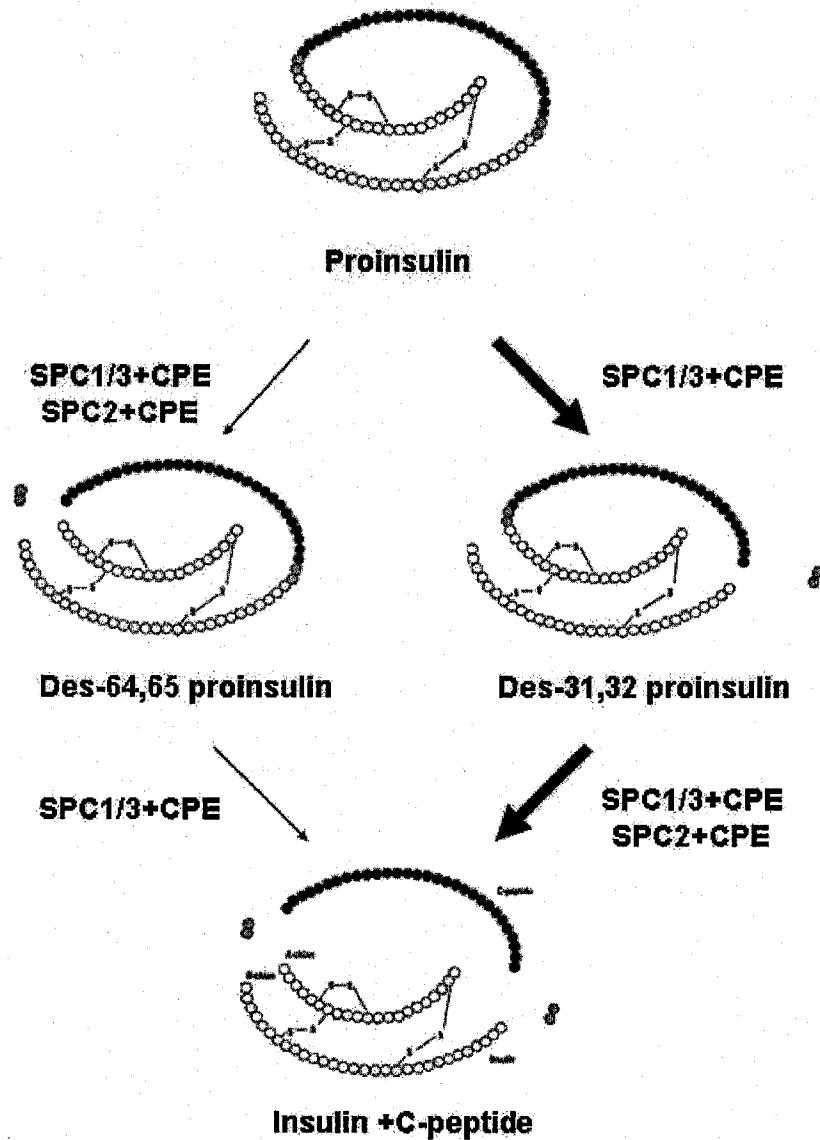
Proinsulin vesicles are classified as regulated secretory vesicles that secrete mature hormone in response to specific signals (23). Unlike constitutive transport vesicles, immature insulin vesicles are coated with the selective protein called clathrin (24). The vesicles also contain proteins such as chromogranin B and secretogranin II, which are thought to help vesicles sort into the regulated pathway (25). The conversion of proinsulin to mature insulin takes place in secretory vesicles as they move away from the trans-Golgi. Enzymatic conversion of proinsulin to insulin requires two classes of enzyme working together, the endopeptidases (referred as SPC1/3 and SPC2) and the carboxypeptidases (carboxypeptidase E or H) (26).



**Figure 1-4:** Insulin synthesis and maturation (taken with permission from (27)).

Endopeptidases involved in proinsulin processing belong to the eukaryotic subtilisin family, which includes Kex-2, furin, and PACE4. Subtilisin-like prohormone convertases (SPC)1/3 and SPC2 share 45-55% amino acid sequence identity with the subtilisin-like catalytic domain of Kex-2 but lack a transmembrane segment, a cytosolic domain and a Ser/Thr rich-region (26). Both SPC1/3 and SPC2 must undergo autocatalytic cleavage at an internal Lys-Arg site to remove a pro-region site and be activated. SPC1/3 rapidly autoactivates, probably in the RER, to yield an 87 kDa proenzyme form. After its segregation into secretory granules, further cleavage in its C-terminal region gives rise to a smaller, active form of SPC1/3 (67kDa) (28). In contrast, the autocatalytic cleavage of SPC2 is much slower than that of SPC1/3. The proteolytic activation of SPC2 most likely occurs within the secretory granules (29;30). Both SPC1/3 and SPC2 have mildly acidic pH optima (pH5.5-6.0) (31) and calcium-dependent activity (32). Compared to SPC1/3, SPC2 has a broader pH activity but a lower calcium requirement (26). SPC1/3 preferentially cleaves proinsulin at the B-chain and C-peptide junction (site 1 or Arg-Arg site) but also at the A-chain and C-peptide junction (site 2 or Lys-Arg site), while SPC2 appears to cleave proinsulin mainly at site 2 (33) (Figure1-5). Carboxypeptidase E removes the residual Lys and Arg residues to yield mature insulin plus C-peptide. Thus, SPC1/3 is the dominant beta cell convertase because it can cleave both sites of proinsulin (34). Also, western blot analysis suggests that there is relatively more SPC1/3 in insulin producing beta cells than in non beta cells (35). After prohormone conversion is

completed, both insulin and C-peptide are stored within the vesicle waiting for a signal to be released.

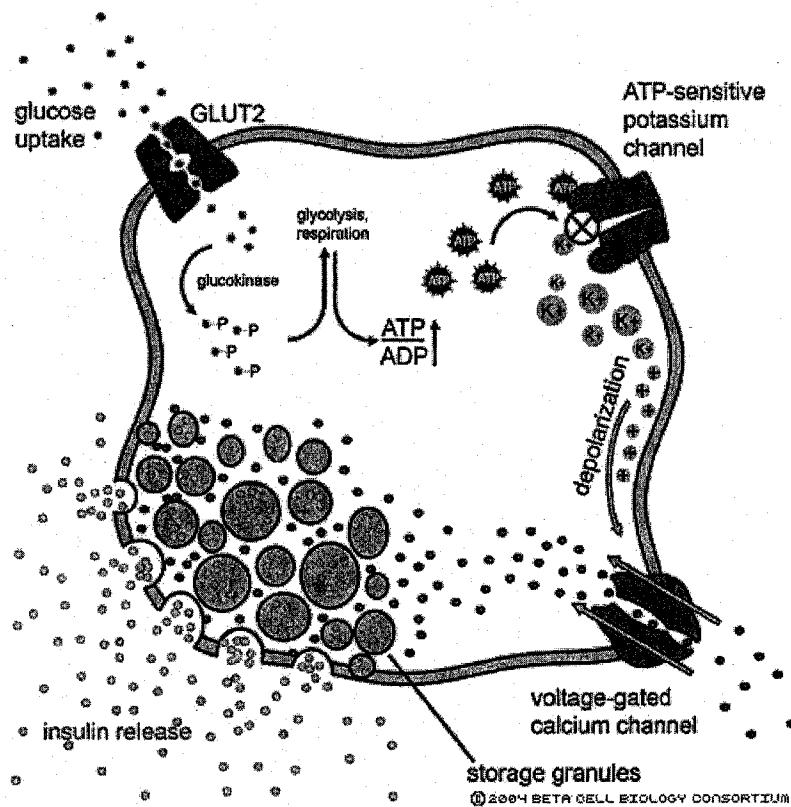


**Figure 1-5:** Proinsulin conversion process (adapted from (36)).

## Insulin secretion

Glucose enters the beta cell via the non-rate limiting, high Km (low glucose affinity) glucose transporter 2 (GLUT2) (Figure 1-6). Once inside the beta cell, glucose undergoes glycolysis and is converted to pyruvate and acetyl CoA. Subsequently, acetyl CoA enters the TCA cycle. Substrate (glucose or fatty acid) oxidation yields NADH and FADH<sub>2</sub>. Electrons donated from NADH and FADH<sub>2</sub> are passed through the electron transport system and generate ATP as a result. It has been noted that [ATP]<sub>i</sub> is 2 pmol/10<sup>3</sup> cells in the absence of glucose and increases to >4 pmol/10<sup>3</sup> cells when glucose is raised to 10 mmol/l (37).

The generation of ATP causes an elevation of the ATP/ADP ratio inside the beta cell. ATP binds to 4 sites on K<sub>ATP</sub> channel proteins, which inactivates the channel. Once the K<sub>ATP</sub> channel is closed, the accumulation of K<sup>+</sup> ions inside the beta cell causes membrane depolarization. Consequently, voltage dependent calcium channels (VDCC) open and allow influx of Ca<sup>2+</sup> into the beta cell cytoplasm. The influx of Ca<sup>2+</sup> that occurs through this channel eventually activates the system responsible for the exocytosis of insulin granules. However, there is huge variation in Ca<sup>2+</sup> concentration changes induced by glucose from beta cell to beta cell (38;39). Therefore it is unlikely that this means of Ca<sup>2+</sup> elevation could drive all events of nutrient induced insulin exocytosis. Ryanodine receptors are found inside pancreatic beta cells and play a role in increasing intracellular Ca<sup>2+</sup> during exocytosis of insulin induced by glucose (40). Moreover, it has been reported that pancreatic mitochondrial Ca<sup>2+</sup> also plays a role in nutrient induced insulin secretion (41).

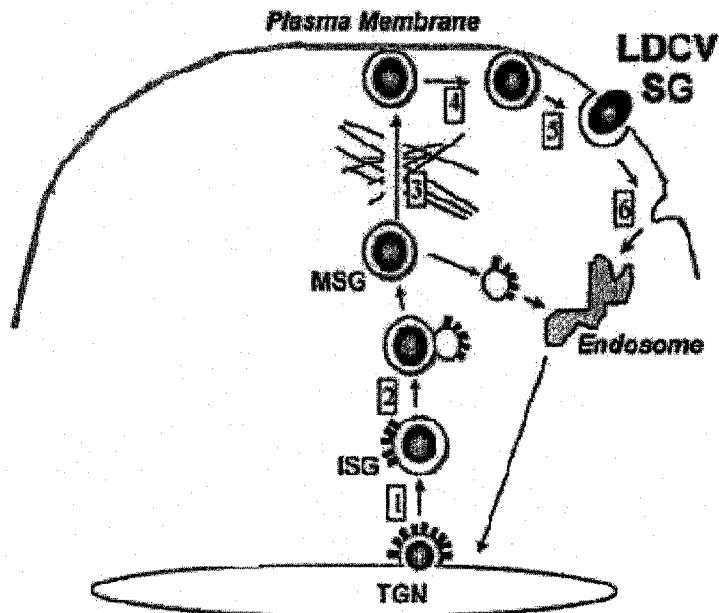


**Figure 1-6: Insulin secretion -** Insulin secretion in beta cells is triggered by rising blood glucose levels. Starting with glucose entering beta cells through the GLUT2 transporter, glucose oxidation causes a rise in the ATP:ADP ratio. The increase in this ratio inactivates the potassium channel, thereby depolarizing the membrane and causing the calcium channels to open, allowing calcium ions to influx. The ensuing rise in levels of calcium leads to the exocytic release of insulin from their storage granules (taken with permission from (42)).

## Insulin exocytosis

The elevation of intracellular  $\text{Ca}^{2+}$  triggers insulin exocytosis. The sequentially activated process includes vesicle recruitment and docking to the plasma membrane, vesicle priming and fusion between vesicles and the plasma membrane (Figure 1-7). To dock and fuse with the plasma membrane, vesicles must first be recruited to the cell surface from intracellular pools. Vesicle recruitment requires the myosin-actin system and ATP, which may serve as an energy donor (43) for molecular motors such as myosin, kinesin, and for protein kinases including  $\text{Ca}^{2+}$ /calmodulin dependent protein kinase II and the myosin light chain kinase (44;45). The concept of vesicle docking has been proposed as the SNARE hypothesis. This process is thought to be induced by the pairing of specific proteins on the vesicle membrane, referred to as v-SNARE, and target membrane, referred to as t-SNARE (46). These SNARE proteins are converted into a multi-subunit aggregate, the 20S particle. Soluble N-ethylmaleimide sensitive factor (NSF) is an ATPase that binds to SNARE complexes via adaptor proteins called SNAPS. NSF functions similar to a chaperone and mediates an ATP-dependent conformational change during the fusion step. Likewise, many phosphorylation reactions in the secretory cascade are thought to use ATP as a phosphate donor (43). In addition to ATP,  $\text{Ca}^{2+}$  is essential for granular fusion to plasma membranes. Before becoming fusion competent, morphologically docked granules undergo an activation process called priming. Priming is the stage when vesicles become competent for fusion-pore opening (47). Synaptotagmin is the most likely candidate for the  $\text{Ca}^{2+}$  sensor of the exocytosis machinery, since at least

two  $\text{Ca}^{2+}$  binding sites are found in this protein (48). Thus, when  $\text{Ca}^{2+}$  is elevated, the fusion pores open and insulin is released into the circulation.



**Figure 1-7:** Schematic representation of exocytosis. Large dense core vesicles (LDCVs) such as secretory granules (LDCV/SG) bud from the trans Golgi network (TGN, 1) yielding immature secretory granules (ISG), which shed their coat proteins 2) and become mature secretory granules (MSG). They have to traverse the subcortical actin network 3) and dock to the plasma membrane 3). Subsequently, LDCVs become primed in an ATP-dependent reaction 4) and their membranes fuse with the plasma membrane 5). The ensuing endocytosis retrieves the membrane material, which is redirected to the TGN through the endocytic pathway 6) (adapted from (47)).

## Glucose Homeostasis

The body receives glucose from three main sources: intestinal absorption, glycogenolysis and gluconeogenesis, while it utilizes glucose via glycolysis and the TCA cycle. There are additional potential metabolic fates for glucose that is transported into cells. The predominant pathway depending on systemic glucose balance is either storage as glycogen (positive balance) or glycogenolysis (negative balance). Hypoglycaemia occurs when the rate of glucose appearance in the circulation fails to keep pace with the rate of glucose disappearance; in contrast, hyperglycaemia happens when glucose appearance exceeds the rate of its disappearance (49).

The regulatory mechanisms that control glucose balance are hormonal, neural and substrate control. Hormones are the main gluco-regulators. Systemic glucose balance is regulated by two sets of hormones; the glucose elevating hormones (glucagon, epinephrine, growth hormone and corticosteroid) and a glucose lowering hormone (insulin). Glucagon is released from the  $\alpha$ -cells of islets of Langerhans and is believed to act exclusively on the liver under physiologic conditions. It activates glycogenolysis, and to some extent gluconeogenesis, and increases hepatic glucose production within minutes (49). The hyperglycaemic effect of the adrenal hormone epinephrine is more complex. Epinephrine is secreted from chromaffin cells of the adrenal medulla in response to falling plasma glucose levels. It raises plasma glucose levels by stimulating hepatic and renal glucose production but limiting glucose utilization (49). The actions of epinephrine are both direct and indirect and are mediated through both  $\alpha$ -adrenergic and  $\beta$ -

adrenergic receptors. Adrenergic ( $\alpha_2$ ) activation also limits insulin secretion (50;51). This is an important indirect hyperglycaemic action of epinephrine because it allows the hyperglycaemic response to occur. However, the increase in insulin secretion that occurs as plasma glucose rises limits the magnitude of the glycaemic response. Adrenergic ( $\beta$ ) stimulation of glucagon secretion also occurs, but its contribution to the hyperglycaemic effect of epinephrine appears to be minor under physiological conditions. In addition to indirect effects, epinephrine acts directly to increase hepatic glycogenolysis and gluconeogenesis. In humans the hepatic effect is mediated predominantly through  $\beta_2$ -adrenergic mechanisms (50), although a small direct  $\alpha$ -adrenergic stimulation of hepatic glucose production has been reported. Epinephrine also mobilizes gluconeogenic precursors (e.g. lactate, alanine, and glycerol). Like glucagon, it acts within minutes to increase glucose production, but unlike glucagon it inhibits glucose utilization and glucose clearance by insulin sensitive tissues (52). Long term elevation of growth hormone and cortisol limit glucose utilization and stimulate glucose production (52). The effect of growth hormone on glucose production starts after several hours of lower glucose levels (53). Similarly, cortisol causes an increase in plasma glucose after two to three hours. In summary, the hyperglycaemic effect of the combination of glucose-elevating hormones is greater than individual effects of any one hormone (54). Conversely, insulin suppresses the endogenous glucose production (both via glycogenolysis and gluconeogenesis) and stimulates glucose utilization by insulin-sensitive tissues. It also converts liver into an organ of net glucose uptake and fuel storage (glycogen and triglycerides).

Insulin suppresses renal glucose production and stimulates glucose uptake, storage, and utilization by tissues such as muscle and fat (52). Glucose production by the liver is regulated primarily by glucagon and insulin, although the nervous system and glucose autoregulation also have an effect on hepatic glucose production (52). The ability of insulin function to maintain hepatic glucose output is the key role of maintaining glucose tolerance. If this ability is lost, it precipitates a diabetic condition.

### **Type 2 diabetes mellitus**

By definition, type 2 diabetes is characterized by two main features, insulin resistance and beta cell dysfunction. Insulin resistance is defined by the requirement for increased insulin concentration to elicit a target cell response (55). Beta cell dysfunction is characterized by impaired insulin secretion in response to glucose stimulation, reduced proinsulin conversion to mature insulin and reduced beta cell mass (56). Type 2 diabetes mellitus is the predominant form of diabetes, accounting for 90% of cases globally (57). The World Health Organization (WHO) estimates that three hundred million people will be diagnosed with type 2 diabetes mellitus by the year 2025. In Canada, according to the National Diabetes Surveillance System, 4.8% of Canadians aged 20 years and older had diabetes (4.6% of women and 5.0% of men) in 1998/99 (58). The prevalence of diabetes by provinces and territories shows that Nova Scotia has the highest prevalence while Yukon has the lowest. In addition, men have a higher prevalence of diabetes compared to women. The prevalence of diabetes increases by 5% to 10% among

adults for every 1 kg increase in population-measured body weight (59;60). Based on a U.S. study, diabetes and its complications cost the Canadian healthcare system an estimated \$13.2 billion every year. By 2010, it has been estimated that these costs will rise to \$15.6 billion a year and by 2020, \$19.2 billion a year (61). However, it is noted that up to 50% of affected people are undiagnosed (62). Type 2 diabetes is diagnosed when the fasting plasma glucose reaches 7 mmol/L (63).

**Table 1-1:** Prevalence of diabetes in Canada (taken from (64)).

	<b>1995</b>	<b>1996-1997</b>	<b>1998-1999</b>	<b>2000-2001</b>
<b>Number of persons with diabetes</b>				
<b>Canada</b>	<b>722,491</b>	<b>787,924</b>	<b>865,908</b>	<b>1,063,698</b>
<b>Male</b>	<b>359,458</b>	<b>423,157</b>	<b>481,833</b>	<b>556,838</b>
<b>Female</b>	<b>363,032</b>	<b>364,766</b>	<b>384,075</b>	<b>506,860</b>

## **Pathogenesis of type 2 diabetes mellitus**

It remains unclear whether insulin resistance or failure of insulin secretion is the primary cause of type 2 diabetes (65). The major sites of insulin resistance in type 2 diabetes mellitus are liver, skeletal muscle, and adipose tissue (66-69). In liver, insulin resistance causes elevation of glucose production after overnight fasting and reduces the suppression of glucose production after a meal (70). In skeletal muscle and adipose tissue, insulin resistance induces a defect in the translocation of glucose transporter 4 (GLUT4) from the cytosol to the plasma membrane (70). In addition, a reduction in tyrosine kinase activity and decreased activation of insulin receptor substrate-1 (IRS-1)-associated PI 3-kinase and protein kinase B are detected (69). Increased circulating levels of FFA and TNF- $\alpha$  may induce insulin resistance (68). The nature of beta cell compensation to insulin resistance involves hyperinsulinaemia even under euglycaemic conditions and increased beta cell mass (71). Moreover, insulin resistance is associated with increased expression of islet low Km hexokinase (72). As a sequence, the glucose-insulin response curve shifts to the left, leading to more insulin secreted in response to less glucose challenge (49). Ultimately, the glucose-insulin secretion relationship is blunted in type 2 diabetic patients and the glucose-insulin curve is shifted to the right because of the loss of first phase insulin secretion in response to glucose (73;74). Interestingly, these characteristics are also found in first degree relatives and normoglycaemic twins of diabetic patients (75). Beta cell abnormalities may therefore precede the development of overt type 2 diabetes by many years. Although diabetic patients have hyperinsulinaemia, the degree of

hyperinsulinaemia is inappropriately low for the prevailing glucose concentration (49).

Type 2 diabetic patients also have hyperproinsulinaemia. This is associated with increases in the proinsulin:insulin ratio in circulation (76). In addition to intact proinsulin, the beta cell also secretes increased amounts of proinsulin intermediates (split 32, 33-, split-65, 66-, des 31, 32- and des 64, 65 proinsulin). These partially processed insulins are produced in the secretory granules of the islets as a result of diminished prohormone convertase activity (76). Prohormone convertase activity depends on the vesicular optimal environment of pH (31) and  $\text{Ca}^{2+}$  concentration (32). It is thus suggested that this optimal environment for the insulin vesicles could be changed in the diabetic condition.

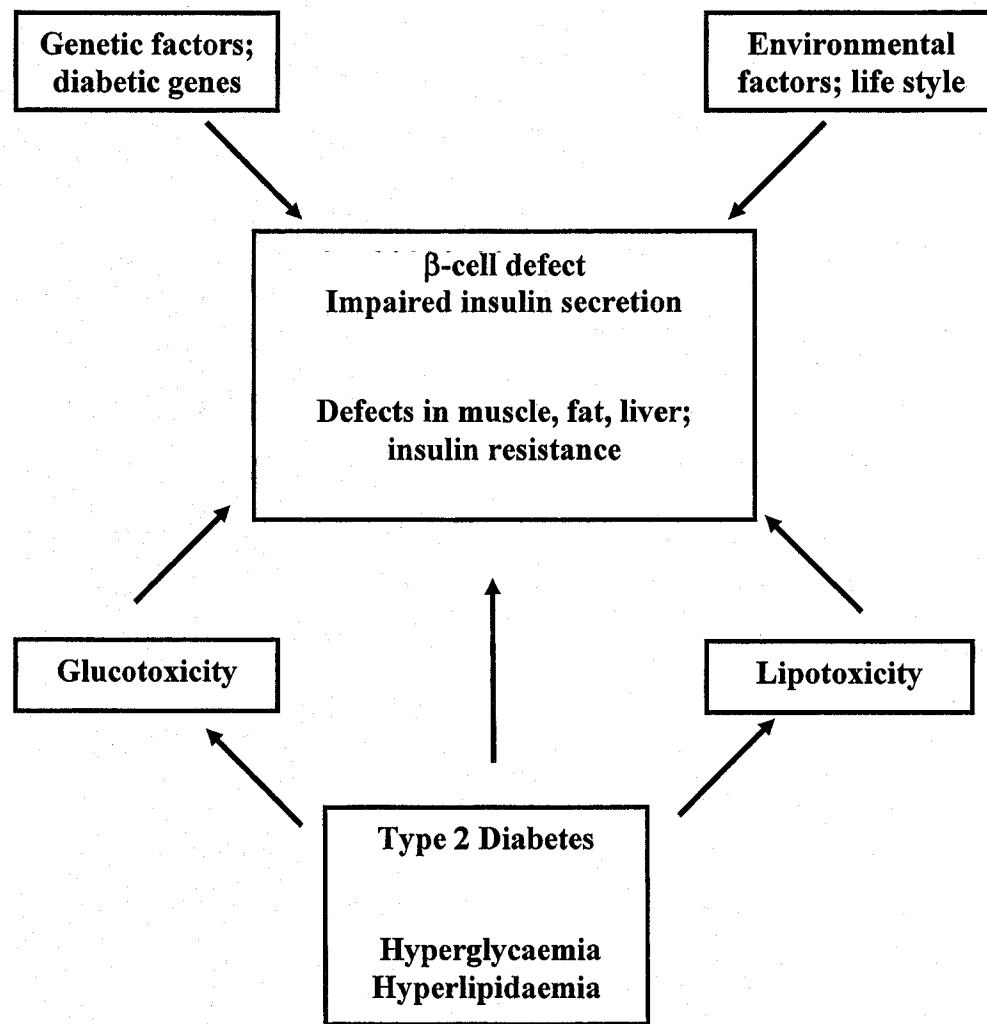
#### **Factors influencing the development of type 2 diabetes mellitus**

Both genetics and environment play a role in the pathogenesis of type 2 diabetes mellitus (77) (Figure 1-8). So far, genetic background has been characterized in only a minor fraction of the patients. Genetically, type 2 diabetes consists of monogenic and polygenic forms. Monogenic forms of diabetes, which are rare, are divided into two classes, those associated with insulin resistance and those associated with defective insulin secretion. Maturity onset diabetes of the young (MODY), the most common monogenic form, is actually caused by 6 distinct genetic mutations of beta-cell proteins (2): hepatocyte nuclear factor-4 $\alpha$  (MODY1), glucokinase (MODY2), hepatic nuclear factor-1 $\alpha$  (MODY3), insulin promoter factor-1 (MODY4), hepatic nuclear factor-1 $\beta$  (MODY5), and neurogenin

differentiation1/beta cell E-box transactivator-2 (MODY6). The most common clinical sign of MODY is a mild, asymptomatic increase in blood glucose in a child or young adolescent. Some patients may have mild hyperglycaemia, while others have varying degrees of glucose intolerance for many years before the onset of persistent hyperglycaemia (2).

The majority of the patients with type 2 diabetes (70-85%) appear to have a polygenic inheritance that acts in concert with environmental factors in the development of the disease. The environmental, or acquired, factors often relate to lifestyle, and comprise overweight, low physical activity (77) and diet (78;79).

The major environmental factors for the development of type 2 diabetes are the combination of a 'sedentary lifestyle' with a 'western-diet pattern', consisting of frequent consumption of red meat, refined carbohydrates, sweets and full-fat dairy products (80;81) High intake of total and saturated fat is associated with an increased risk of type 2 diabetes (81). Conversely, increased intake of cereal fibres and an elevated ratio of the intake of polyunsaturated/saturated fat and a low glycaemic load have a protective effect against this disease (82). As mentioned earlier, a sedentary life style is also a risk factor for type 2 diabetes. Elevation of VLDL is found in obese subjects with hyperlipidaemia, which can be accompanied by chylomicronaemia, LDL accumulation, hypertriglyceridaemia and hypercholesterolaemia (83) The molecular mechanisms of the effects of fat on type 2 diabetes will be discussed later.



**Figure 1-8:** Proposed pathogenesis of type 2 diabetes (adapted from (70)).

## **Nutrient interactions in the pancreatic beta cell**

The stimulus-response coupling system in pancreatic beta cells requires nutrient metabolism in order to induce insulin secretion. Metabolism of the main fuels, glucose and fatty acids, generates the signals for eliciting  $\text{Ca}^{2+}$  entry and allows insulin secretion. Generally, inside the beta cell the metabolism of energy source nutrients such as glucose and fatty acids are interrelated but reciprocal. Beta cells use fatty acids as a major fuel under low glucose conditions whereas they use glucose as a main source of energy when glucose levels in the environment are high. The details of the metabolism of these two major sources of energy and their interaction are as follows.

### **Glucose metabolism**

Glucose enters pancreatic beta cells via the high capacity glucose transporter 2 (GLUT 2) (84). Glucose is then phosphorylated by glucokinase (GK) and converted to glucose-6-phosphate. GK has a  $K_m$  for glucose of 10 mmol/L (84). This first enzyme in the glycolytic pathway acts as the glucose sensor for beta cells while the glucose regulator for beta cell glycolysis is phosphofructokinase. The end product of glycolysis is pyruvate, which is converted into acetyl CoA by pyruvate dehydrogenase (PDH). Consequently, acetyl CoA enters the tricarboxylic acid (TCA) cycle and then passes the reducing equivalents through the electron transport system to generate ATP as a final product.

### **Fatty acid $\beta$ -oxidation**

The complete hydrolysis of triacylglycerols yields glycerol and three free fatty acids. Free fatty acids undergo metabolism called  $\beta$ -oxidation in mitochondria. Following entry into the cells, the free fatty acid is first activated by coenzyme A and fatty-acyl CoA synthetase to become fatty acyl CoA. Fatty acyl CoA must bind to carnitine at the cytoplasmic side of the outer mitochondrial membrane in order to enter the mitochondria with catalysis from carnitine palmitoyl transferase-I. Subsequently, carnitine is removed, fatty acyl CoA is released into the matrix and carnitine cycles back to the cytoplasm. Once fatty acyl CoA is in the matrix side,  $\beta$ -oxidation occurs and ATP is generated from the acetyl CoA endproduct.

### **Lipid synthesis**

The pathway for fatty acid synthesis exists in the cytoplasm. Mitochondrial citrate is transported to the cytoplasm where citrate lyase converts citrate to oxaloacetate and acetyl CoA. The synthesis of malonyl CoA is the first committed step of fatty acid synthesis. The enzyme that catalyzes this reaction, acetyl CoA carboxylase (ACC), is the major site of regulation of fatty acid synthesis. The enzymes involved in fatty acid synthesis are an enzyme complex called the fatty acid synthase system. The normal product of the fatty acid synthase system is palmitic acid. Generally, the activity of fatty acid synthase in pancreatic beta cells is low, thus malonyl CoA synthesized inside beta cell will not be used as a substrate for fatty acid synthesis, but used as the switch signal for inhibiting the

fatty acid oxidation pathway to promote glucose oxidation. In contrast, when both glucose and fatty acids are present, high glucose stimulates the expression of fatty acid synthase (85) and hormone sensitive lipase (86) while inhibiting the expression of peroxisome proliferators-activated receptor  $\alpha$  (87). As a result, *de novo* synthesis of fatty acids can occur in beta cells.

### **Glucose-fatty acid cycle**

The relationship between plasma fatty acids and glucose metabolism was first reported in 1963 (88). It was proposed that increased free fatty acid (FFA) oxidation restrains glucose oxidation in muscle by altering the redox potential of the cell and by inhibiting key glycolytic enzymes. Briefly, fatty acid oxidation increases the NADH/NAD ratio, causing a slowing of the TCA cycle, thereby altering redox potential. The oxidation of fatty acids and ketone bodies released into the circulation in diabetes or starvation inhibits the catabolism of glucose in muscle. These effects of fatty acid and ketone bodies are mediated by inhibition of phosphofructokinase-1, hexokinase and the PDH complex. The essential mechanism is an increase in the mitochondrial ratio of [acetyl-CoA]/[CoA], which inhibits the PDH complex and leads to inhibition of phosphofructokinase-1 by citrate and of hexokinase by glucose-6-phosphate. The block in glucose transport results in a build-up of intracellular free glucose, which restrains glucose transport into the cell via the GLUT4 (GLUT2 in beta cells) transporter. The resultant decrease in glucose transport impairs glycogen synthesis. This proposed mechanism, which occurs in beta cells as well as other tissues, clearly explains the

glucose-fatty acid interaction in a euglycaemic and hyperlipidaemic condition, but may not be the operative mechanism in hyperglycaemia.

Under conditions in which glucose and fatty acid rise simultaneously, glucose diminishes fatty acid oxidation (89) and shifts the beta cell from fatty acid to glucose as an oxidative fuel (90). Glucose metabolism in the TCA cycle generates citrate, which can be converted into acetyl CoA and malonyl CoA (91) via two successive reactions catalyzed by citrate lyase and acetyl CoA carboxylase (ACC). Malonyl CoA serves as a signalling molecule to inhibit fatty acid oxidation by allosteric interaction with carnitine palmitoyl transferase 1 (CPT1) (92). Therefore fatty acid  $\beta$  oxidation is blocked because long chain acyl CoA cannot be transported to the mitochondria (93).

Once long chain acyl CoA (LC CoA) accumulates in the pancreatic beta cell cytoplasm, it acts as a signalling molecule for insulin secretion (94) or has direct effects on insulin trafficking (95). Alternately, LC CoA can react with glycerol-3-phosphate and be converted into other bioactive metabolites such as diacylglycerol (DAG) or phospholipids. DAG serves to activate PKC, an enzyme implicated as a player in the insulin secretory process (96), while phospholipids might be required for reworking of the cell membrane during exocytosis (94). In addition, the esterification of LC CoA (89;97) or de novo synthesis of ceramide (98) could occur in islets with the concomitant presence of fatty acids and glucose. However, fatty acids also have an effect on glucose metabolism. Inhibition of PDH but augmentation of pyruvate dehydrogenase kinase occurs in 48 h palmitate

cultured islets, resulting in the reduction of pyruvate conversion to acetyl CoA and hence, a reduction in glucose oxidation (99).

In summary, long chain fatty acids exert pleiotropic effects on the pancreatic beta cells. They can augment insulin secretion via the activation of LC CoA and can suppress beta cell function via esterification of fatty acid. More detail of the effect of fatty acid on insulin secretion and insulin synthesis is as follows.

### **The effect of fatty acids on insulin secretion**

It is now well established that the effects of exogenous free fatty acid on insulin secretion are dose-dependent (100) and also time-dependent. Likewise, elevated fatty acids only negatively affect the beta cell in the presence of hyperglycaemia (91). Short term exposure to high free fatty acid stimulates insulin secretion (102-104) whereas long term exposure increases basal secretion but suppresses insulin secretion in response to glucose (99;105).

The acute stimulatory effect of fatty acids on glucose-stimulated insulin secretion (GSIS) has been demonstrated both *in vitro* (106) and *in vivo* (104). It is accepted that fatty acids are necessary for insulin secretion in response to glucose (107). As mentioned earlier, GSIS requires an increase in the ATP/ADP ratio, the closure of  $K_{ATP}$  channels and an increase in intracellular  $Ca^{2+}$  ions. When infused with 10% Intralipid for 3 h, rat islets increased insulin secretion by 86% in response to 27 mmol/L glucose (101). Moreover, it has been reported that culturing rat islets with 0.625 mmol/L palmitate can increase  $Ca^{2+}$  influx (104;108) and cause a small mobilization of intracellular  $Ca^{2+}$  (104). Palmitate inhibits  $Ca^{2+}$

uptake and stimulates  $\text{Ca}^{2+}$  efflux from the endoplasmic reticulum (109). In addition, LC CoA can be converted to complex lipids such as phosphatidic acid, diacylglycerol and phospholipids. These complex lipids can activate PKC, consequently stimulating insulin secretion (110). Palmitate and myristate can augment insulin secretion by protein acylation (111). Fatty acylation of protein seems to be essential for the process of signalling through a subunit of G protein and Src family tyrosine kinases (112;113). As a result, the regulation of enzymatic activities in mitochondria is changed (114). However, it should be noted that the insulinotropic potency of fatty acids is influenced profoundly by their chain length and degree of saturation (115). Acute perfusion of fatty acids at 0.5 mmol/L revealed elevated stimulation of insulin secretion (115). Moreover, the longer the length of fatty acids, the higher potency in insulin secretion in response to 12.5 mmol/L glucose was seen. In contrast, unsaturated fatty acids had lower potency to affect insulin secretion compared with the saturated fatty acid at the same carbon number.

The long-term effects of FFAs on GSIS, however, are still controversial. Chronic plasma FFA elevation has been found both to increase (116) and decrease GSIS (117). Six weeks of soybean and olive oil feeding to rats induced an increase in insulin secretion when challenged by high glucose (116). Other studies with unsaturated fats (soybean oil) had similar results (115). As mentioned earlier, the beta cell response depends on the FA chain length and on their degree of saturation. In the perfused pancreas, the very different effectiveness of the two lipid emulsions, soybean and lard oil, in enhancing GSIS clearly suggests a

different potency of SFAs as compared with  $\omega$ -6 PUFAs (116). Thus, for any given level of glucose, insulin secretion will be greatly influenced not only by the free FA circulating levels but also by the percentage of unsaturation of the fatty acids (115).

The mechanisms by which the chronic exposure to high fatty acids could impair GSIS are many. Firstly, FFA-treated beta cells have decreased ACC levels (102) and increased LC CoA content (118). LC CoA activate  $K_{ATP}$  channels (119;120), which causes impaired insulin secretion. The activation of PKC by LC CoA (110;121) may be down regulated by chronic FFA exposure, thus contributing to impairment in secretory activity. Also, cytosolic LC CoA is the precursor for triglycerides, diacylglycerol and phospholipids. When LC CoA accumulates this will markedly favour lipid esterification and fat deposition in islet tissue via the condensation of glucose derived glycerol-3-phosphate with fatty acyl CoA. The activation of TG synthesis impairs GSIS in diacylglycerol-acyltransferase-1 overexpressing islets (122).

Another possible mechanism is that excessive fuel supply is associated with mitochondrial uncoupling via increased expression of uncoupling protein 2 (UCP2). This in turn is expected to decrease GSIS (91) simply by lowering intracellular ATP content of beta cells. Indeed, UCP2 expression is increased by high fat feeding in various tissues (123). PPAR $\alpha$  is induced by high concentrations of fatty acid in rat islets (124). PPAR $\alpha$  knockout mouse islets have a greater insulin secretion in response to glucose than wild-type (WT) mouse islets (125). In addition, the suppression of GSIS and induction of UCP2 expression detected in

PPAR $\alpha$  transfected INS-1 cells (126) implies that PPAR $\alpha$  has an effect on UCP2 induction, which in turn could suppresses GSIS. In contrast, adenoviral coexpression of PPAR $\alpha$  and retinoid X receptor  $\alpha$  leads to the induction of UCP2 expression and potentiates GSIS in rat islets and INS-1E cells without affecting the mitochondrial membrane potential (127). However, in the latter investigation INS1E cells were cultured in oleate-containing medium, which is an unsaturated fatty acid. As mentioned above, the effect of fatty acid on beta cell function depends on the saturation and length of fatty acid. In summary, chronic exposure to high levels of lipids initially boosts the insulin response in hyperinsulinaemic conditions (104); later, it selectively desensitizes the  $\beta$ -cell to glucose (128) and causes beta cell lipotoxicity. Fatty acids would appear to play a significant role in the progression of  $\beta$ -cell failure in patients at risk of developing type 2 diabetes. The mechanisms leading to a decrease in GSIS with prolonged exposure of beta cells to elevated FFA are only partially elucidated, however, it is suggested that this decrease is likely to occur via mechanisms such as changes in expression of UCPs, GLUT2, glucokinase, insulin (129) and transcription factors as well.

### **The effect of fatty acid on insulin biosynthesis**

Proinsulin, the prohormone of insulin is packaged into the secretory granules as described above. Within this granule proinsulin continues the maturation process. During this maturation or conversion process, proinsulin is cleaved by prohormone convertases and carboxypeptidases, yielding C-peptide and insulin. Under normal conditions, this conversion is almost complete, leaving only

1-2% of proinsulin intact (130). Generally, glucose is the predominant stimulator of preproinsulin synthesis (15). In contrast, it has been demonstrated that palmitate inhibits preproinsulin mRNA levels in isolated rat islets after 72 h of exposure (131). Palmitate does not affect basal insulin promoter activity, but strongly inhibits its stimulation by glucose (131). The mechanisms of this transcriptional inhibition involve two key transcription factors of the insulin gene, PDX-1 and MafA (132). In contrast to an inhibitory effect of high fatty acids on insulin gene transcription, hyperproinsulinaemia has been reported in obese diabetic patients (133-135). High plasma free fatty acid is a well known marker in obese and diabetic conditions (136). However, the relationship between high free fatty acid and proinsulin synthesis has yet to be demonstrated convincingly. Long term high fatty acid exposure leads to a reduction of insulin content and marked elevation of proinsulin in beta cells (137). In addition, SPC1/3 and SPC2 protein expression are reduced by 15% and 23% without any change in mRNA levels (137). As mentioned before, impairment of GSIS induced by chronic high fatty acid might be correlated with the expression of UCP2. UCP2 has been reported to impair insulin secretion in response to glucose (138-140). Moreover, islets from mice fed a high fat diet had increased UCP2 mRNA (117) and protein (141). UCP2 is a negative regulator of ATP generation (139). Both GSIS and proinsulin processing are dependent on ATP availability. It is thus hypothesized that UCP might be implicated in the impaired GSIS and proinsulin processing induced by chronic high FFA. A summary of UCP2 effects on insulin synthesis and secretion will be in the next section.

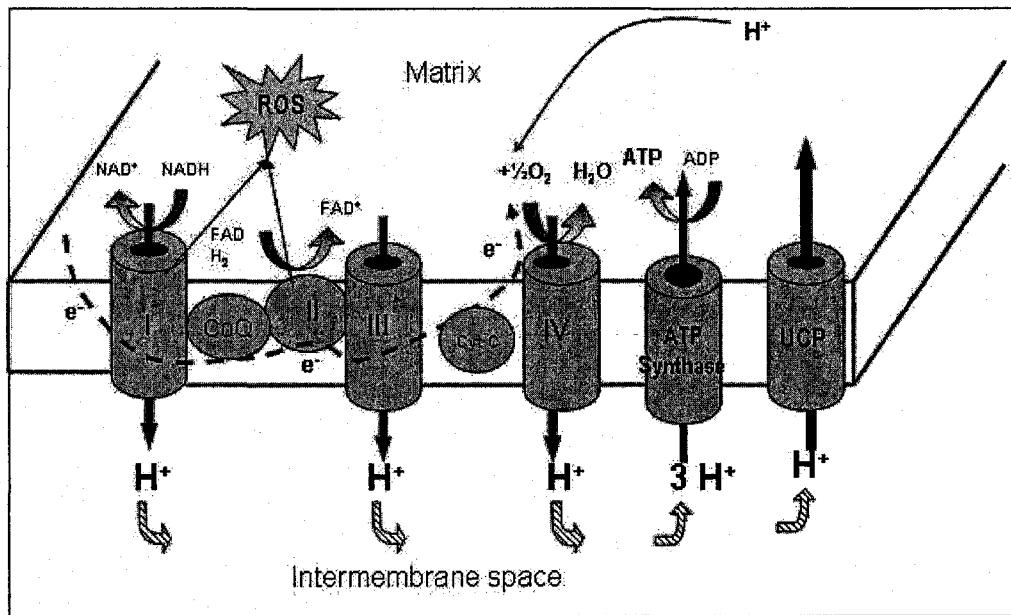
## **Uncoupling protein 2**

Uncoupling protein 2, an inner membrane protein of mitochondria, was discovered in 1997 (123). The human UCP2 gene is on chromosome 11 and the rodent UCP2 gene is on chromosome 7. The loci of the UCP2 gene on these chromosomes are in regions that display linkage to obesity (123). Human UCP2 gene spans over 8.4 kb distributed on 8 exons (142) with the 6 translated exons starting at the third exon (143). The localization of the exon/intron boundaries within the coding region matches precisely those found in the human UCP1 gene (142).

Uncoupling protein 2 shares about 56% identity with UCP1 (144). Based on UCP1's structure, the UCP2 monomer is predicted to have six membrane spanning alpha helices, with both the C- and N-termini protruding from the cytosolic side of the mitochondrial inner membrane (145). The functional UCP2 is thought to be a homodimer (146), in which the third matrix side loops of each monomer contain a purine nucleotide binding domain, which regulates proton translocating activity (146). Expression of UCP2 mRNA is more-or-less ubiquitous (123) and includes pancreatic islets (139;147). Interestingly, UCP2 mRNA expression does not always correlate with that of the protein (148). It is possible that UCP2 gene expression is partially controlled at the transcriptional level but UCP2 protein expression is modulated at the translational level (148).

UCP2 is a negative regulator of ATP generation (139). Theoretically, the mitochondrial electrochemical proton gradient, generated as electrons pass down

the respiratory chain, is the major potential energy source for ATP synthesis. The mitochondria have five respiratory enzyme complexes localized to the inner membrane. Complexes I, II and IV (NADH-dehydrogenase, succinate-coenzyme Q dehydrogenase and cytochrome c oxidase, respectively) pump protons out to the mitochondrial space while the last complex; called  $F_0$ - $F_1$  ATP synthase (149), lets the protons move back to the mitochondrial matrix and generate ATP as a product (150). At least three protons are needed to pass through the  $F_0$  subunit of ATP synthase to synthesize one ATP.  $H^+$  fluxes through the  $F_0$  subunit back to the matrix, leading to rotation of the  $F_1$  subunit and inducing a conformational change (149). The coupling of substrate (glucose, fatty acid) oxidation to ATP synthesis from  $ADP + Pi$  is called oxidative phosphorylation. Alternatively, to protons re-entering the matrix through ATP synthase, they could flux through uncoupling proteins without producing ATP. As a result, ATP synthesis is diminished. Ordinarily, these uncoupling pathways accelerate respiration and overcome the limitation of ATP synthase capacity, such that the overall effect of uncoupling on cellular ATP is small.



**Figure 1-9:** UCP2 functions as an ATP regulator in cellular respiration. Generally, in a coupled state, H<sup>+</sup> re-enters the mitochondrial matrix through ATP synthase to generate ATP. Alternatively, in uncoupled state, H<sup>+</sup> can leak back to the mitochondrial matrix via the uncoupling protein (UCP) without ATP formation (taken with permission from (151)).

There are two mechanisms proposed to explain the uncoupling process. The first mechanism is based on the gate domain and pore domain structure. These two domains of the uncoupling proteins allow  $H^+$  to pass through. Carboxyl groups of fatty acids in the mitochondrial inner membrane participate in proton transport by providing  $H^+$  buffering capacity (152) while UCP mediates proton translocation by functioning as an ion channel. The second mechanism is based on the flip flop model (153). In this model, UCP allows fatty acids in the mitochondrial inner membrane to function as a cycling protonophore. Fatty acid anions are transported by UCP from the matrix side to the intermembrane space. The anions then combine with protons to become electrically neutral and flip back through the membrane, releasing the proton into the matrix (153).

To date, the hypothesized functions of UCP2 are as a thermoregulator (154), a metabolic regulator (155) or part of the innate defense against infection (156). Because the main topic of this study is the effect of fatty acids on insulin synthesis and processing, the following section will therefore focus on UCP2 function as a metabolic regulator.

UCP2 mRNA expression in white adipose tissue (WAT) is elevated after excessive fat intake (157;158). Five day caloric restriction associated with elevated plasma free fatty acids induces significant increases in adipose UCP2 mRNA (147). Moreover, in response to a diet high in fat content, an obesity resistant mouse strain doubled their UCP2 mRNA expression in WAT, while an obesity prone strain only slightly increased UCP2 mRNA (123). In pancreatic islets, chronic culture of islets with free fatty acids induces UCP2 mRNA expression

(117;139;159). Both unsaturated and saturated fatty acids induce UCP2 mRNA transcription in tissue-specific fashion. In addition to white adipose tissue (123;160) and islets (117;139), high fat diet also increases UCP2 mRNA in liver (161) but no change is seen in type IIA (oxidative-glycolytic type) skeletal muscle and brown adipose tissue (160). In contrast to abundant evidence of free fatty acid induced UCP2 mRNA expression, only a few studies specifically reported increased expression of UCP2 protein. Joseph et al. (141) showed an elevation of UCP2 protein in 48 h palmitic acid (PA)-cultured islets. Medvedev et al (162) also reported a two fold elevation of UCP2 protein in 48 h oleate exposed INS-1 cells, but an internal control was not mentioned in this study. Likewise, UCP2 mRNA and protein are shown to be increased after chronic exposure of INS-1 cells to oleic acid (159;163). High fat diet increased UCP2 by two folds in hyperglycaemic GK rats concomitant with impaired insulin secretion in response to glucose, whereas when this hyperglycaemia was normalized by insulin administration, high fat no longer had an effect on islet UCP2 expression (164). Taken together, these data suggest that UCP2 may play a role in lipid handling or substrate partitioning.

### **The relationship of UCP2 with glucose stimulated insulin secretion**

Insulin secretion in response to glucose is dependent on ATP availability. Patients with mitochondrial DNA mutations exhibit impaired beta cell function and overt diabetes (165). In addition, blockade of mitochondrial metabolism inhibits insulin secretion (166). Moreover, GSIS is impaired in diabetic patients (167). Overexpression of UCP2 in clonal beta cell lines (168;169) and rodent islets (139)

suppresses GSIS and diminishes the ability of glucose to increase cellular ATP content (139). Conversely, UCP2 overexpressing islets of Zucker diabetes rats have a greater ATP:ADP ratio and improved GSIS (170). Chronic exposure to high glucose or high fatty acid also induces UCP2 protein (171) as well as reducing insulin release in response to glucose. ATP-dependent potassium ( $K_{ATP}$ ) channel activity is inhibited by a high glucose challenge, leading to  $K^+$  accumulating inside beta cells and consequently stimulating insulin secretion. In UCP2-overexpressing islets, high glucose failed to significantly suppress  $Rb^+$  efflux (a  $K^+$  tracer) compared with low glucose, showing that control of  $K^+$  flux by metabolism was impaired (139). As a result,  $K_{ATP}$  channels remain open and insulin secretion is inhibited.

In contrast to overexpression models, UCP2 knockout (-/-, KO) mice have a greater intra-islet ATP content (172) and ATP/ADP ratio (141) compared to WT mice. Moreover, UCP2 KO islets have enhanced glucose-stimulated increases in the ATP/ADP ratio (141). When UCP2 KO islets are “rescued” with adenovirally transfected UCP2, the enhancement of glucose-stimulated increases in ATP/ADP is lost (141). As mentioned earlier, GSIS is impaired in UCP2 transfected islets, in contrast, insulin secretion is elevated *in vivo* and *in vitro* in UCP2 KO mice (172). Given that UCP2 is a negative regulator of insulin secretion and that relatively small changes in UCP2 levels significantly impact on insulin secretion, it is hypothesized that increased UCP2 expression (or activity) in obesity might be an important link between obesity and the development of type2 diabetes (172). UCP2 mRNA and UCP2 protein were found to be markedly increased in islets of *ob/ob*

mice, a model of obesity-induced type 2 diabetes (172). All these studies suggest a role for UCP2 in the regulation of insulin secretion in response to glucose, particularly when free fatty acids are elevated.

### **The effect of UCP2 expression on insulin synthesis**

To date there is little known about the effect of UCP2 on proinsulin synthesis and processing. The only information is that higher preproinsulin mRNA is detected in UCP2 overexpressing islets of Zucker diabetic rats (170) but a causal relationship has not been established. Fatty acid exposure is reported to reduce beta cell insulin content (137), while markedly elevating proinsulin in beta cells. In addition, SPC1/3 and SPC2 protein expression are reduced without any change in their mRNA levels (137). Chronic exposure to palmitate inhibits preproinsulin mRNA expression in isolated rat islets (131). Proinsulin synthesis and processing are dependent on ATP, so it is possible that factors affecting ATP generation, such as UCP2, could regulate proinsulin synthesis and processing. However, the relationship between fatty acids, UCP2 and proinsulin synthesis and processing is unexplored.

### **Objectives of this study**

In this thesis, it is hypothesized that high fatty acid conditions will induce impairment of GSIS and proinsulin synthesis and processing, associated with the induction of UCP2 expression and reduction of ATP synthesis. To test this hypothesis three studies were performed. The first objective was to show that

UCP2 up-regulation could reduce GSIS along with proinsulin transcription and processing. Therefore, beta cells were transfected with UCP2 to induce overexpression and allow direct comparisons of GSIS, insulin gene transcription and processing in control and UCP2-induced conditions. The second objective was to replicate conditions in which fatty acids had previously been shown to induce UCP2 protein expression (141) and specifically examine insulin gene transcription and proinsulin processing. The third objective was to determine if increasing fat consumption *in vivo* would up-regulate UCP2 expression and induce changes in GSIS, insulin gene transcription and proinsulin processing.

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## CHAPTER 2

### THE EFFECT OF UCP2 OVEREXPRESSION ON INSULIN SYNTHESIS, PROCESSING AND SECRETION

#### Abstract

Two cardinal features found in diabetic patients are impaired glucose stimulated insulin secretion (GSIS) and hyperproinsulinaemia. Moreover, an increased proinsulin to insulin ratio in apparently healthy individuals predicts future development of type 2 diabetes but the causes of increased release of proinsulin have yet to be elucidated. It is well recognized that GSIS and proinsulin processing to mature insulin depends on ATP availability. UCP2 is a negative regulator of ATP production and its expression is elevated in diabetic models. The amount of ATP a cell is able to generate is inversely proportional to UCP2 expression. Therefore it is plausible that the defective insulin secretion and proinsulin processing found in diabetic patients might result at least partially from increased expression of beta cell UCP2. To test this hypothesis, we measured insulin secretion and ATP content in UCP2 overexpressing rat insulinoma cell lines (INS-1 cells) in addition to insulin mRNA and protein expression and proinsulin processing capacity. This study demonstrated that UCP2 induction reduced ATP availability inside beta cells, consequently impairing GSIS. UCP2 increased insulin mRNA and protein expression but, under prolonged aggressive stimulation, UCP2 overexpression could impair proinsulin processing. We conclude that under conditions where the demand for insulin secretion by beta-cells is increased, up-

regulation of UCP2 expression contributes to defective conversion of proinsulin to insulin, which could result in hyperproinsulinemia.

## Introduction

Type 2 diabetes mellitus is defined by defects in both insulin secretion and function (1). The first detectable abnormality in type 2 diabetic patients is the inability of insulin sensitive tissues to respond to normal levels of circulating insulin, called insulin resistance. In the beta cell, loss of glucose-stimulated insulin secretion (GSIS) (2-4) is associated with changes in oscillatory insulin secretion (5). Multiple changes in beta cell biochemistry are likely to cause the impairment in glucose responsiveness (6,7). Previous studies have identified uncoupling protein-2 (UCP2) as a contributor to beta cell dysfunction (8-11).

UCP2 is an inner mitochondrial membrane protein expressed widely in many tissues including pancreas (12;13). UCP2 transports either unprotonated free fatty acids (14) or protons (15) to dissipate the proton motive force and consequently reduce the potential energy available for conversion of ADP to ATP (16). Notably, the amount of ATP a cell is able to generate is inversely proportional to UCP2 expression (8;17). Adenovirally mediated overexpression of UCP2 in isolated pancreatic islets leads to lower ATP content (8) and UCP2 knockout mice have greater ATP concentrations in islets (10). The reduction in beta cell ATP impairs closure of ATP-dependent K channels to reduce GSIS (8). However, because of the importance of ATP as a signalling molecule in the beta cell (18), additional aspects of metabolism-signalling coupling are likely to be affected.

As mentioned above, both proinsulin biosynthesis and conversion of proinsulin to insulin depend on ATP availability (19). Granule acidification (19)

and uptake of calcium (20) both rely upon ATP-dependent ion pumps. It has been proposed that intracellular energy homeostasis is an important determinant of insulin biosynthesis and processing (21). In a rat model of diabetes, the proinsulin to insulin ratio was 2-fold greater than in nondiabetic rats (22). In humans there is a wealth of evidence that proinsulin secretion is elevated in type 2 diabetic patients (23). Moreover, an increased proinsulin to insulin ratio in apparently healthy individuals predicts future development of type 2 diabetes (24-26) but the causes of increased release of proinsulin have yet to be elucidated. It is plausible that the defective proinsulin processing found in diabetic patients might result at least partially from increased expression of beta cell UCP2. To test this hypothesis, we measured insulin secretion and ATP content in UCP2 overexpressing INS-1 cells and correlated them with insulin gene transcription and processing capacity.

## **Material and Methods**

### **Cell culture**

INS-1 cells were cultured overnight in RPMI 1640 medium (Fisher, Ottawa, Canada) with 11.1 mM D-glucose, 10 mM HEPES, 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, 1 mM sodium pyruvate and 50  $\mu$ M 2-mercaptoethanol. Cells were plated in 24 well plates (250,000 cells/well) and cultured at 37°C in a humidified 95% air-5% CO<sub>2</sub> atmosphere for 24 h before conducting UCP2 plasmid transfection. For experiments in which high K<sup>+</sup> conditions were required, KCl up to 20 mM was added to RPMI 1640. Osmolality was less than 300 mOsm/kg after adding KCl. The day after transfection, cells were cultured in either regular K<sup>+</sup> RMPI or high K<sup>+</sup> RMPI for 24 h before conducting experiments.

### **UCP2 transfection**

Plasmid (pcDNA 3.1, Invitrogen, Burlington, Canada) containing the full length cDNA sequence for human UCP2 (8) was transfected into overnight cultured INS-1 cells at a concentration of 0.375  $\mu$ g/well using lipofectamine (Invitrogen, 1:3, DNA:lipofectamine). Transfected INS-1 cells were then incubated for 6 h in Opti-MEM (Invitrogen) before adding RPMI with 20% FBS. For control transfections, INS-1 cells were incubated 6 h in Opti-MEM solution without lipofectamine and UCP2 DNA before adding RPMI with 20% FBS. After 24 h, both control and UCP2 transfected cells were incubated for another 24 h in either

RPMI with 10% FBS or RPMI supplemented with 20 mM KCl and 10% FBS, before using in further experiments. In all cases, the RPMI contained 5.5 mM glucose.

### **Insulin secretion and insulin content assay**

INS-1 cells cultured 48 h after UCP2 transfection were then washed and preincubated twice for 30 min in a modified Krebs Ringer bicarbonate buffer (KRB, 115 mM NaCl, 5.0 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.0 mM MgCl<sub>2</sub>, 24 mM NaHCO<sub>3</sub>, and 10 mM HEPES, pH 7.4) with 0.1% bovine serum albumin (BSA). After a third brief wash, the cells were incubated for 2 h in KRB containing 2.8, 11 or 22 mM glucose. Following incubation, the KRB was transferred to microcentrifuge tubes and the adherent cells were extracted with 3% acetic acid, with both fractions frozen at -20° C until assayed for insulin. Insulin secretion in response to glucose was quantified as a percent of total beta cell insulin content using a radio-immunoassay (RIA) with rat insulin as standard. The antibody utilized in the RIA detects both insulin and proinsulin with similar affinity (C. Chan, unpublished data). Thus, the values of both the insulin release and cellular fractions were added to determine the proinsulin and insulin content. Content was normalized by the average cell number in each group. Cell number measurement was conducted in the parallel experiments in which numbers of cells were counted using a hemocytometer 48 h after transfection.

### **ATP content**

INS-1 cells were incubated exactly as described above for insulin secretion before measuring ATP content using ATPlite (Perkin Elmer, Woodbridge, ON), which is based on the production of light caused by the reaction of ATP with added luciferase and D-luciferin. Briefly, after trypsinization and washing in 0.1 M BSA-free phosphate buffered saline, pH 7.4 (PBS), one portion of INS-1 cells was lysed with mammalian lysis solution with agitation at 700 rpm for 5 min. Cells then were added to the substrate buffer, agitating again for 5 min. The samples were kept in the dark for 10 min before measuring luminescence on an I450 Micro Beta Scintillation and Luminescence detector (Perkin Elmer). The rest of the cell samples were measured for protein concentration using the Lowry method (Sigma Aldrich, Oakville, Canada) as the normalization.

### **Western blotting**

Proinsulin-insulin ratio – Total INS-1 cell lysates were prepared using 3% acetic acid. The protein concentration was determined using the Lowry method. Protein samples (100 µg of each cell lysate) were separated on 15% polyacrylamide gels under non-reducing conditions, then electrophoretically transferred onto nitrocellulose membranes (Trans-Blot, Bio-Rad Laboratories (Canada) Ltd, Missisauga, Canada), followed by blocking of nonspecific binding in 5% skim milk-1% Tween 20/PBS overnight at 4°C with agitation. The membranes were then incubated at room temperature with guinea pig anti-insulin (1:1000, gp06) for 1 h. After washing extensively, membranes were incubated for 1 h at

room temperature with anti-guinea pig IgG peroxidase (1:15,000, Sigma Aldrich). Specific signals were detected using an enhanced ECL Plus reagent according to the manufacturer's instructions (GE Healthcare, Piscataway, NJ). The intensity of reactive band was quantified using Kodak Image station 440 (Perkin Elmer). Subsequently, after stripping, membranes were incubated with goat anti C-peptide (1:1000, Linco, St. Charles, MI) followed by rabbit anti-goat IgG peroxidase (1:15,000, Sigma Aldrich) in order to resolve the identity of mature insulin versus proinsulin bands.

Prohormone convertase (SPC1/3) -- Cells were lysed with N-tris[hydroxymethyl] methyl-2-aminoethane-sulfonic acid (TES) buffer containing 0.1% sodium dodecyl sulphate (SDS), and aprotinin (1 µg/ml) and bestatin (10 µg/ml) as protease inhibitors. Total protein was separated by 12% SDS-polyacrylamide gel electrophoresis. The proteins were electrotransferred onto nitrocellulose membranes and blocked with 5% skim milk containing 1% Tween-20 in PBS for 45 min. After extensively washing, the membranes were incubated in anti-PC1/3 (SPC1/3-fusion protein antibody, SPC1/3/K51, a gift from Dr. N.P. Birch, Auckland, New Zealand, 1:1000) overnight at 4°C. Subsequently, the membrane was incubated in goat anti-rabbit IgG peroxidase (1:20,000, Sigma Aldrich.) for 1 h at room temperature. Specific signals were detected using the enhanced ECL Plus reagent. The protein loading was normalized using a housekeeping gene antibody (mouse anti-β-actin, 1:5000, Sigma Aldrich) and appropriate secondary antibody (anti-mouse IgG peroxidase, 1:5000, Sigma Aldrich).

ATP synthase enzyme -- The same protocol as that for SPC1/3 was used except anti-ATP synthase subunit  $\alpha$ , 1:2000, Molecular Probes, Eugene, OR) was used as the primary antibody and rabbit anti-mouse IgG peroxidase was used as the secondary antibody (1:5000, Sigma Aldrich).

Uncoupling protein 2 (UCP2) -- Total protein (100  $\mu$ g/sample) from cell lysates was separated by 12% SDS-polyacrylamide gel electrophoresis. Samples were subsequently transferred onto nitrocellulose membranes at 100 V for 1 h. To reduce non-specific signal, membranes were blocked in 5% skim milk in 1% Tween-PBS overnight at 4°C. Membranes then were incubated in goat anti-UCP2 antibody (1:2500, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C and rabbit anti-goat IgG peroxidase (1:10,000, Sigma Aldrich) for 2 h at room temperature. The visualized bands were revealed using enhanced ECL Plus reagent and quantified as described above.

#### **RNA extraction and cDNA synthesis**

Total RNA was extracted from  $10^6$  cells of either control or UCP2 overexpressing cells using TRIzol reagent (Invitrogen) according to manufacturer's protocol. Briefly, 1 ml of TRIzol was added directly to cells plated in 3.5 cm diameter wells and triturated by pipetting multiple times before transfer to a microcentrifuge tube. Cell lysate was incubated for 5 min at room temperature and then 200  $\mu$ l of chloroform was added. Lysate was shaken vigorously for 15 s, then incubated for 2 min and centrifuged at 12,000 rpm at 4°C for 15 min. Clear aqueous phase was collected, transferred to a new tube and mixed with 500  $\mu$ l

isopropanol. The mixture was incubated for 10 min, and then centrifuged at 12,000 rpm at 4°C for 10 min. The supernatant was discarded. The pellet was washed with 75% ethanol in DEPC treated water. The pellet was subsequently centrifuged at 9,000 rpm at 4° C for 5 min, then air dried for 15 min, dissolved in DEPC water and incubated 10 min at 65° C.

cDNA synthesis was carried out using the Cloned AMV First-Strand cDNA Synthesis kit (Invitrogen). RNA (1-5 µg) was added to the mixture containing 50 ng of random hexamer primer, 2 µl of 10 mM Deoxyribonucleotide triphosphate (dNTP), 4 µl of 5X cDNA Synthesis buffer, 1 µl of 0.1 M DTT, 1 µl or 40 U RNase OUT and 1 µl or 15 U of Clone AMV reverse transcriptase. DEPC treated water was added up to 20 µl in total volume. The reaction tube was transferred to a thermal cycler, and incubated sequentially at 25°C for 10 min followed by 50° C for 50 min. The reaction was terminated by incubating at 85° C for 5 min.

### Quantitative RT-PCR

All cDNA samples were amplified in PCR reactions using specific primers as follows: UCP2 Forward: 5'CAGCCAGCGCCCAGTACC3'; UCP2 Reverse: 5'CAATGCGGACGGAGGCAAAGC3'; (27) rat preproinsulinI Forward: 5'GGAACGTGGTTCTTCTACAC3'; rat preproinsulinI Reverse: 5'GGGAGTGGTGGACTCAG3'; (28) rat preproinsulinII Forward: 5'GACCAGCTACAGTCGGAAAC3'; rat preproinsulinII Reverse: 5'TCCACGATGCCCGCGTTCTG3'; (28)

(28)

18S Forward: 5'CTTGTCGCTCGCTCCTC3';

18S Reverse: 5'CTGACCGGGTTGGTTTGAT3' (29)

Real time PCR reactions were carried out using 1  $\mu$ l of cDNA (1 $\mu$ g/ $\mu$ l) in a solution containing 12.5  $\mu$ l of SYBR green (Invitrogen), 0.5  $\mu$ l of specific forward primer, 0.5  $\mu$ l of specific reverse primer and 10.5  $\mu$ l of de-ionized distilled water. Reactions were carried out with heating to 95°C for 10 min, followed by 40 cycles of melting at 95°C for 15 s, annealing at 53°C for 45 s and extension at 72°C for 20 s (for UCP2 and 18S), 40 cycles of melting at 95°C for 20 s, annealing at 55°C for 20 s and extension at 72°C for 30 s (for preproinsulin I), and 40 cycles of melting at 95°C for 20 s, annealing at 58°C for 20 s and extension at 72°C for 30 s (for preproinsulin II). Melt curve analysis was performed following cycling as a method of validation. The PCR products (expected sizes UCP2 -127 bp, rat preproinsulin I - 216 bp, rat preproinsulin II - 326 bp and 18S - 130 bp) were run on agarose gels to further verify that the PCR reaction generated the expected product. The expression of UCP2 and preproinsulin mRNAs was normalized to 18S for comparison of expression between treatment groups.

#### Statistical analysis

All the data are presented as means  $\pm$  SEM. Significant differences were determined using 2-way ANOVA with Bonferroni post tests unless otherwise stated. Differences were considered significant if  $P<0.05$  or better.

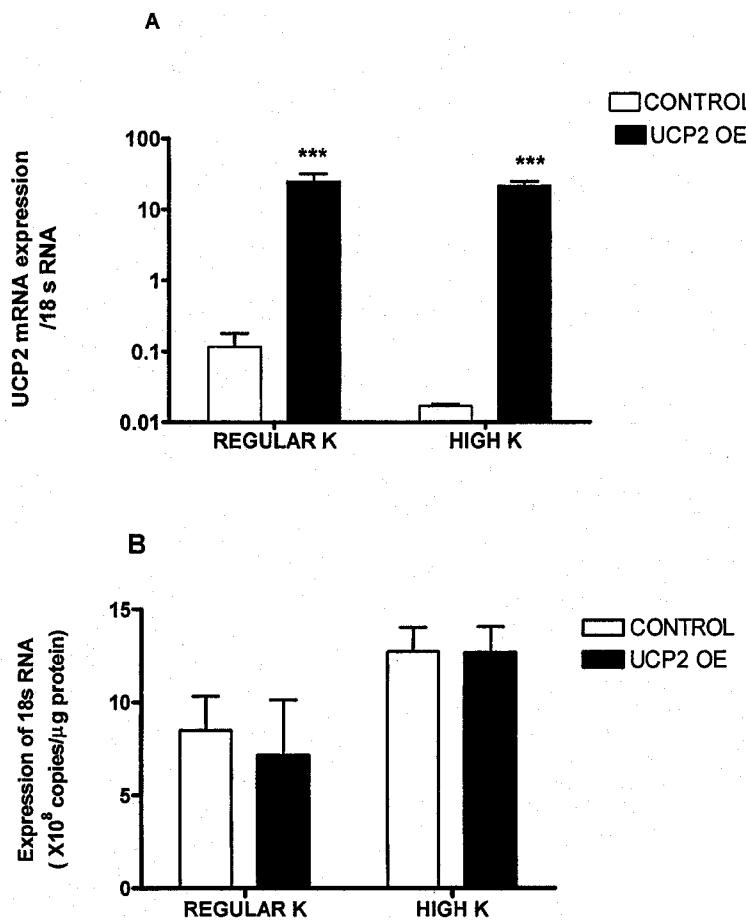
## Results:

### Quantification of UCP2 mRNA

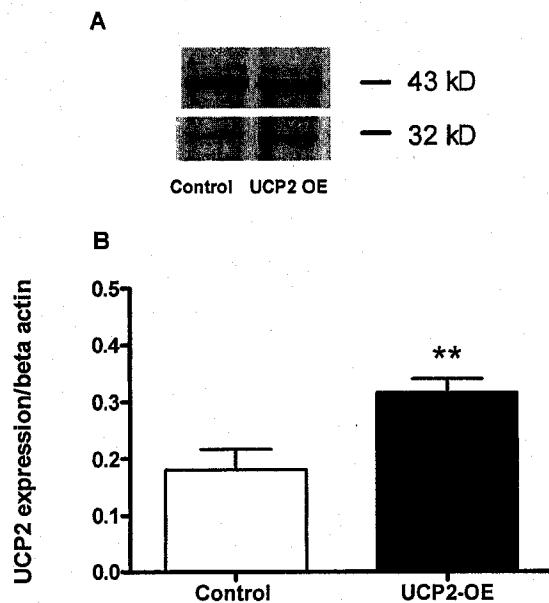
Real time PCR was performed to confirm the successful transfection of the UCP2-expressing plasmid in INS-1 cells. Figure 2-1A shows a 240- and 2000-fold increment of UCP2 mRNA expression relative to 18S rRNA in UCP2 overexpressing cells ( $0.12 \pm 0.06$  versus  $24.49 \pm 6.74$ ,  $P<0.0001$  for regular  $K^+$  medium;  $0.02 \pm 0.001$  versus  $21.38 \pm 2.98$ ,  $P<0.0001$  for high  $K^+$  medium; control and UCP2 overexpressing cells, respectively). 18S rRNA expression was not changed by UCP2 transfection (Figure 2-1B,  $p>0.05$ ).

### Western blotting of UCP2

Western blot analysis was performed in order to confirm the amount of UCP2 protein present in UCP2 overexpressing cells. There was a significant 1.78 fold ( $P=0.0076$  using Student's t-test) elevation of this protein in overexpressing cells cultured in regular potassium medium compared to control cells ( $0.18 \pm 0.04$ ,  $n=10$  and  $0.32 \pm 0.02$ ,  $n=9$ , control and UCP2 overexpressing cells respectively) (Figure 2-2).



**Figure 2-1.** Effect of transfecting a UCP2-expressing plasmid into INS-1 cells on UCP2 mRNA expression, measured by real time PCR. (A) UCP2 mRNA expression, relative to 18S rRNA expression in control and UCP2 overexpressing cells cultured with either regular  $\text{K}^+$  or high  $\text{K}^+$  medium. A significant increase ( $P < 0.0001$ ,  $n=10$ ) was detected in UCP2 overexpressing cells under both  $\text{K}^+$  conditions. (B) 18S rRNA expression in both control and UCP2 overexpressing cells. Similar expression was demonstrated in both groups ( $P > 0.05$ ,  $n \geq 11$  ).



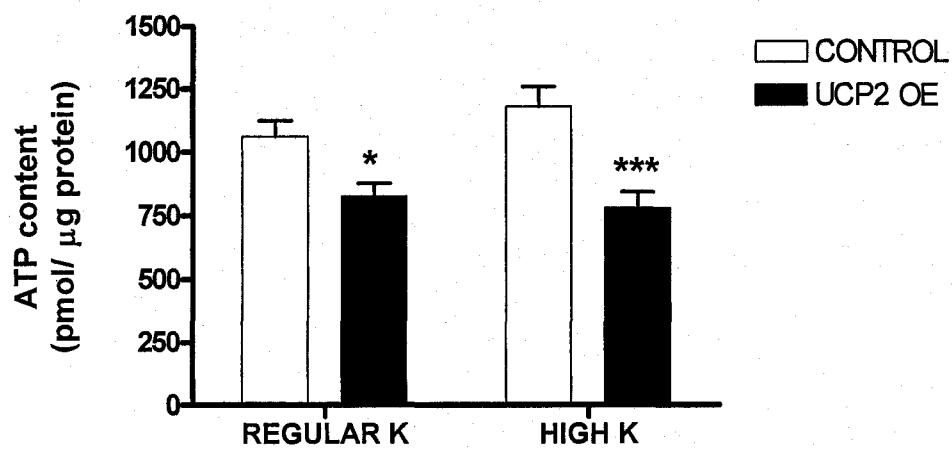
**Figure 2-2:** UCP2 protein expression in UCP2 overexpressing cells. A) Representative blot showing UCP2 (32 kD) compared to beta actin (43kD). B) Cells cultured in medium with regular  $K^+$  concentration had significantly increased UCP 2 expression (1.78 fold) 48 h after UCP2 transfection ( $P=0.0076$  using student t-test,  $n=9$ ) compared to control cells ( $n=10$ ).

### **ATP content**

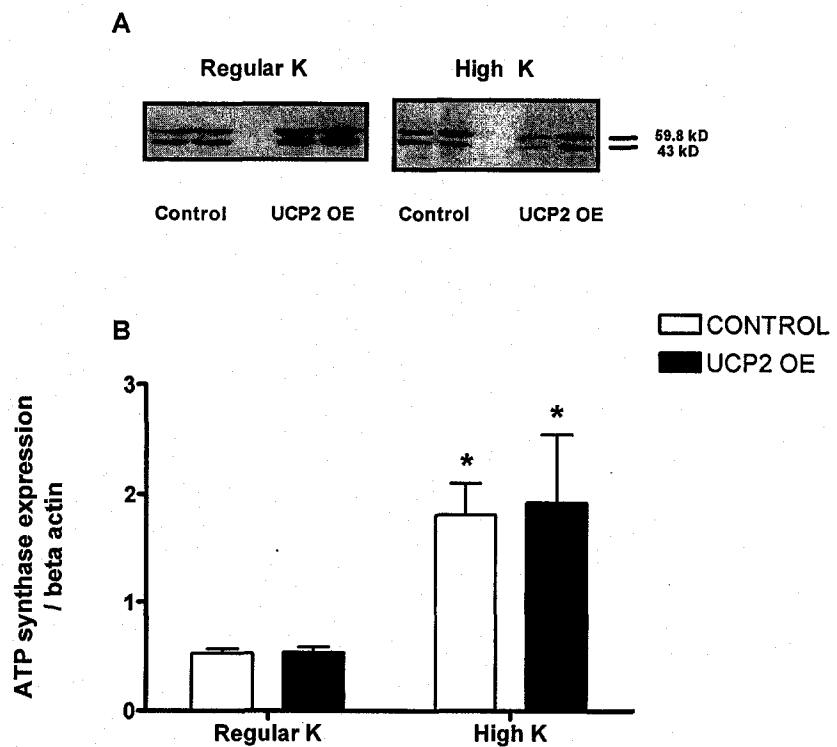
Because UCP2 mRNA and UCP2 protein are significantly increased in UCP2 overexpressing cells, we investigated whether UCP2 could function properly. Since the activity of UCP2 is well known to negatively regulate ATP content, the measurement of cellular ATP content was thus performed. The results are shown in Figure 2-3. Potassium concentration did not have an effect on intracellular ATP content, while ATP content inside UCP2 overexpressing cells both under regular (~ 22%) and high K<sup>+</sup> (~33%) was significantly reduced (P<0.05 and P<0.001 in regular K<sup>+</sup> and high K<sup>+</sup> respectively) compared to control cells under the same conditions.

### **ATP synthase**

To test if the reduction in ATP generation was due to a decline in ATP synthase expression, we performed western blotting. Similar protein expression of ATP synthase was detected in both control and UCP2 overexpressing cells cultured in the same K<sup>+</sup> medium (Figure 2-4), but 3.5 fold elevation of ATP synthase was found in high K<sup>+</sup> RPMI cultured cells ( P< 0.05 ) compared to cells cultured in regular K<sup>+</sup>.



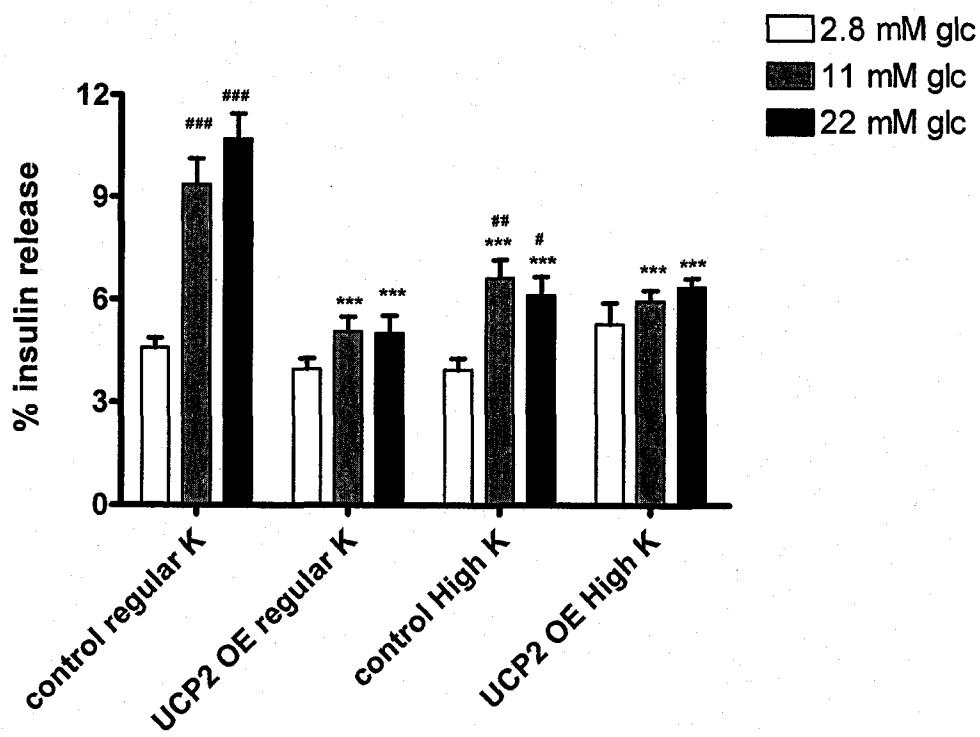
**Figure 2-3.** ATP content (pmol/μg protein) in control and UCP2 overexpressing cells. UCP2 overexpressing cells had significantly lower ATP content than control cells under both regular K<sup>+</sup> and high K<sup>+</sup> conditions (\*P<0.05 and \*\*\*P<0.001, n=24).



**Figure 2-4:** Comparison of ATP synthase in control and UCP2 overexpressing cells cultured in either 5.33 or 20 mM KCl. A) Representative blot showing ATP synthase (~60 kD) expression to beta actin (43kD). B) Both control and UCP2 overexpressing cells cultured in high K<sup>+</sup> had significantly higher ATP synthase than respective controls (P<0.05, n =8-9).

### **Glucose stimulated insulin secretion**

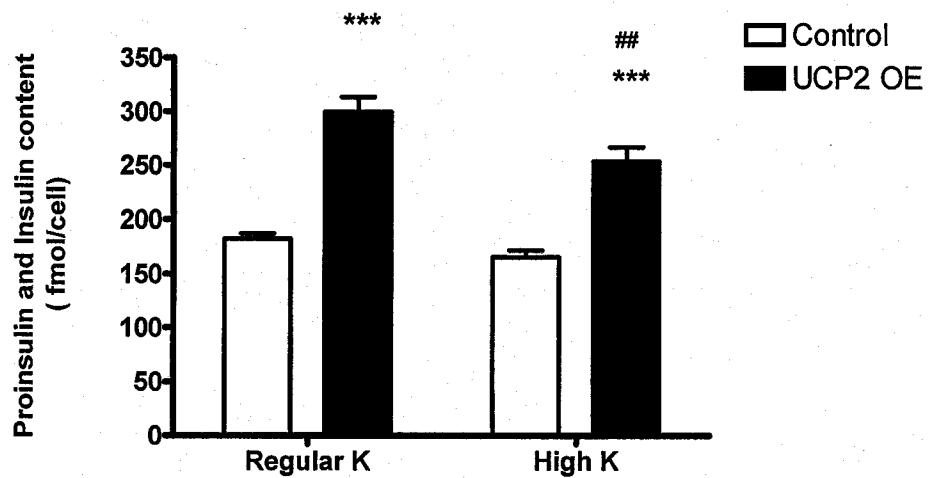
To confirm the inhibitory effect of UCP2 overexpression on glucose stimulated insulin secretion, control and UCP2 overexpressing INS-1 cells cultured in RPMI with regular  $K^+$  (5.3 mM) were incubated in various glucose concentrations (2.8, 11 or 22 mM) in KRB. Figure 2-5 shows insulin secretion as a % of total insulin content in response to glucose in control and UCP2 overexpressing cells. In control cells, insulin release increased significantly between 2.8 and 11 mM glucose ( $P<0.0001$ ). Insulin release was still significantly increased ( $P<0.01$  and  $P<0.05$  in 11 mM glucose and 22 mM glucose, respectively) in control cells cultured in high  $K^+$  RPMI compared to 2.8 mM glucose, but the magnitude of response was significantly smaller. In UCP2 overexpressing cells cultured in both regular  $K^+$  and high  $K^+$  RPMI, glucose did not stimulate any increase in insulin secretion, even at 22 mM glucose ( $p>0.05$ ).



**Figure 2-5.** Effects of UCP2 overexpression on insulin secretion in INS-1 cells cultured in either regular or high K<sup>+</sup> medium. Insulin release as a % of total cell insulin content was calculated. \*\*\*P< 0.001, \*P<0.01 compared with 2.8 mM glucose concentration in the same treatment, \*\*\*P< 0.001 compared with control cells cultured in regular K<sup>+</sup> at the same glucose concentration (n > 25 in each group).

### **Proinsulin and insulin content**

Inhibition of insulin secretion in response to a glucose challenge occurred in UCP2 overexpressing cells in either regular or high K<sup>+</sup> condition. We investigated whether the reduction in insulin secretion was due to the diminution of insulin content or inhibition of exocytosis. Therefore, assay of intracellular proinsulin and insulin content was carried out (Figure 2-6). An increase in proinsulin and insulin content was demonstrated in UCP2 overexpressing cells (62% and 53% increased in regular K<sup>+</sup> and high K<sup>+</sup>, respectively) compared to control cells in the same K condition ( $P<0.001$ ,  $n>90$ ). Moreover UCP2 overexpressing cells under regular K<sup>+</sup> ( $P<0.01$ ,  $n>90$ ) had more proinsulin and insulin content than those cultured in the high K<sup>+</sup> condition.

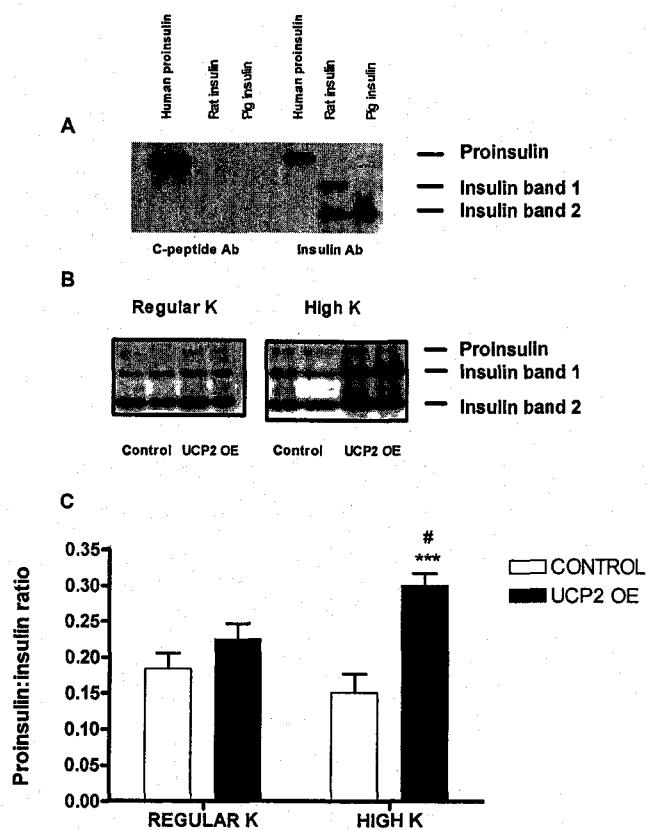


**Figure 2-6** Effect of UCP2 and K<sup>+</sup> on proinsulin and insulin content in INS-1 cells.

Following UCP2 transfection, proinsulin and insulin content of INS-1 cells increased significantly compared to respective controls (\*\*\* P< 0.001 compared to control cells under the same K<sup>+</sup> condition, n>90 in each group). UCP2 overexpressing cells cultured in high K<sup>+</sup> condition had less proinsulin and insulin content than those cultured in regular K<sup>+</sup> medium ( ## P<0.01).

### Proinsulin processing

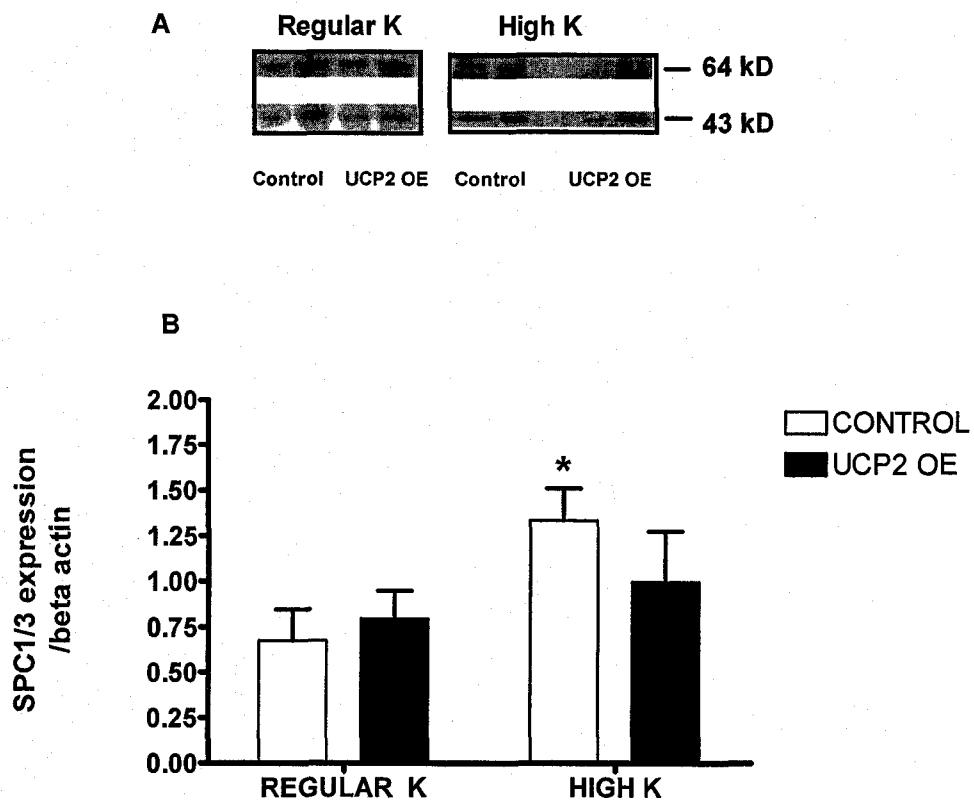
The impairment of insulin secretion in response to a glucose challenge is not the only outcome that depends on ATP. The processing of proinsulin to insulin also depends on having sufficient ATP. To determine whether proinsulin processing was impaired in UCP2 overexpressing cells, western blot analysis for proinsulin and insulin was performed. In addition to using guinea pig anti-insulin antibody for detecting both proinsulin and insulin bands, the proinsulin bands were differentiated from mature insulin bands using anti-C-peptide antibody (Figure 2-7A). Proinsulin:insulin ratios were calculated after measuring the intensity of proinsulin and insulin bands. The proinsulin:insulin ratios were similar ( $0.18 \pm 0.02$ ;  $n=11$ , control cells vs.  $0.22 \pm 0.02$ ;  $n=10$ , UCP2 overexpressing cells,  $p>0.05$ ) in both groups when the cells were cultured in regular RPMI (Figure 2-7B), but this ratio was significantly increased in the UCP2 overexpression group ( $0.30 \pm 0.02$ ,  $n=9$ ,  $P=0.0002$ ) compared to the control cells ( $0.22 \pm 0.02$ ,  $n=6$ ) when cells were cultured in RPMI supplemented with 20 mM KCl for 24 h (Figure 2-7C). Furthermore, the increase in proinsulin detected in UCP2 overexpressing cells under high KCl was also significantly greater than in UCP2 overexpressing cells cultured in regular medium ( $P<0.05$ ). The increase in this ratio implied an impairment in processing of proinsulin to mature insulin under conditions where the cells were chronically stressed to secrete insulin.



**Figure 2-7: Effect of UCP2 overexpression on the proinsulin:insulin ratio.** A) representative blots showing the distinction between proinsulin and insulin bands as detected by C-peptide antibody and insulin antibody. B) representative blots from control and UCP2 overexpressing cells were incubated in regular RPMI or high K<sup>+</sup> medium for 24 h. C) Similar proinsulin:insulin ratios were found in control and UCP2 overexpressing groups ( $P>0.05$ ) cultured in regular RPMI, while under supplementation with high KCl, a significantly increased proinsulin:insulin ratio was seen in UCP2 overexpressing cells ( $***P=0.0002$ ,  $n=9$ ) compared to control. Moreover, this elevation was also significantly higher than the ratio found in UCP2 overexpressing cells under regular conditions ( $^*P<0.05$ ).

### **Prohormone convertase-1 (SPC1/3) expression**

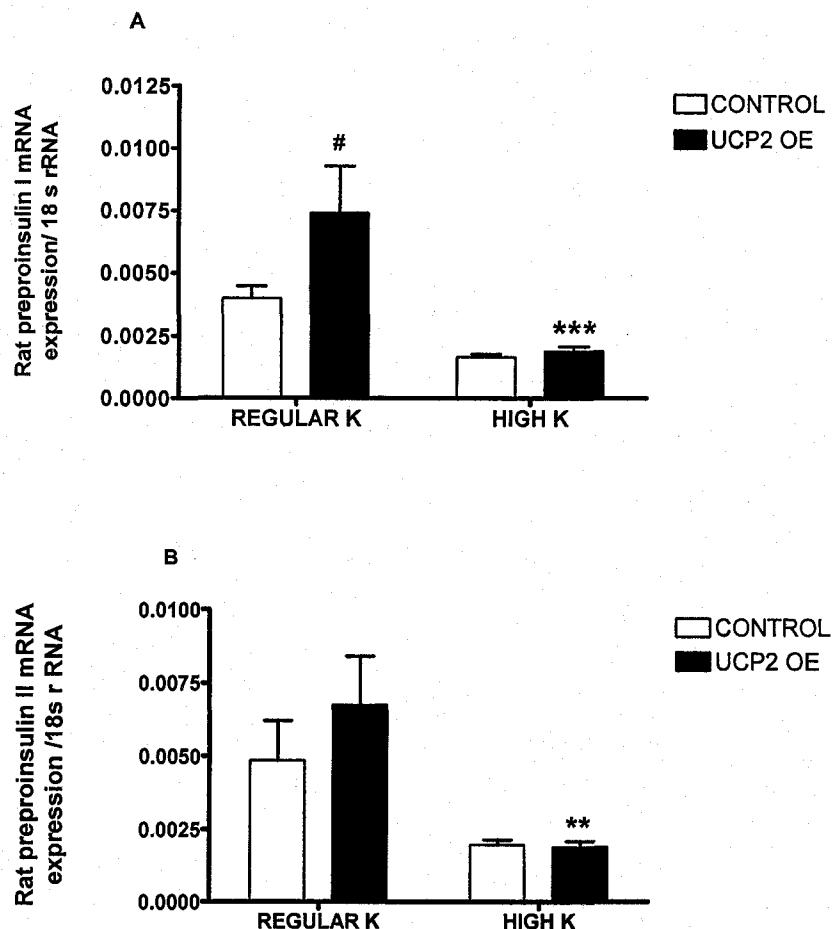
Proinsulin processing depends on the activity of prohormone convertase enzyme (SPC1/3), thus western blot analysis for SPC1/3 was performed and normalized to  $\beta$ -actin expression. SPC1/3 in both groups was similar ( $0.68 \pm 0.17$ , n=10 vs.  $0.80 \pm 0.15$ , n=11, control cells vs UCP2 overexpressing cells, respectively,  $p>0.05$ ) when cells were incubated in regular RPMI for 24 h (Figure 2-8A). Furthermore, when cells were stimulated to secrete more insulin using high  $K^+$ , SPC1/3 expression was still similar in both groups (Figure 2-8B). However, comparing regular with high  $K^+$  medium showed that SPC1/3 was increased in high  $K^+$  cultured control cells but not UCP2 overexpressing cells ( $1.34 \pm 0.18$ , n=9,  $p<0.05$ ).



**Figure 2-8:** Effects of UCP2 overexpression and  $K^+$  on SPC1/3 expression. A) Representative blot showing SPC1/3 band (64 kD) and beta actin (43 kD). B) prohormone convertase expression was similar in control cells and UCP2 overexpressing cells under regular  $K^+$  condition. When cells were stimulated to secrete insulin by culturing in high  $K^+$ , prohormone convertase was increased (\* $P<0.05$ ,  $n>9$ ) only in control cells supplemented with 20 mM KCl.

### Preproinsulin mRNA expression

Similar expression of prohormone convertase was demonstrated in UCP2 overexpressing cells regardless of the  $K^+$  concentration in which they were cultured, but the ratio of proinsulin:insulin was elevated only in the high  $K^+$  group. To substantiate whether the increased proinsulin:insulin ratio in UCP2 overexpressing cells supplemented with 20 mM KCl resulted from an alteration of proinsulin synthesis, measurement of preproinsulin mRNA was performed (Figure 2-9). Preproinsulin I mRNA was increased in UCP2 overexpressing cells ( $0.007 \pm 0.002$ ,  $n=10$ ) under regular  $K^+$  condition ( $^{\#} P<0.05$ ,  $n=10$ ) compared to control cells ( $0.004 \pm 0.0005$ ,  $n=10$ ). Preproinsulin mRNA I and II expression in UCP2-OE cells supplemented with high  $K^+$  were significantly less than those of UCP2-OE cells cultured in regular  $K^+$  medium ( $0.002 \pm 0.0002$ ,  $n=10$ ,  $P<0.001$  for preproinsulin I and  $0.002 \pm 0.0002$ ,  $n=10$ ,  $P<0.01$  for preproinsulin II ).



**Figure 2-9:** Effect of UCP2 overexpression and  $K^+$  on preproinsulin mRNA expression. Real time PCR was carried out in control and UCP2 overexpressing cells cultured under either regular or high  $K^+$  condition. A) preproinsulin I mRNA and B) preproinsulin II mRNA. Preproinsulin I mRNA was significantly increased in UCP2 overexpressing cells under regular condition ( ${}^{\#}P<0.05$ ,  $n=10$ ) compared to control cells under the same condition. UCP2 overexpressing cells under high potassium condition have less preproinsulin I ( ${}^{\#\#\#}P<0.001$ ) and II ( ${}^{\#\#}P<0.01$ ) mRNA compared to UCP2-OE cells in regular  $K^+$ .

## Discussion

In the present study, full length human UCP2 cDNA was transfected into INS-1 cells to investigate the contribution of UCP2 to regulation of proinsulin processing to mature insulin. The transfection procedure significantly increased UCP2 mRNA and protein in UCP2-OE cells. As expected, the intracellular ATP content was reduced in UCP2-OE cells. Although both ATP and GSIS were reduced in UCP2-OE cells, there was no effect of UCP2-OE on the proinsulin:insulin ratio unless the INS-1 cells were chronically depolarized and stimulated to secrete by increasing the K<sup>+</sup> concentration in the culture medium.

Impairments of GSIS and hyperproinsulinaemia are usually found in patients with type 2 diabetes mellitus. Moreover, many steps in insulin synthesis and secretion are dependent on ATP. We hypothesized that insufficient ATP availability could induce defects in insulin processing. UCP2 is a well known negative regulator of ATP synthesis (8) and has been implicated in the pathogenesis of beta cell defects in diabetes (18;30;31). Thus we investigated whether induction of UCP2 inside beta cells could induce an impairment of insulin synthesis, processing and secretion.

As expected, an elevation of UCP2 mRNA was detected in UCP2-OE cells. Moreover, this mRNA was translated into protein in UCP2-OE cells. As a consequence, a reduction of ATP content was observed in UCP2-OE cells, consistent with previous data from rat islets (8). ATP synthase expression was not changed by UCP2-OE, confirming that UCP2 is the negative regulator of ATP generation. Although a reduction of ATP content was also found in UCP2-OE

cells after culture in high  $K^+$  medium, significantly greater ATP synthase expression was detected in these cells compared to those cultured under regular  $K^+$  conditions. Moreover, ATP synthase expression was also increased in control cells after culture in high  $K^+$ , without affecting cellular ATP content. When pretreated with 70 mM KCl for 5 min, glucose-stimulated islets produced higher ATP (32). In contrast, ATP content of islets was significantly reduced when incubated with either low (3 mM) or high (20 mM) glucose containing 30 mM KCl for 60 min (33). Both studies showed an acute effect of high  $K^+$  on ATP content. In contrast, in the present study, when ATP content from INS-1 cells was measured after culture of islets in RPMI media supplemented with 20 mM KCl for 24 h, no effect was seen. Thus, it appears that elevated  $K^+$  changes ATP content inside the beta cell in a time-dependent fashion. In addition, it is well known that high  $K^+$  can increase insulin secretion from beta cells by depolarizing the plasma membrane to allow voltage-dependent  $Ca^{2+}$  channels to open. Insulin exocytosis also depends on ATP availability. Hence, the ATP requirement of INS-1 cells under high  $K^+$  condition would be increased. Consequently, the augmentation of ATP synthase expression might be a response to an increased ATP requirement. However, a dynamic investigation of ATP content as well as ATP synthase expression needs to be done in order to clarify the relationship between  $K^+$ , ATP synthase and ATP content.

As mentioned earlier, the absence of GSIS is one of the cardinal features of type 2 diabetes mellitus. Moreover, it has been shown that UCP2-OE in islets is associated with the impairment of GSIS (8;9;34). Ordinarily, glucose metabolism

within pancreatic beta cells leads to the increased ratio of ATP/ADP, which in turn inhibits  $K_{ATP}$  channel activity. Consequently, extracellular  $Ca^{2+}$  ions influx into beta cells to stimulate insulin exocytosis. As mentioned above, induction of UCP2 inside INS-1 cells reduces ATP content in UCP2-OE cells under both regular and high  $K^+$  conditions. As a consequence, the present study also demonstrated the impairment of GSIS in UCP2-OE cells under both  $K^+$  conditions. Interestingly, although GSIS was still detected in control cells under high  $K^+$  condition, the magnitude of the response was significantly reduced. Acutely, an increase in extracellular  $K^+$  availability increases GSIS (8;35;36). High extracellular  $K^+$  induces depolarization of beta cells (37) thereby increasing in  $Ca^{2+}$  influx and stimulating GSIS (38) even when  $K_{ATP}$  channels are still open. Moreover, high extracellular  $K^+$  restores the impaired GSIS induced by UCP2-OE (8). However, these investigations were performed acutely, unlike the investigation of chronic effects of high extracellular  $K^+$  on GSIS in the present study. Beta cell exhaustion occurs when beta cells are repeatedly or chronically exposed to stimulant; in this case, high  $K^+$  condition. In beta cell exhaustion, the releasable insulin granule pool becomes depleted. This phenomenon is a physiological state of beta cell refractoriness (39) and is readily reversible after discontinuation of the exposure (40). The reduction in GSIS that occurred in control cells under high  $K^+$  condition was likely due to beta cell exhaustion. In contrast to control cells, UCP2-OE cells cultured in high  $K^+$  had similar glucose-responsiveness as those cultured in regular RPMI. As mentioned earlier, GSIS is dependent on ATP availability and a reduction of ATP content was found in UCP2-OE cells under high  $K^+$  condition. In

addition, the beta cell exhaustion may occur in this condition as well. Taken together, the impairment of GSIS in UCP2-OE cells under high K<sup>+</sup> condition could be due to the effect of UCP2 accompanied by beta cell exhaustion.

In addition to GSIS, proinsulin synthesis and processing are other processes that depend on ATP availability. It was hypothesized that ATP insufficiency induced by the induction of UCP2 would reduce proinsulin synthesis and impair proinsulin processing in UCP2-OE cells. Gene transcription and translation are ATP-dependent processes. An important paradigm in transcription regulation is the influence of the chromatin organization and remodelling (41). Chromatin remodelling factors act by reformation of the nucleosome in an ATP dependent manner (42). Additionally, transcription initiation begins with the ATP-dependent phosphorylation of RNA polymerase II by transcription factor for polymerase II H (43). These two steps are stimulated under conditions of elevated ATP. Proinsulin is converted to mature insulin by prohormone convertases, with the process occurring inside the secretory vesicles. The activity of prohormone convertase depends on an optimal pH (19) and intragranular Ca<sup>2+</sup> concentration (20), which is dependent on ATP. Taken together, a reduction of proinsulin synthesis and impaired proinsulin processing could occur in ATP-insufficient conditions.

To test whether the reduction in cellular ATP induced by UCP2 could induce a reduction of proinsulin biosynthesis and impair processing measurements of preproinsulin mRNA, proinsulin and insulin content, proinsulin:insulin ratio and prohormone convertase expression were performed. In UCP2-OE cells under regular K<sup>+</sup> condition, preproinsulin I mRNA and proinsulin and insulin content

were increased significantly compared to control cells, while the proinsulin:insulin ratio and prohormone convertase expression were similar to control cells. These results suggested that proinsulin synthesis was normal or increased despite lower availability of ATP. The ATP concentration requirement for maximal mitochondrial DNA transcription activity is 0.5 to 1.0 mM (44). Moreover, the  $K_m$  for ATP in transcription initiation by RNA Pol II is 5-10  $\mu$ M (45), whereas the  $K_m$  for helicase activity is nearly 200  $\mu$ M (45). The ATP concentration in beta cell cytoplasm is estimated to be ~1 mM (46). Therefore the 20% reduction of ATP availability in this current study was likely not enough to affect preproinsulin gene transcription. However, the present study did not investigate dynamic changes in transcription and translation processes or directly examine insulin biosynthesis; further studies using pulse-chase methodology could be done to address this latter point (47). As mentioned above, impairment of GSIS occurred in UCP2-OE cells. Insulin is a positive auto-regulator of preproinsulin gene transcription and translation (48). Therefore, lower insulin secretion leads to a lower requirement for proinsulin synthesis.

To further investigate whether preproinsulin transcription and proinsulin processing in UCP2-OE cells would be altered under chronic stimulation of insulin secretion that was not dependent on glucose metabolism or UCP2 expression, INS-1 cells were cultured in medium containing an elevated  $K^+$  concentration. Under this aggressive stimulation, preproinsulin I and II mRNA and proinsulin and insulin content were lower whereas the proinsulin:insulin ratio was greater when compared to UCP2-OE cells cultured under regular  $K^+$  condition. Moreover, the

expression of prohormone convertase was similar in both groups. If the conversion of proinsulin to insulin was normal in UCP2-OE cells cultured in high  $K^+$  medium, then the proinsulin:insulin ratio should be similar to control cells. Altogether, under aggressive stimulation, the reduction in intracellular ATP induced by UCP2 was sufficient to reduce the conversion of proinsulin to insulin. We speculate that this occurred by disrupting the optimal regulation of pH and  $Ca^{2+}$  in the granule, which would reduce the activity of prohormone convertases. Because INS-1 cells were repeatedly stimulated by  $K^+$  under high K condition, it is likely that granules were no longer retained within the beta cell to allow sufficient time for processing in a sub-optimal environment.

Exposure to high  $K^+$  affected mRNA levels of the insulin genes I and II in this study.  $K^+$  dependent gene expression is reported in plants (49) but has not been reported in mammals. Despite unavailable information in mammals, it is possible that preproinsulin gene expression is dependent on  $K^+$ , because preproinsulin mRNA I and II were lower than in UCP2 overexpressing cells cultured in regular  $K^+$  medium. Moreover, there was a tendency to reduced preproinsulin I and II mRNA in control cells supplemented with high  $K^+$ . Prolonged exposure to high  $K^+$  of UCP2-OE cells led to an elevation in insulin secretion, whereas preproinsulin gene expression was decreased. After 24 h, insulin content and secretion was decreased significantly, consistent with beta cell exhaustion. However, control cells cultured under high  $K^+$  conditions maintained insulin content and increased the expression of prohormone convertase, thereby partially compensating for the greater demand for insulin secretion, while UCP2-OE cells under this condition

could not. If  $K^+$  dependent gene expression exists in beta cell, it is suggested that not all genes are dependent on  $K^+$ , as shown by null effect on expression of prohormone convertase. Moreover, it is possible that UCP2 could affect the expression of this enzyme and other proteins. Under the higher demand for prohormone convertase in high  $K^+$  condition, this enzyme was not increased in UCP2-OE cells, but was increased in control cells. Another possibility is that high  $K^+$  could affect expression of genes not examined in this study. Recently, the transcription factor PDX-1 was shown to influence proinsulin processing independent of glucose metabolism or changes in UCP2 mRNA expression (50). Further studies of the effects of  $K^+$  and UCP2 on gene transcription therefore need to be carried out.

The method used to quantify proinsulin and mature insulin in this current study was based on separating the proteins based on their isoelectric point followed by immunodetection with specific antibodies. Electrophoresis was run under non-reducing conditions in order to retain the integrity of disulphide bonds in the proinsulin and insulin molecules. Thus, peptide species could not be identified by molecular weight, necessitating the use of C-peptide antibody to distinguish between insulin and proinsulin. A more common method that can be used to identify proinsulin and insulin is high performance liquid chromatography (22;51). However, the method we used is an accepted strategy for quantifying processing for other hormones such as islet amyloid polypeptide (52). Moreover, this method can be combined with radiolabelling and immunoprecipitation to detect processing of newly synthesized proinsulin relative to total in the future.

In summary, the present study demonstrated that UCP2 could reduce ATP availability inside beta cells, consequently impairing GSIS. Moreover, under prolonged aggressive stimulation, UCP2-OE could impair proinsulin processing. Type 2 diabetic patients almost universally exhibit impaired GSIS and hyperproinsulinaemia. The findings presented in this study implicate UCP2 in the development of these defects in diabetes.

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## CHAPTER 3

### THE EFFECT OF HIGH FREE FATTY ACID ON INSULIN PROCESSING AND SECRETION

#### **Abstract**

Chronic saturated free fatty acid exposure is detrimental to beta cell function. Free fatty acids may affect insulin synthesis and processing but the mechanisms are unclear. We hypothesize that free fatty acids induce uncoupling protein 2, a negative regulator of ATP generation, thereby impairing insulin biosynthesis and processing. INS-1 cells were exposed to either 0.25 or 0.4 mM palmitic acid (PA) for 48 h before measuring glucose stimulated insulin secretion (GSIS), ATP content and proinsulin biosynthesis and processing. Uncoupling protein 2 expression was increased only in INS-1 cells treated with 0.4 mM PA. As predicted, basal insulin hypersecretion with impairment of GSIS was found after 0.4 mM PA treatment. ATP content was reduced by both 0.25 and 0.4 mM PA. The proinsulin and insulin contents were elevated only in 0.4 mM PA-treated cells. Likewise, the proinsulin: insulin ratio was increased by 0.4 mM but not 0.25 mM PA. It is thus concluded that 48 h exposure to 0.4 mM PA induced uncoupling protein 2 expression, which was associated with an increased proinsulin:insulin ratio, elevated total insulin content and impaired GSIS. However, because 0.25 mM PA also reduced ATP, the changes in insulin gene expression and processing were not clearly found to be ATP-dependent.

## Introduction

The coexistence of insulin resistance and beta cell dysfunction is widely recognized as a feature of type 2 diabetes mellitus. While insulin resistance is an early clinical sign related to obesity (1), beta cell dysfunction plays a major role in the disease progression (2-4). Beta cell function declines gradually prior to the presentation of elevated fasting plasma glucose levels (5-8). Circulating lipids are proposed to be important factors contributing to the loss of beta cell glucose responsiveness. Hyperlipidaemia, commonly found in diabetic patients, leads to the perturbation of insulin action in peripheral tissues and the impairment of glucose-stimulated insulin secretion (GSIS) (9). People experimentally exposed chronically (10;11), but not acutely (10-12) to high circulating fatty acids have reduced islet GSIS. Glucose stimulated insulin secretion *in vitro* is reduced by 40% in 48 h palmitate (PA) cultured islets (13).

Further studies have partially elucidated the so-called "lipotoxic" effect. ATP-dependent potassium ( $K_{ATP}$ ) channel activity, the key regulator of insulin secretion (14), is increased after fatty acid exposure (15). This channel is regulated by the ATP/ADP ratio. When the ATP/ADP ratio is increased, it inhibits the activity of  $K_{ATP}$  channels on the beta cell membrane, resulting in beta cell depolarization and insulin secretion. Therefore, an increase in channel activity would impair insulin secretion. Beta cell ATP is reduced in 72 h oleate cultured insulin-secreting MIN6 cells (16). Therefore, reduction of the ATP/ADP ratio in islets exposed to high concentrations of fatty acids might provoke the impairment of GSIS. Interestingly, the intra-islet ATP/ATP ratio is diminished in diabetic

human beta cells stimulated with high glucose (17). If reduction of ATP production is an important consequence of a lipotoxic environment, then other ATP-dependent functions of beta cells should also be affected, for example, proinsulin biosynthesis and processing to insulin. Hyperproinsulinemia is commonly detected in both non-diabetic obese subjects (18) and diabetic subjects (19-22). Glucose induced insulin biosynthesis is decreased and the proinsulin:insulin ratio is enhanced in long term fatty acid exposed islets (23). Also, glucose induced preproinsulin gene expression is inhibited by culture of beta cells in palmitate (24).

The conversion of proinsulin to mature insulin depends predominantly on the ATP-dependent activity of subtilisin-like prohormone convertases (SPC1/3 and SPC2), and reduced function of prohormone convertases has been implicated in models of diabetes. The expression of these enzymes was decreased in beta cells of streptozotocin treated rats (25). Moreover, mice lacking SPC2 had elevated circulating proinsulin and des-31,32-proinsulin levels with a reduction of mature insulin (26). Therefore, it might be hypothesized that the reduction of intracellular ATP induced by high fatty acids impairs insulin biosynthesis and processing as well as insulin secretion. However, the mechanism by which fatty acids reduce beta cell ATP still needs to be fully explained.

Uncoupling protein 2 (UCP2), a key regulator of ATP generation, is a mitochondrial inner membrane protein expressed in many tissues including pancreatic beta cells (27-29). In general, UCP2 functions to decrease metabolic efficiency by dissociating substrate oxidation in the mitochondrion from ATP synthesis. Hence, the level of intracellular ATP is diminished when UCP2 is

increased. UCP2 mRNA expression is induced by free fatty acids in non-islet tissues, such as skeletal muscle and adipocytes (30), islets (31;32) and clonal  $\beta$  cells (33;34). It is well recognized that UCP2 has a negative effect on beta cell function. GSIS is impaired in UCP2 overexpressing islets (29;31). In contrast, UCP2 knockout mice have higher insulin secretion compared to wild type counterparts (32;35). Thus, the reduction of intracellular ATP induced by free fatty acids may result from increased UCP2 expression. The aim of the present study was to investigate whether chronic free fatty acid exposure induces UCP2 expression, reduces intracellular ATP and, consequently, impairs insulin synthesis, processing and secretion.

## **Material and Methods**

### **Cell culture**

Rat insulinoma cells (INS-1 cells) were cultured overnight in RPMI 1640 medium (Fisher, Ottawa, Canada) with 11.1 mM D-glucose, 10 mM HEPES, 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, 1 mM sodium pyruvate and 50  $\mu$ M 2-mercaptoethanol (control medium). Cells were plated in 24 well plates (250,000 cells/well) and cultured at 37°C in a humidified 95% air-5% CO<sub>2</sub> atmosphere for 24 h. The culture medium was then changed to either control or fatty acid medium containing 0.25 mM or 0.4 mM PA in 1% fatty acid free bovine serum albumin (BSA) for a further 48 h.

### **Insulin secretion and insulin content assay**

INS-1 cells cultured 48 h in either control or fatty acid medium were washed and preincubated twice for 30 min in a modified Krebs Ringer bicarbonate buffer (KRB, 115 mM NaCl, 5.0 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.0 mM MgCl<sub>2</sub>, 24 mM NaHCO<sub>3</sub>, and 10 mM HEPES, pH 7.4) with 0.1% BSA. After the third wash, cells were incubated for 2 h in KRB containing 2.8, 11 or 22 mM glucose. Following incubation, the KRB was transferred to microcentrifuge tubes and the adherent cells were extracted with 3% acetic acid, with both fractions frozen at -20° C. Insulin secretion in response to glucose was quantified using a radio-immunoassay with rat insulin as standard and data were expressed as a percent of the total cell insulin content. The antibody utilized in the RIA detects both insulin and proinsulin

with similar affinity (C. Chan, unpublished data). Thus, the values of both the insulin release and cellular fractions were added to determine the proinsulin and insulin content. Content was normalized by the average cell number in each group. Cell number measurement was conducted in the parallel experiments in which numbers of cells were counted using a hemocytometer 48 h after exposure to high free fatty acid.

### **ATP content**

INS-1 cells were incubated exactly as described above before measuring ATP. ATP content was measured using ATPlite (Perkin Elmer, Woodbridge, Canada), which is based on the production of light caused by the reaction of ATP with added luciferase and D-luciferin. Briefly, after trypsinization and washing in 0.1 M BSA-free phosphate buffered saline, pH 7.4 (PBS), one portion of INS-1 cells was lysed with mammalian lysis solution with agitating at 700 rpm for 5 min. Cells then were added to the substrate buffer with agitation again for 5 min. The samples were kept in the dark for 10 min before measuring luminescence on an I450 Micro Beta Scintillation and Luminescence detector (Perkin Elmer). The rest of the cell samples were assayed for protein concentration using the Lowry method (Sigma Aldrich, Oakville, Canada) as the normalization.

### **Western blotting**

Proinsulin and insulin – Total INS-1 cell lysates were prepared using 3% acetic acid. The protein concentration was determined using the Lowry method.

Protein samples (100 µg) were separated on 15% polyacrylamide gels under non-reducing conditions, then electrophoretically transferred onto nitrocellulose membranes (Trans-Blot, Bio-rad, Hercules, CA), followed by blocking of nonspecific binding in 5% skim milk-1% Tween 20/PBS overnight at 4°C with agitation. The membranes were then incubated at room temperature with guinea pig anti-insulin (1:1000, a gift of R.A. Pederson, Vancouver, Canada) for 1 h. After extensive washing, membranes were incubated for 1 h at room temperature with anti-guinea pig IgG peroxidase (1:15,000, Sigma Aldrich). Specific signals were detected using an enhanced ECL Plus reagent according to the manufacturer's instructions (GE Healthcare, Piscataway, NJ). The intensity of reactive bands was quantified using Kodak Image station 440 (Perkin Elmer). Subsequently after stripping, membranes were incubated with goat anti rat C-peptide (1:1000, Linco, St. Charles, MI) followed by rabbit anti-goat IgG peroxidase (1:15,000, Sigma Aldrich) in order to resolve the identity of mature insulin versus proinsulin bands. The proinsulin:insulin expression ratio was calculated for each sample.

Prohormone convertase (SPC1/3) -- Cells were lysed with N-tris[hydroxymethyl] methyl-2-aminoethane-sulfonic acid (TES) buffer containing 0.1% sodium dodecyl sulphate (SDS), and aprotinin (4 µg/ml) and bestatin (5 µg/ml) as protease inhibitors. Total protein (100 µg/sample) was separated by 12% SDS-polyacrylamide gel electrophoresis. Then the proteins were electrotransferred onto nitrocellulose membranes and blocked with 5% skim milk containing 1% Tween-20 in PBS for 45 min. After extensive washing, the membranes were incubated in anti-SPC1/3 (SPC1-fusion protein antibody, SPC3/K51, a gift from

Dr. N.P. Birch, Auckland, New Zealand, 1:1000) overnight at 4°C. Subsequently, the membrane was incubated in goat anti-rabbit IgG peroxidase (1:20,000, Sigma Aldrich) for 1 h at room temperature. Specific signals were detected using enhanced ECL Plus reagent. The protein loading was normalized using a house keeping gene antibody (mouse anti- $\beta$ -actin, 1:5000 and anti-mouse IgG peroxidase, 1:5000, Sigma Aldrich).

ATP synthase -- The same protocol as that for SPC1/3 was used except anti-ATP synthase subunit  $\alpha$  (1:2000, Molecular Probes, Eugene, OR) was used as the primary antibody and rabbit anti-mouse IgG peroxidase was used as the secondary antibody (1:5000, Sigma Aldrich).

Uncoupling protein 2 (UCP2) -- Lysates were electrophoresed and transferred as described for SPC1/3 except that membranes were blocked overnight at 4°C. Specific UCP2 signal was acquired by incubating membranes in goat anti-UCP2 antibody (1:2500, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C, followed by rabbit anti-goat IgG peroxidase (1:10,000, Sigma Aldrich) for 2 h at room temperature.

#### **RNA extraction and cDNA synthesis**

Total RNA was extracted from  $10^6$  cells of either control or fatty acid exposed cells using TRIzol reagent (Invitrogen, Burlington, Canada) according to the manufacturer's protocol. Briefly, 1 ml of TRIzol was added directly to cells plated in 3.5 cm diameter wells and triturated by pipetting multiple times before transfer to a microcentrifuge tube. Cell lysate was incubated for 5 min at room

temperature and then 200  $\mu$ l of chloroform was added. Lysate was shaken vigorously for 15 s, then incubated for 2 min and centrifuged at 12,000 rpm at 4°C for 15 min. Clear aqueous phase was collected, transferred to a new tube and mixed with 500  $\mu$ l isopropanol. The mixture was incubated for 10 min and then centrifuged at 12,000 rpm at 4°C for 10 min. The supernatant was discarded. The pellet was washed with 75% ethanol in Diethylpyrocarbonate (DEPC) treated water. The pellet was subsequently centrifuged at 9,000 rpm at 4°C for 5 min, then air dried for 15 min, dissolved in DEPC water and incubated 10 min at 65°C.

cDNA synthesis was carried out using Cloned AMV First-Strand cDNA Synthesis kit (Invitrogen). RNA (1-5  $\mu$ g) was added to a mixture composed of 50 ng random hexamer primers, 2  $\mu$ l of 10 mM dNTP, 4  $\mu$ l of 5X cDNA synthesis buffer, 1  $\mu$ l of 0.1 M dithiothreitol (DTT), 40 U RNase OUT and 15 U of Clone AMV reverse transcriptase (RT). DEPC-treated water was added up to 20  $\mu$ l in total volume. The reaction tube was transferred to a thermal cycle and incubated sequentially at 25°C for 10 min followed by 50°C for 50 min. The reaction was terminated by incubating at 85°C for 5 min.

### Quantitive RT-PCR

All cDNA samples were amplified in PCR reactions using specific primers as follows:

UCP2 forward: 5'-CAGCCAGCGCCCAGTACC-3';

UCP2 reverse: 5'-CAATGCGGACGGAGGCAAAGC-3' (36)

rat preproinsulinI forward: 5'-GGAACGTGGTTCTTCTACAC-3';

rat preproinsulinI reverse: 5'-GGGAGTGGTGGACTCAG-3' (37)

rat preproinsulinII forward:5'-GACCAGCTACAGTCGGAAAC-3'

rat preproinsulinII reverse:5'-TCCACGATGCCGCGCTTCTG-3'

(37)

18S forward: 5'-CTTGCGCTCGCTCCTC-3';

18S reverse: 5'-CTGACCGGGTTGGTTTGAT-3' (38)

Real time PCR reactions were carried out using 1  $\mu$ l of cDNA (1 $\mu$ g/ $\mu$ l) in a solution containing 12.5  $\mu$ l of SYBR green (Invitrogen), 0.5  $\mu$ l of specific forward primer, 0.5  $\mu$ l of specific reverse primer and 10.5  $\mu$ l of de-ionized distilled water. Reactions were carried out with heating to 95°C for 10 min, followed by 40 cycles of melting at 95°C for 15 s annealing at 53°C for 45 s and extension at 72°C for 20 s (for UCP2 and 18S), 40 cycles of melting at 95°C for 20 s, annealing at 55°C for 20 s and extension at 72°C for 30 s (for preproinsulin I), and 40 cycles of melting at 95°C for 20 s, annealing at 58°C for 20 s and extension at 72°C for 30 s (for preproinsulin II). Melt curve analysis was performed following cycling as a method of validation. The PCR products (expected sizes UCP2 - 127 bp, rat preproinsulin I - 216 bp, rat preproinsulin II - 326 bp and 18S - 130 bp) were run on agarose gels to further verify that the PCR reaction generated the expected product. The expression of UCP2 and preproinsulin mRNAs was normalized to 18S for comparisons of expression between treatment groups.

### **Statistical analysis**

All the data are presented as means  $\pm$  SEM. Significant differences were determined using 1-way ANOVA with Bonferroni post-tests unless otherwise indicated. Differences were considered significant if  $P < 0.05$  or better.

## Results

### Glucose stimulated insulin secretion

To confirm the effect of fatty acid exposure on GSIS, control, 0.25 or 0.4 mM PA-treated INS-1 cells were incubated in various glucose concentrations (2.8, 11 or 22 mM) in KRB. Figure 3-1 shows insulin secretion as a percent of total insulin in response to glucose. In control cells and 0.25 mM PA-treated cells, insulin release increased significantly ( $P<0.0001$ ) between 2.8 and 11 mM glucose but had no further increase in response to 22 mM glucose. In the 0.4 mM PA-treated group, there was elevated basal insulin secretion ( $P<0.001$ ) but higher glucose failed to induce any further insulin secretion when compared to 2.8 mM glucose.

### ATP content

Because GSIS is ATP-dependent, we measured the effect of chronic fatty acid exposure on cellular ATP content. The results are shown in Figure 3-2A. The intracellular ATP content was significantly reduced ( $P<0.0001$ ) in both 0.25 and 0.4 mM PA- treated cells compared to control cells.

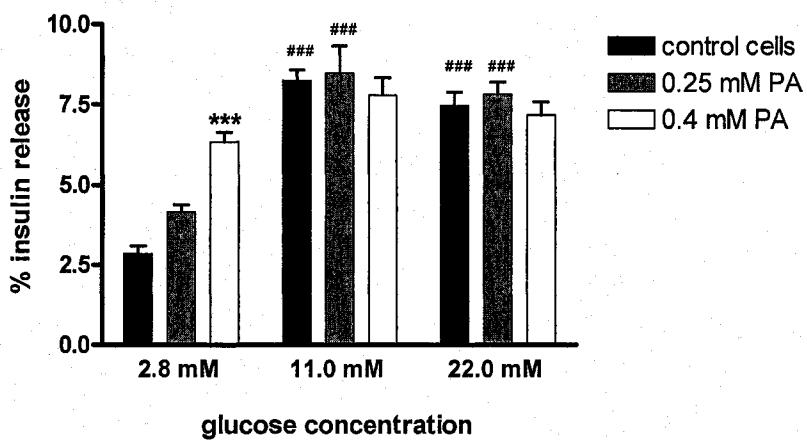
### ATP synthase expression

To assess whether the reduction in intracellular ATP was caused by an abnormality of ATP synthase expression, western blot analysis for detecting this

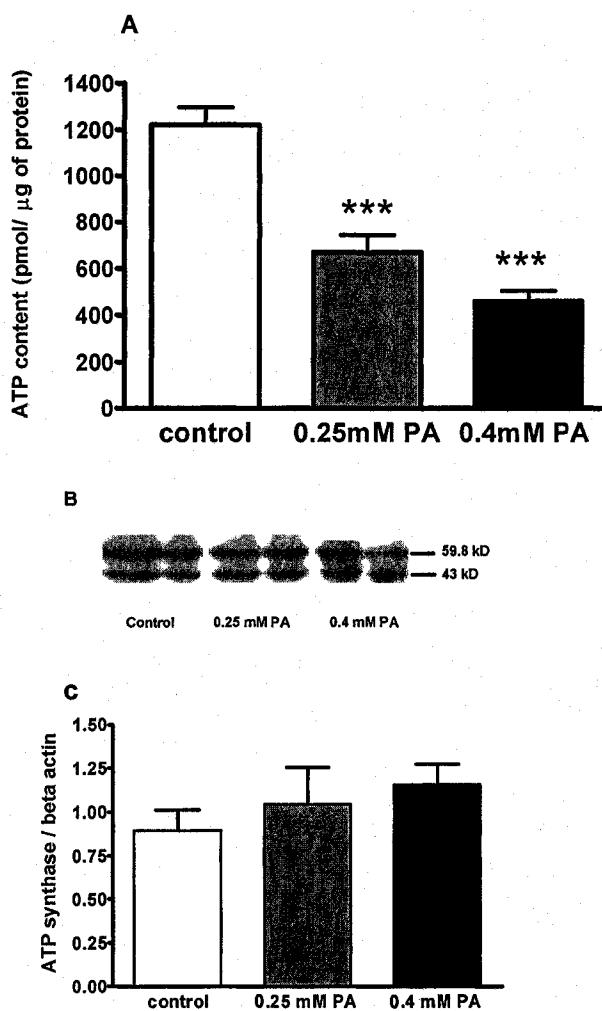
enzyme was carried out. All treatment groups had similar expression of ATP synthase (Figure 3-2B,C) after normalizing with beta actin ( $p>0.05$ ).

### **Proinsulin processing**

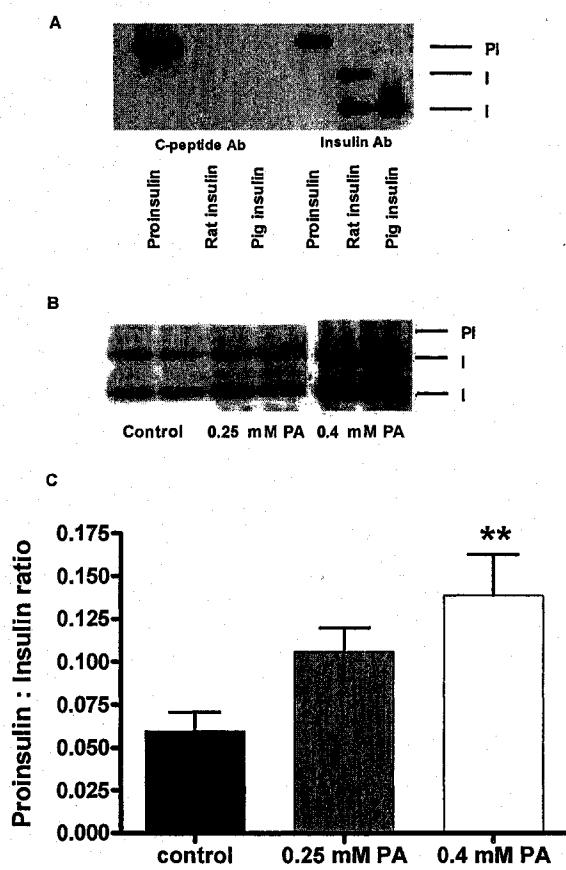
Not only is insulin secretion in response to a glucose challenge an ATP dependent event, but proinsulin processing also depends on ATP availability. To determine whether proinsulin processing was impaired in PA-treated cells, western blot analysis for proinsulin and insulin was performed. In addition to using guinea pig anti-insulin antibody for detecting both proinsulin and insulin bands, proinsulin bands were differentiated from mature insulin bands using goat anti-C-peptide antibody (Figure 3-3A). Proinsulin:insulin ratios were calculated after measuring the intensity of proinsulin and insulin bands (Figure 3-3B). Figure 3-3C shows that proinsulin:insulin ratios were increased in 0.4 mM PA-treated cells ( $0.06 \pm 0.01$ ,  $0.11 \pm 0.01$ ,  $0.14 \pm 0.02$  ( $P<0.01$ ) for control, 0.25 and 0.4 mM PA-treated cells, respectively).



**Figure 3-1.** Effect of culturing INS-1 cells for 48 h in high fatty acid on insulin secretion measured acutely over 90 min. Insulin release, expressed as a % of total cell insulin content, was calculated. \*\*\* $P<0.001$  compared with control cells and 0.25 mM palmitic acid (PA) treated cells, #### $P<0.0001$  compared with 2.8 mM glucose concentration by 2-way ANOVA with Bonferroni post tests.  $N\geq 30$  for all groups.



**Figure 3-2.** A) Intracellular ATP content in control, 0.25 and 0.4 mM palmitic acid (PA) treated cells. Both 0.25 and 0.4 mM PA treated cells had significantly reduced intracellular ATP content when compared to control cells. \*\*\*p<0.001, n=60 for all groups. B) Representative blot showing ATP synthase (~60 kD) and beta actin (43 kD) after 48 h exposure to control or fatty acid medium. C) Quantification of ATP synthase. Expression of ATP synthase, relative to beta actin, is similar in all treatment groups, p>0.05, n=10 for each.



**Figure 3-3.** Effect of culturing INS-1 cells in fatty acid on the proinsulin:insulin ratio, as an index of processing of hormone to its mature form. A) Representative blot showing control staining that allowed differentiation of proinsulin from insulin on the blots. Proinsulin cross-reacted with both C-peptide (left) and insulin antiserum (right) whereas mature insulin cross-reacted with only insulin antibodies (right). B) Representative blot showing the effects of culturing INS-1 cells in medium containing 0.25 or 0.4 mM PA, compared with control medium. C) 0.4 mM PA-treated cells had reduced proinsulin processing compared with control cells, \*\*p<0.01, n=9 for each group.

### **Prohormone convertase (SPC1/3) expression**

Proinsulin processing depends on the expression of prohormone convertase (SPC1/3), thus western blot analysis for SPC1/3 was conducted (Figure 3-4). SPC1/3 (relative to beta actin) was similar in all treatment groups ( $0.68 \pm 0.08$ ,  $0.67 \pm 0.07$  and  $0.926 \pm 0.160$  for control, 0.25 mM and 0.4 mM PA-treated cells, respectively,  $p>0.05$ ).

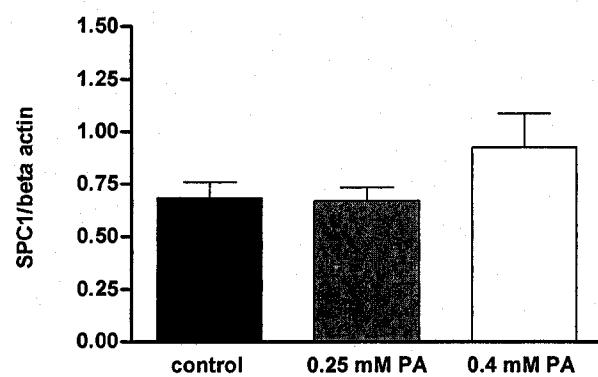
### **Proinsulin and insulin content**

In order to evaluate the effect of fatty acid treatment on total proinsulin plus insulin content per cell, both portions (release + total) of the samples from insulin secretion experiments were added together. The data are shown in Figure 3-5. After normalizing with cell number, proinsulin and insulin content in 0.4 mM PA-treated cells ( $0.43 \pm 0.02$  fmol/cell,  $n=93$ ) was significantly ( $p<0.001$ ) higher than both control ( $0.23 \pm 0.02$  fmol/cell,  $n=88$ ) and 0.25 mM PA-treated cells ( $0.23 \pm 0.01$  fmol/cell,  $n=91$ ).

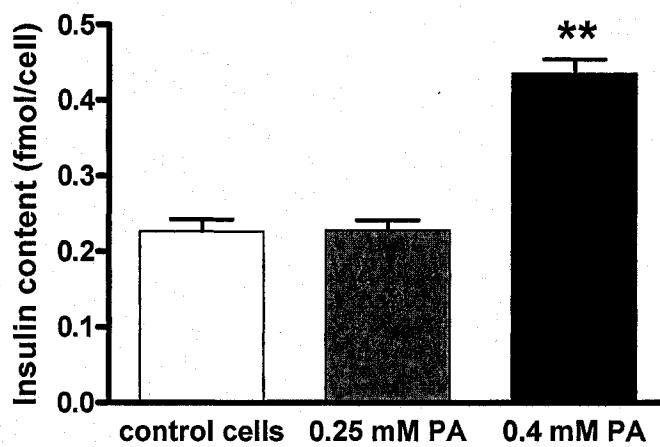
A



B



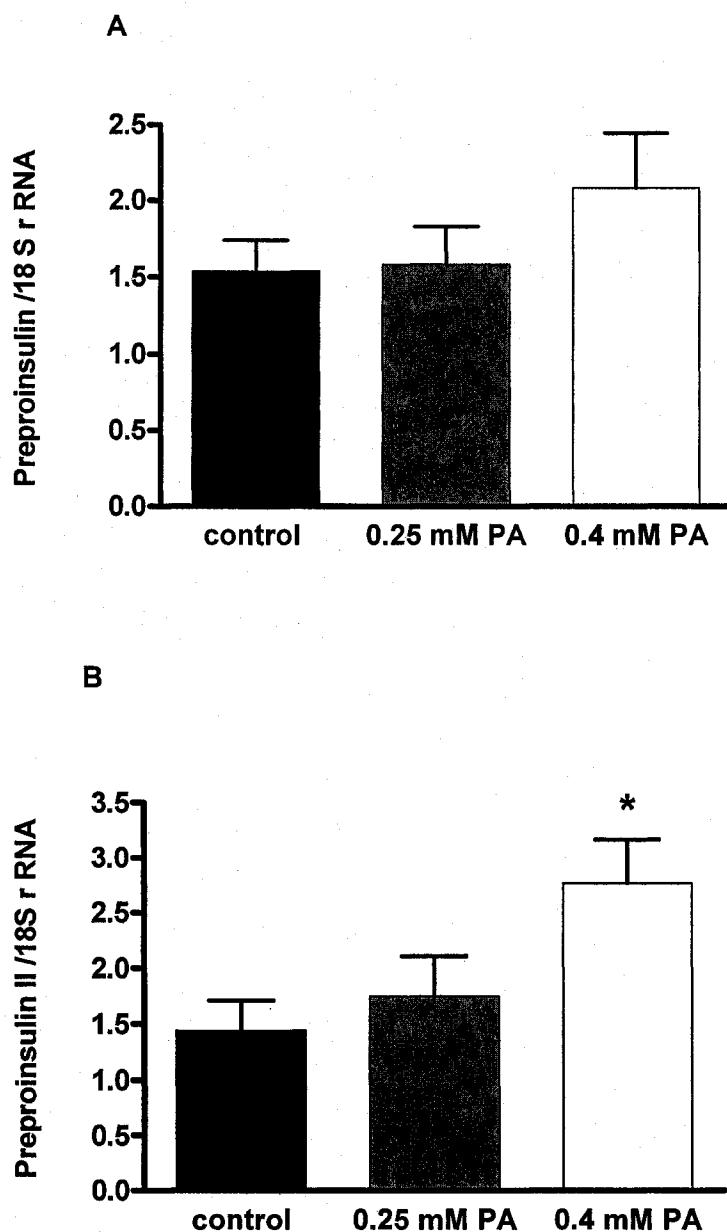
**Figure 3-4.** The effect of fatty acid exposure on prohormone convertase (SPC1/3) protein expression in control, 0.25 and 0.4 mM palmitic acid treated cells. A) Representative blot showing the effect of PA (0.25 or 0.4 mM) exposure of INS-1 cells on prohormone convertase (64 kD) relative to beta actin (43 kD). B) All treatment conditions had similar expression of this enzyme ( $p>0.05$ ,  $n=8$  for all groups).



**Figure 3-5.** Total proinsulin plus insulin content from control, 0.25 and 0.4 mM PA- treated groups. Proinsulin and insulin content of 0.4 mM PA-treated cells was significantly greater than control and 0.25 mM PA-treated cells (\*\*p<0.01, n≥88 for all groups).

### Quantitative PCR of preproinsulin mRNA

Higher proinsulin:insulin ratios and proinsulin plus insulin content were found in 0.4 mM PA-exposed INS-1 cells but the expression of prohormone convertase was similar in all groups. This situation might be caused either by a reduction in the activity of prohormone convertase or by an elevation of insulin synthesis. Thus quantitative PCR of preproinsulin mRNA was performed and normalized to 18S rRNA, the expression of which did not differ between groups (data not shown). Figure 3-6a shows similar expression of preproinsulin I mRNA in all groups. However, preproinsulin II mRNA, as shown in Figure 3-6b, was significantly increased in 0.4 mM PA-treated cells ( $2.76 \pm 0.48$ , n=10, P< 0.05) compared to control cells ( $1.44 \pm 0.27$ , n=10).



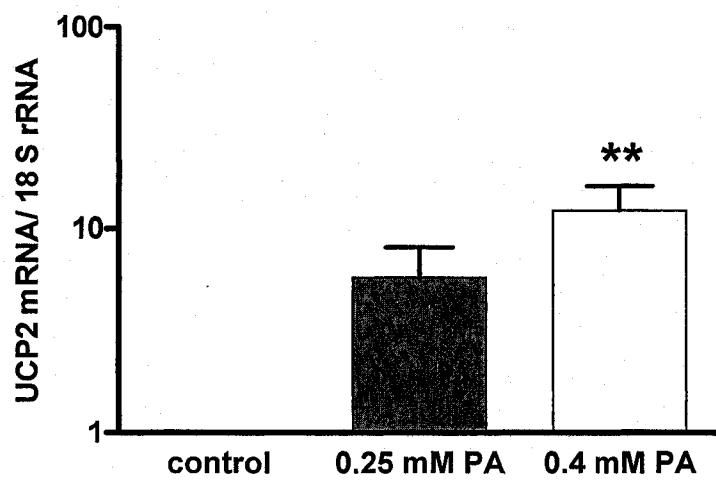
**Figure 3-6.** Relative expression of A) preproinsulin I and B) preproinsulin II mRNA in control and fatty acid treated cells. Preproinsulin I mRNA is similar in all groups ( $P>0.05$ ,  $n=10$  for each), but preproinsulin II mRNA is significantly higher in 0.4 mM PA-treated cells compared to controls ( $P<0.05$ ,  $n=10$  each).

### **Quantitative RT-PCR of UCP2 mRNA**

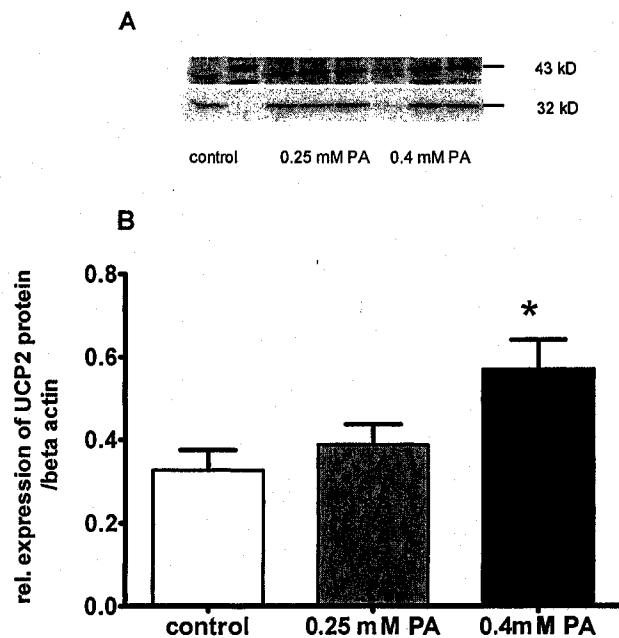
To test whether the impaired insulin secretion and reduced proinsulin processing in fatty acid exposed beta cells might be due to effects of UCP2, quantitative PCR was performed on mRNA from INS-1 cells exposed to control, 0.25 or 0.4 mM PA medium. There was a tendency for UCP2 mRNA to be increased by both concentrations of fatty acid, but this achieved significance ( $P<0.01$ ) only in the 0.4 mM PA-treated group (Figure 3-7).

### **Western blot analysis of UCP2**

To test whether UCP2 mRNA, which was elevated in 0.4 mM PA-treated cells, could be translated into protein, western blot analysis of UCP2 was carried out. Significantly ( $P<0.05$ ) higher expression of UCP2 relative to  $\beta$ -actin was detected in 0.4 mM PA-treated beta cells (Figure 3-8) when compared to control and 0.25 mM PA-treated groups.



**Figure 3-7.** The effect of fatty acid exposure on UCP2 mRNA expression. As indicated (\*\* $p<0.01$ ), 0.4 mM PA-treated INS-1 cells had significantly greater expression of UCP2 mRNA than control cells ( $n=9$  for all groups). The expression level of the control group was  $0.12 \pm 0.03$  relative to 18S rRNA.



**Figure 3-8.** The effect of free fatty acid exposure on the induction of UCP2 expression. A) Representative blot showing the relative expression of UCP2 ( 32 kD) to beta actin (43 kD) in control cells and PA-exposed cells. B) Significantly greater UCP2 ( $0.57 \pm 0.07$ , n=14) was detected in 0.4 mM PA-exposed INS-1 cells when compared to control cells ( $0.33 \pm 0.05$ , n=13).

## Discussion

The main hypothesis tested in this study was that a consequence of exposing beta cells to saturated free fatty acid (palmitic acid, PA) would be increased expression of UCP2, leading to lowering of cellular ATP content and contributing to a reduction in proinsulin processing. A secondary aim was to confirm that elevated UCP2 was associated with blunted GSIS. However, although a reduction in ATP generation was noted in both 0.25 and 0.4 mM PA-exposed cells, a reduction in proinsulin processing and impaired GSIS were detected in only 0.4 mM PA-treated cells. Furthermore, quantitative PCR showed that PA exposure increased UCP2 mRNA by 50- and 106-fold in 0.25 mM PA and 0.4 mM PA-treated cells, respectively; but significantly increased expression of UCP2 protein could be detected only in 0.4 mM PA-treated cells. Therefore, although markers of beta cell dysfunction were clearly associated with the higher PA concentration, these were not clearly associated with the effects of PA on beta cell ATP content.

Generally, the normal level of plasma free fatty acids in rodent circulation is 0.1 mmol/l (39). Rats fed a high fat diet (42% Kcal as fat) for 50 days have increased plasma free fatty acids level of 0.35 mmol/l (39). This feeding protocol is similar to a diet study that found the effect of high fat on beta cell dysfunction (36). Moreover, in mice the levels of fatty acids would fluctuate. These suggested that the level of plasma free fatty acid (0.25 and 0.4 mM) used in this study should be high enough to see its effect.

It is well documented that chronic culture of beta cells in free fatty acid induces UCP2 mRNA expression (31;34;36). Both unsaturated and saturated fatty acids induce UCP2 mRNA transcription in tissue-specific fashion. High fat diet increases UCP2 mRNA in white adipose tissue (40;41), liver (42) and pancreatic islets (31;36) but no change is seen in type II A(oxidative-glycolytic type) skeletal muscle and brown adipose tissue (41). Moreover, there is some evidence showing that free fatty acids also induce UCP2 expression at the protein level. Joseph et al. (32) showed an elevation of UCP2 protein in 48 h PA-cultured mouse islets. Medvedev et al. (43) also reported a two-fold elevation of UCP2 protein in 48 h oleate-exposed INS-1 cells. As mentioned above, elevation of UCP2 mRNA expression was detected in both 0.25 and 0.4 mM PA-treated cells, but a significant increase in UCP2 protein expression was achieved in only 0.4 mM PA-exposed cells. It is documented that UCP2 mRNA levels do not always reflect the expression of the protein (44). Similar levels of UCP2 mRNA were measured in spleen and stomach but ten times less protein expression was displayed in stomach than in spleen (44). Translational regulation of UCP2 is associated with an open reading frame in exon 2 of the UCP2 gene (44). It is likely that there is independent, tissue-specific regulation of both transcription and translation for UCP2.

Reduction of ATP production was detected in 0.25 mM PA-treated cells without a change in UCP2 protein expression. How could ATP generation be reduced without elevation of UCP2 protein expression? There are three possible mechanisms. Firstly, the diminishment of ATP generation may be due to a fatty

acid induced reduction in ATP synthesis, for example inhibition of ATP synthase expression. However, ATP synthase expression was not affected by fatty acid treatment. Although the present study cannot confirm that activity of ATP synthase was normal, to date there is no evidence that free fatty acids modulate ATP synthase activity. Secondly, free fatty acid itself could act as an uncoupler (45). The increase in free fatty acids in the cell environment could change the cell membrane composition (46), thereby promoting an induction of uncoupling, while generating less ATP production. Free fatty acids act as a protonophore uncoupler via the ADP/ATP antiporter (45). In those studies, PA stimulated respiration and reduced the mitochondrial membrane potential. This effect was abolished by an ATP/ADP antiporter inhibitor (CAtr). Interestingly, CAtr did not abolish the effect of the artificial uncoupler FCCP. The molecular mechanism of the uncoupling effect of fatty acids is likely to be similar to uncoupling protein (47). The anionic head group of free fatty acids is driven from one side of the membrane to the opposite side by the electric field generated by the electron transport system. When fatty acid carboxylate reaches the outerside the of inner mitochondrial membrane, it picks up a proton and rapidly flips back to the inner side to release the proton (48). Thirdly, UCP2 activity is enhanced in the absence of a change in expression. Of interest, it has been demonstrated that reactive oxygen species (ROS) activate UCP2 activity. Mitochondrial membrane potential is increased and proton leak is reduced in thymocytes from mice treated with superoxide dismutase mimetic agents, suggesting that superoxide regulates respiratory coupling. Mitochondrial membrane potential is not changed in thymocytes from UCP2 knockout mice

incubated with or without this superoxide scavenger (49). Moreover, addition of exogenous superoxide activates proton leak in wild type but not UCP2 knockout mitochondria (49). Both hyperglycaemia (49) and hyperlipidaemia (16) can induce ROS generation. In 0.4 mM PA-treated beta cells, both an increase in UCP2 activity and expression could contribute to lowering cellular ATP. UCP2 over-expressing islets have 50% less intra-islet ATP than control islets (31). On the other hand, UCP2 knockout mice have greater intra-islet ATP level than wild type mice (35).

Induction of UCP2 expression follows activation of specific transcription factors. Fatty acids including PA are natural ligands for peroxisome proliferator-activated receptors (PPAR) (50). Both PPAR $\alpha$  and PPAR $\gamma$  are present in pancreatic beta cells, but PPAR $\alpha$  is the most abundant subtype in rat pancreatic islets (51). A peroxisome proliferator response element (PPRE) is found in the UCP2 promoter (52) and PPAR $\alpha$  induces UCP2 in insulinoma cells (53). Moreover, free fatty acids also promote lipogenesis via sterol regulatory element binding protein 1c (SREBP1c) activation. SREBP1c is detected in pancreatic islets and its activation leads to enhanced fatty acid synthesis and esterified fatty acid. SREBP1c overexpressing beta cells have higher UCP2 expression compared to normal beta cells (54).

In the present study, it could be concluded that free fatty acid induced UCP2 expression and/or increased UCP2 activity, which was associated with reduced ATP production. ATP-dependent beta cell functions were in turn investigated. Impaired GSIS was detected in 0.4 mM PA-treated cells. In addition,

the proinsulin:insulin ratio was greater, an effect not attributable to any change in prohormone convertase. The mechanisms of insulin exocytosis and the activity of prohormone convertase are ATP-dependent. With respect to GSIS, the metabolism of high energy nutrients, such as glucose and free fatty acids, occurring in pancreatic beta cells leads to the elevation of the ATP/ADP ratio. Consequently, this greater ATP/ADP ratio inactivates ATP sensitive potassium ( $K_{ATP}$ ) channels, resulting in increased intracellular calcium ion and stimulated insulin exocytosis. In contrast, induction of UCP2 expression reduces the ATP/ADP ratio, inhibits  $K_{ATP}$  channel activity and impairs insulin secretion (31). Moreover, UCP1-overexpressing cell lines exhibited a significantly greater activity of  $K_{ATP}$  channels compared to control cells, even in the presence of  $K_{ATP}$  channel openers (55). This suggested that uncoupling proteins not only inhibit  $K_{ATP}$  channel inactivation but also affect events downstream. However, we found a reduction of ATP availability in 0.25 mM PA-treated cells without any change in beta cell GSIS or proinsulin processing. It is apparent that other factors induced by higher concentrations of fatty acids contribute to impairment of beta cell function.

Besides free fatty acid induced beta cell dysfunction induced via the activation of UCP2 activity, other plausible mechanisms of the effect of chronic high free fatty acid on beta cell dysfunction have been proposed. Firstly, free fatty acid itself (56) or its activated form, long chain acyl CoA (57), could directly activate the  $K_{ATP}$  channel and inhibit GSIS. Secondly, free fatty acid may also act directly to insulin exocytosis machinery. Chronically elevated fatty acid up-regulated SNAP-25, which is associated with a decline in insulin secretion in

mouse islets (58). This effect was restored when non-esterified fatty acid was removed.

The main hypothesis tested in this study was that PA-related induction of UCP2 would result in impairment of proinsulin processing. The activity of prohormone convertases (SPC1/3 and SPC2), enzymes which convert proinsulin to mature insulin, is ATP dependent. SPC1/3 preferentially cleaves proinsulin at the B-chain and C-peptide junction (site 1 or Arg-Arg site) but also at the A-chain and C-peptide junction (site 2 or Lys-Arg site), while SPC2 appears to cleave proinsulin mainly at site 2 (59). Subsequently, carboxypeptidase E, another endopeptidase found in insulin vesicles, cleaves the C-terminus of those basic residues resulting in des 31, 32 intermediates and des 64, 65 intermediates. Thus, SPC1/3 is the dominant beta cell convertase because it can cleave both sites of proinsulin (60). Moreover, western blot analysis suggests that there is relatively more SPC1/3 in insulin producing beta cells than in non-beta cells (61).

The activity of convertase enzymes also depends on pH (62) and  $\text{Ca}^{2+}$  ions (63). Of note, both optimal pH and  $\text{Ca}^{2+}$  ion maintenance inside insulin vesicles are ATP-dependent. After proinsulin is synthesized in the RER, it is transported to the trans-Golgi network where it is packaged into secretory granules with its converting enzymes. At this point, the secretory granules become acidic and have millimolar concentrations of calcium, providing an optimum environment for converting enzyme activity. To investigate whether ATP depletion induced by chronic free fatty acid could alter proinsulin processing, measurement of proinsulin:insulin ratios was therefore carried out. In addition, SPC1/3 expression

was measured. The present study revealed a significantly greater ratio of proinsulin:insulin in 0.4 mM PA-exposed cells compared with control cells. The reduced processing of proinsulin to insulin was not due to reduced SPC1/3 expression suggesting that perhaps the granule microenvironment or SPC1/3 activity was affected by fatty acid exposure. Although enzyme activity was not determined in this study, factors that can alter enzyme activity have been determined. SPC1/3 activity is reduced when the vesicle pH is increased (62). This suggests that reduction in beta cell ATP availability could reduce the activity of ATP-dependent proton pumps found in vesicular granule membranes, causing an increase in vesicular pH and a decrease in enzyme activity. As described above, convertase enzyme activity also depends on intracellular  $\text{Ca}^{2+}$ . Plasma membrane-related  $\text{Ca}^{2+}$  ATPase and sarcoplasmic reticulum  $\text{Ca}^{2+}$  ATPase (SERCA) play a role in calcium homeostasis in RER, Golgi complex and insulin vesicles (64). However, our data exhibits, dissociation between beta cell ATP content and proinsulin processing. Like for GSIS, other fatty acid-dependent mechanisms must contribute to reduced insulin maturation.

Insulin gene transcription and translation has also been reported to be negatively affected by exposure of beta cells to fatty acids (24;65). However, the effects are time and concentration dependent. Bollheimer et al. (66) reported that the inhibition of insulin gene expression by free fatty acids was noted up to 24 h, thereafter, there was no effect. An important paradigm in transcription regulation is the influence of the chromatin organization and remodeling, which is an ATP dependent process (67). Additionally, transcription initiation begins with the

phosphorylation of serine 5 (SER5) on the C-terminal domain of the largest subunit of RNA polymerase II by Transcription factor for polymerase II-H (TFIIF) (68). These two steps might be altered under an ATP depleted condition. Not only does glucose stimulate insulin biosynthesis, but it also regulates the conversion of proinsulin to insulin, at least in part by affecting prohormone convertase biosynthesis (69). If free fatty acids inhibit glucose-stimulated beta cell function, they could also regulate prohormone convertase biosynthesis. However, although preproinsulin II mRNA expression (with a non-significant increasing trend for preproinsulin I), the proinsulin:insulin ratio and proinsulin and insulin content were increased in this study, prohormone convertase expression did not change, showing that under some conditions, expression of insulin and its converting enzymes is not coordinately regulated. Moreover, insulin itself is a positive auto-regulator of preproinsulin gene transcription (70). Under chronic exposure to 0.4 mM PA, high basal insulin release was detected in this study, which could positively influence gene transcription. Perhaps the higher basal insulin secretion detected in the 0.4 mM PA condition could overcome both the putative inhibitory effect of ATP depletion on preproinsulin gene transcription and the regulatory effect of free fatty acids on insulin translation, but not overcome the effect of fatty acids on proinsulin processing.

In summary, this study verified the effect of high free fatty acid on beta cell dysfunction. Chronic exposure to saturated fatty acid induced UCP2 expression and/or activity, which in turn affected ATP generation. However, the reduction in ATP generation was detected in both 0.25 mM and 0.4 mM PA treatment groups,

while the elevation of UCP2 expression and the changes in beta cell function were detected in only the 0.4 mM PA treated group. Our findings are consistent with the conclusion that lowering of cellular ATP is not sufficient to induce the changes in beta cell dysfunction observed.

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## CHAPTER4

### THE EFFECT OF HIGH FAT INTAKE ON INSULIN PROCESSING AND SECRETION

#### Abstract

The prevalence of type 2 diabetes mellitus in Asian people (3.1%) is less than that in the North American population (5.1%), but the prevalence of this disease in Asians who immigrate to North America is significantly higher than that of those who remain in their native homeland. This implied that in addition to genetic factors, dietary changes also influence disease prevalence. Diets high in fat have an effect on both of the cardinal features of type 2 diabetes mellitus. However, the mechanism by which high fat diet is detrimental to beta cell function still needs to be defined. Glucose stimulated insulin secretion and hyperproinsulinaemia are well documented in this disease. In addition, these two characteristics are ATP dependent. Therefore there might be some defect in ATP availability under these conditions. Moreover, chronic exposure of islets to high free fatty acid concentrations induces UCP2 mRNA and protein expression. The present study hypothesized that high fat diet would induce UCP2, a negative regulator of ATP generation, subsequently reducing ATP and impairing glucose stimulated insulin secretion (GSIS), proinsulin synthesis and conversion to mature insulin. A five week intake of either standard rodent diet (SRD) or high fat diet (45% of calories from fat, HFD) was assigned to both wild type (WC-wild type control diet and WF-wild type high fat diet) and UCP2 KO (KC-knockout control

diet and KF-knockout high fat diet) mice. Thereafter, islets were isolated and evaluated for fatty acid induced beta cell dysfunction. The results showed that GSIS was impaired and proinsulin to insulin ratio was increased in wild type fed with high fat diet (WF) even though UCP2 expression and intra-islet ATP content were not changed. However, UCP2 KO mice fed the high fat diet maintained normal GSIS. This implies that fatty acids induce beta cell dysfunction by mechanisms other than the reduction of ATP production. Free fatty acids appear to impair glucose stimulated insulin secretion by a mechanism involving UCP2 activity without altering ATP synthesis. Of note, UCP2 KO mice have a greater ATP level and proinsulin and insulin content than wild type mice; however, the proinsulin:insulin ratio is higher in UCP2 KO than in wild type mice fed with SRD (WC). This result showed that some factors other than ATP availability could influence the expression of insulin and its converting enzymes. Moreover, these factors should be regulated by UCP2. In summary, the present study demonstrated that five weeks of a high fat diet intake did not induce UCP2 expression or reduce ATP generation. However, high fat diet affected beta cell function by mechanisms under UCP2 regulation but not directly related to ATP content.

## Introduction

The prevalence of type 2 diabetes mellitus in Asian people was only 3.1% in 2003, less than that in the North American population (5.1%) (1), but the prevalence of this disease in Asians who immigrated to North America was significantly higher than in those who remained in their native homeland (2). In addition to genetic factors, this implies that dietary changes also influence disease prevalence.

Diets high in fat, rather than high in sugars, have an effect on both of the cardinal features of type 2 diabetes mellitus: insulin resistance and insulin insufficiency(3). The mechanism by which high fat diet is detrimental to beta cell function still needs to be elucidated. Acute culture of islets in media supplemented with high free fatty acid concentration stimulates glucose-stimulated insulin secretion (GSIS) (4), but chronically culturing Zucker diabetic islets (5) or an insulinoma cell line (6) in media with increased free fatty acids results in the reduction of GSIS. Palmitate, the most common saturated free fatty acid from diet, causes increased release of insulin at basal glucose concentrations but suppresses the secretion of insulin at higher concentrations of glucose (7). When perfused with various glucose concentrations ranging from 1.4 mM to 20 mM, GSIS was blunted in islets from mice fed with a high fat diet for 4.5 months (8). On the other hand, hamsters receiving a hypercaloric diet with 52.5% of calories as fat for 20 weeks showed elevation of plasma glucose by 22% but no change in plasma insulin compared with controls (9). Although many mechanisms have been proposed to

explain these changes in beta cell function (10;11), a complete appreciation of the biochemical basis of this defect remains elusive.

A key molecule regulating insulin secretion in response to glucose is ATP. In normal circumstances, ATP generated from nutrient metabolism will stimulate insulin secretion by inhibiting the activity of the ATP sensitive potassium ( $K_{ATP}$ ) channel, but under conditions of chronically elevated free fatty acids or their CoA derivatives,  $K_{ATP}$  channel activity is increased (12;13). This evidence suggests that high fatty acid concentration induces a reduction in beta cell GSIS (7;14-16) due to activation of the  $K_{ATP}$  channel. Beta cell ATP is reduced in 72 h oleate-cultured insulin-secreting MIN6 cells (6) and 48 h palmitate-cultured mouse islets (8). Moreover, when cultured for 48 h in media with both a high glucose and a high palmitate concentration, rat islets lose the ability to release insulin in response to a glucose challenge (17). However, GSIS was normal in islets cultured with low glucose and high fatty acid concentrations(17). Thus, the deleterious effects of chronic high fatty acid on beta cell function are worst when accompanied by a high concentration of glucose. Of interest, after chronic high free fatty acid was used to reduce ATP generation, the glucose stimulated increase of the ATP/ADP ratio was blunted (18). Therefore, a reduction of the ATP/ADP ratio in islets chronically exposed to high concentrations of fatty acids might provoke the impairment of GSIS.

In addition to insulin secretion, other biochemical and physiological steps of beta cell function related to insulin biosynthesis and availability are also ATP dependent, such as phosphorylation of proteins involved in transcription (19),

chromatin remodelling (20), and proinsulin processing (21). The conversion of proinsulin to mature insulin depends on the activity of ATP dependent enzymes, prohormone convertases 1/3 and 2 (SPC1/3 and SPC2). In diabetic patients, hyperproinsulinaemia is present (22-25). A reduction of SPC 1/3 and SPC2 protein expression is found in islets from streptozotocin (STZ)-induced diabetic rats (26). Likewise, chronic exposure to free fatty acid causes beta cells to elevate SPC1/3 and SPC2 mRNA expression, but reduces SPC1/3 and SPC2 protein content by 15% and 23% respectively (27). Moreover, palmitate is also reported to inhibit insulin promoter activity in a beta cell line (28) and decrease preproinsulin mRNA of rat islets after 72 h exposure (29). However, the effect of high fatty acid on insulin biosynthesis and processing *in vivo* still needs to be elucidated. We speculate that the abnormalities in insulin synthesis and processing induced by chronic high fat intake are related to the reduction in ATP availability. The linkages between fatty acids, cellular ATP and insulin biosynthesis and processing need to be established.

Uncoupling protein-2 (UCP2), a negative regulator of ATP generation, is a mitochondrial inner membrane protein expressed in many tissues including pancreatic beta cells (30-32). UCP2 mRNA expression is induced by free fatty acids in non-islet tissues, such as skeletal muscle and adipocytes (33), islets (18;34) and clonal beta cells (35;36). It is well recognized that UCP2 has an effect on beta cell function. GSIS is impaired in UCP2 overexpressing islets (30;34). In contrast, UCP2 knockout (KO) mice have higher insulin secretion compare to their wild type (WT) counterparts (18;37). However, *in vivo* effects of high fat on UCP2

activity and other biochemical defects in beta cell function are still unclear. Of interest, Joseph and co-workers (8) reported a greater ATP content in UCP2 KO mouse islets compared to WT mouse islets. Moreover, mouse islets lacking UCP2 conserved ATP levels even when chronically exposed to high fatty acid. UCP2 KO mice also had enlarged beta-cell mass, more insulin content as well as greater insulin release in response to glucose (8) but whether insulin biosynthesis and processing were affected was not determined. In contrast to UCP2 KO mice, WT mice fed with high fat diet for 4.5 months showed increased UCP2 mRNA and protein expression compared to mice fed normal chow (18). Moreover, GSIS was lost and ATP levels were reduced in chronically high fat fed WT mice. Similarly, UCP2 overexpressing beta cells have less ATP content and impaired GSIS compared to control cells but have a higher proinsulin:insulin ratio when stimulated to secrete (unpublished data, see Chapter 2). It is therefore of interest to investigate whether chronic high fat consumption can induce UCP2 expression and/or activity, reduce ATP generation and consequently impair beta cell ATP dependent processes such as proinsulin synthesis, processing, and insulin secretion.

In the present study UCP2 WT and KO mice were fed with either standard rodent chow or high fat diet for 5 weeks in order to investigate the long term effect of high fat consumption on insulin synthesis, processing and secretion. It was hypothesized that long term high fat consumption could induce UCP2 expression, and as a result, impair insulin synthesis, processing and secretion by reduction of ATP availability. UCP2 KO mice were expected to be protected from these effects.

## **Materials and Methods**

### **Animals**

Female UCP2  $+/+$  (wildtype, WT) or  $-/-$  (knockout, KO) mice were used at three months of age. Mice (5 mice/cage) were housed in standard rodent cages, maintained on 12h dark-light cycles and were fed *ad libitum* with either standard rodent diet (SRD) with 4.05 calories/g (Cat# 8664, Harlan Teklad, Indianapolis, IN) or a high fat diet (HFD) for 5 weeks. The fat source was lard and comprised 45% of the total calories (4.7 calories/g) (Cat#D12451, Research Diets Inc., New Brunswick, NJ). All experiments were performed in accordance with the approval of the local animal care committee, following the guidelines of the Canadian Council on Animal Care.

### **Body weight gain and food consumption**

Each mouse was weighed weekly in order to monitor body weight gain. Food was weighed three times per week to measure food consumption per cage. Food consumption data were averaged over the entire 5 week period and presented as caloric intake/mouse/day.

### **Plasma insulin and glucose**

Ambient plasma samples for insulin and glucose assay were collected at the beginning of diet study and the day that islet isolation experiments were performed. Blood was drawn from the tail vein of conscious mice into anti-coagulant-treated

microcapillary tubes. Plasma was collected by centrifuging blood samples at 6000 rpm for 10 min at 4°C. Glucose concentrations were determined using a glucometer (Ascensia Elite<sup>TM</sup> XL, Bayer Health Care LLC, Mishawaka, IN) and insulin concentrations were determined using a rat insulin ELISA kit (Crystal Chem Inc., Chicago, IL). The cross reactivity of this kit for mouse insulin is 105%.

### **Islet isolation and culture**

Mice were anaesthetized using sodium pentobarbital intraperitoneally (65 mg/kg). After anaesthetization, the abdominal cavity was exposed by laparotomy and 1-2 ml collagenase type XI (1145.6 units/ml DEM media) (Sigma Aldrich, Oakville, Canada) was injected into the pancreatic duct using polyethylene tubing (PE-50). The pancreas was excised, chopped into small pieces and digested twice in collagenase solution for 15-20 min at 37°C. The digestions were stopped by adding Hank's balanced salt solution containing 5.5 mM glucose (Sigma Aldrich) followed by centrifugation at 1500 rpm for 5 min at 4°C. After the last wash, islets were separated from the other tissue using a dextran discontinuous gradient technique and centrifugation at 1500 rpm for 15 min at 4°C. The top two layers of the gradient were collected and islets were harvested using hand picking. All islets were cultured in DME medium (Invitrogen, Burlington, Canada) containing 8.3 mM glucose, 10 mM HEPES and 10% calf serum, overnight before, doing further experiments.

### **Glucose stimulated insulin secretion**

After overnight culture three islets were used in each sample. Duplicate samples were incubated in 2.8, 5.5, 11, 16.5 or 22 mM glucose in DME for 90 min. Samples were then centrifuged at 3500 rpm for 3 min. Supernatants were collected for quantifying the insulin release fraction. The pellets were lysed with 3% acetic acid and assayed for total insulin. The percent of total insulin secreted in response to glucose was quantified using a radio-immunoassay with human insulin as standard.

### **Islet ATP content**

After overnight culture, ATP content from 20 islets was measured using ATPlite (Perkin Elmer, Woodbridge, Canada), which is based on the production of light caused by the reaction of ATP with added luciferase and D-luciferin. Briefly, 20 islets were hand-picked into 100  $\mu$ l 8.3 mM glucose DME medium. Mammalian lysis solution (50  $\mu$ l) was added to samples in order to lyse the islets; the samples were then agitated at 700 rpm for 5 min at room temperature. Subsequently, 50  $\mu$ l substrate buffer was added, agitating again for 5 min. The samples were kept in the dark for 10 min before measuring luminescence on an I450 Micro Beta Scintillation and Luminescence detector (Perkin Elmer). Data were presented as ATP content per islet.

### Western blot analysis

Proinsulin-insulin ratio – Cell lysates were prepared using TES buffer (N-tris[hydroxymethyl] methyl-2-aminoethane-sulfonic acid buffer) containing aprotinin (4 µg/ml) and bestatin (5 µg/ml) as protease inhibitors. Protein samples (50 islets) were separated on 15% polyacrylamide gels under non-reducing conditions, then electrophoretically transferred onto nitrocellulose membranes (Trans-Blot, Bio-Rad Laboratories (Canada) Ltd, Mississauga, Ontario), followed by blocking of nonspecific binding in phosphate buffered saline (PBS) containing 5% skim milk and 1% Tween 20 overnight at 4°C with agitation. The membranes were then incubated at room temperature with guinea pig anti-insulin (1:2500; gp06) for 6 h. After washing extensively, membranes were incubated for 1 h at room temperature with anti-guinea pig IgG peroxidase (1:15,000, Sigma Aldrich). Specific signals were detected using an enhanced ECL Plus reagent according to the manufacturer's instructions (GE Healthcare, Piscataway, NJ). The intensity of reactive bands was quantified using a Kodak Image station 440 (Perkin Elmer). In order to distinguish between proinsulin and mature insulin bands, membranes were stripped and subsequently incubated with goat anti-C-peptide (1:1000, Linco, St. Charles, MI) followed by rabbit anti-goat IgG peroxidase (1:15,000, Sigma Aldrich).

Prohormone convertase (SPC1/3) – After washing three times in 0.1 mM PBS, 150 islets/sample were lysed with TES buffer containing 0.1% sodium dodecyl sulphate (SDS), and aprotinin (4 µg/ml) and bestatin (5 µg/ml) as protease inhibitors. Lysates were loaded into wells and separated by electrophoresis on

12% SDS-polyacrylamide gels. The proteins were electrotransferred onto nitrocellulose membranes and blocked with 5% skim milk containing 1% Tween-20 in PBS at 4°C overnight. After extensive washing, the membranes were incubated in anti-SPC1/3 (SPC1-fusion protein antibody, SPC3/K51, a gift from Dr. N.P. Birch, Auckland, New Zealand, 1:1000) for 2 h at room temperature. Subsequently, the membranes were incubated in goat anti-rabbit IgG peroxidase (1:20,000, Sigma Aldrich) for 1 h at room temperature. Specific signals were detected using the enhanced ECL Plus reagent. The protein loading was normalized using antibody to a housekeeping protein (mouse anti- $\beta$ -actin, 1:5000, Sigma Aldrich) and appropriate secondary antibody (anti-mouse IgG peroxidase, 1:5000, Sigma Aldrich).

ATP synthase – The same protocols for lysate preparation, protein separation and transference as those for SPC1/3 were used. After blocking overnight at 4°C, membranes were incubated in primary antibody (anti-ATP synthase subunit  $\alpha$ ; 1:2000, Molecular Probes, Eugene, OR) for 1 h at room temperature. Rabbit anti-mouse IgG peroxidase was used as the secondary antibody (1:5000, Sigma Aldrich). Bands of the appropriate molecular weight (59.8 kilodaltons) were identified by comparing to a standard protein ladder (Magic Mark<sup>TM</sup>, Invitrogen). Specific signal was visualized and quantified as for SPC1/3.

Uncoupling protein 2 – Islet lysates separated as described above were also blotted for UCP2 expression. Specific signals were developed by incubating in goat anti-UCP2 antibody (1:2500, Santa Cruz Biotechnology, Santa Cruz, CA)

overnight at 4°C and rabbit anti-goat IgG peroxidase (1:10,000, Sigma Aldrich) for 2 h at room temperature.

### **RNA extraction and cDNA synthesis**

Total RNA was extracted from 30 islets of each mouse per sample using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Briefly, 1 ml of TRIzol was added directly to the sample. Cell lysate was incubated for 5 min at room temperature and then 200 µl of chloroform was added. Lysate was shaken vigorously for 15 s, then incubated for 2 min and centrifuged at 12,000 rpm at 4°C for 15 min. The clear aqueous phase was collected, transferred to a new tube and mixed with 500 µl isopropanol. The mixture was incubated for 10 min, and then centrifuged at 12,000 rpm at 4°C for 10 min. The supernatant was discarded. The pellet was washed with 75% ethanol in DEPC treated water. The pellet was subsequently centrifuged at 9,000 rpm at 4°C for 5 min, then air dried for 15 min, dissolved in RNase-free water and incubated 10 min at 65°C. The extracted RNA samples were kept at -80°C until cDNA synthesis was carried out.

cDNA synthesis was performed using a Cloned AMV First-Strand cDNA Synthesis kit (Invitrogen). RNA (1-5 µg) was added to the mixture containing 50 ng of random hexamer primer, 2 µl of 10 mM dNTP, 4 µl of 5X cDNA Synthesis buffer, 1 µl of 0.1 M DTT, 1 µl or 40 U RNase OUT and 1 µl or 15 U of Clone AMV RT. RNase-free water was added up to 20 µl in total volume. The reaction tube was transferred to a thermal cycler, and incubated sequentially at 25°C for 10

min followed by 50°C for 50 min. The reaction was terminated by incubating at 85°C for 5 min.

### Quantitative RT-PCR

All cDNA samples were amplified in PCR reactions using specific primers as follows:

UCP2 Forward: 5'CAGCCAGCGCCCAGTACC3';

UCP2 Reverse: 5'CAATGCGGACGGAGGCAAAGC3' (8)

Mouse Preproinsulin Forward: 5'CCATCAGCAAGCAGGTTAT3'

Mouse Preproinsulin Reverse: 5'GGGTGTGTAGAAGAAGCCA3'

(38)

Mouse Glyceraldehyde-3 phosphate dehydrogenase (GAPDH)

Forward: 5'GTGGCAGTGATGGCATGGAC3'

Mouse Glyceraldehyde-3 phosphate dehydrogenase (GAPDH)

Reverse: 5'CAGCACCAAGTGGATGCAGGG3' (8)

Real time PCR reactions were carried out using 1 µl of cDNA (0.1 µg/µl) in a solution containing 12.5 µl of SYBR green (Bio-Rad Laboratories (Canada) Ltd, Missisauga, Canada), 0.5 µl of specific forward primer, 0.5 µl of specific reverse primer and 10.5 µl of de-ionized distilled water. Reactions were carried out with heating to 95°C for 10 min, followed by 40 cycles of melting at 95°C for 15 sec, annealing at 57°C for 45 sec and extension at 72°C for 20 sec (for UCP2), 50 cycles of melting at 95°C for 5 sec, annealing at 60°C for 10 sec and extension at

72°C for 15 sec (for GAPDH), 40 cycles of melting at 95°C for 20 sec, annealing at 58°C for 20 sec and extension at 72°C for 30 sec (preproinsulin). Melt curve analysis was performed following cycling as a method of validation. The PCR products (expected sizes UCP2 - 127 bp, mouse preproinsulin - 307 bp and GAPDH - 193 bp) were run on 1% agarose gels to further verify that the PCR reactions generated the expected products. The expression of UCP2 and preproinsulin mRNAs was normalized to GAPDH for comparison of expression between treatment groups.

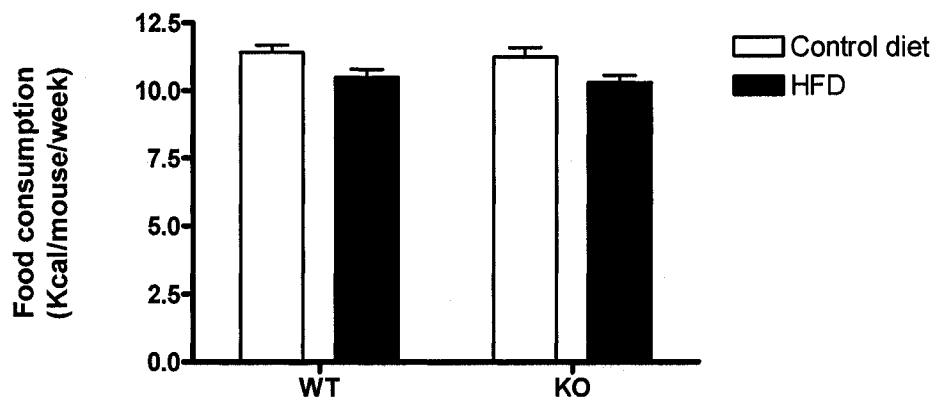
#### **Statistical analysis**

All data are presented as means  $\pm$  SEM. Significant differences were determined using 2-way ANOVA with Bonferroni post-tests for comparisons of genotype and diet treatment. Student's unpaired t-test was used to detect effects of diet on UCP2 expression of WT mice. Differences were considered significant if  $P<0.05$  or lower.

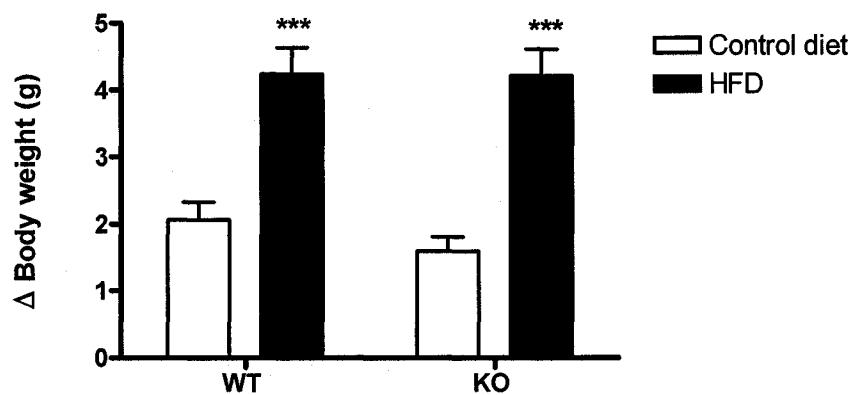
## Results

### **Food consumption and body weight gain after 5 weeks on diets.**

To determine whether or not all mice consuming either standard rodent diet (SRD) or high fat diet (HFD) received the same amount of calories/week, weekly food intake was measured in a sub-group of each diet treatment. Figure 4-1 shows that both WT and UCP2 KO mice fed either SRD or HFD consumed a similar amount of calories per week ( $P>0.05$ ,  $n = 13-15$ ). Furthermore, after five weeks of diet intake, both WT and KO mice fed HFD had a greater body weight increase (Figure 4-2) than mice fed SRD ( $P<0.05$ ,  $n \geq 70$ ).



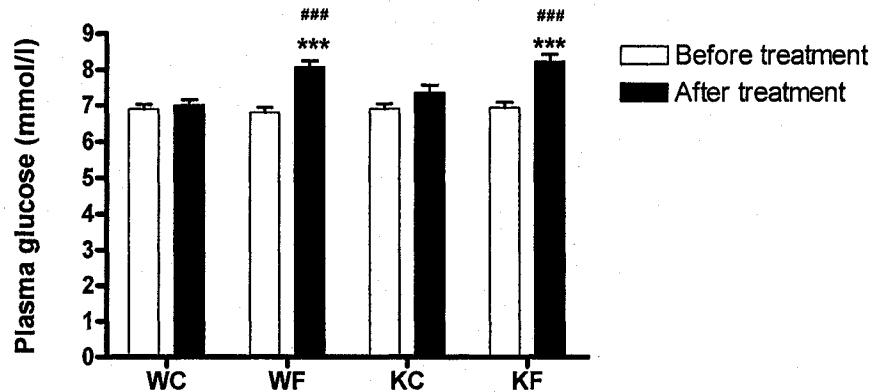
**Figure 4-1:** Food consumption of diet treatment groups. No significant differences in caloric intake were detected ( $P>0.05$ ,  $n=13-15$  in each group).



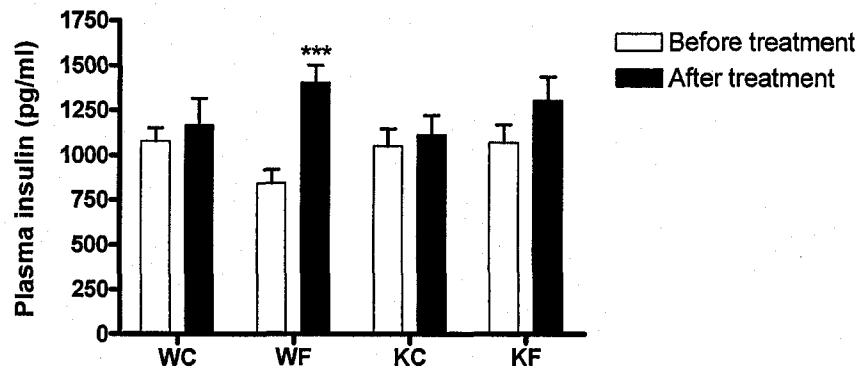
**Figure 4-2:** Body weight gain of diet treatment groups. Both wild type ( $4.2 \pm 0.4$  g, n=83) and knockout ( $4.2 \pm 0.4$  g, n=90) mice fed with a HFD had significantly increased body weight compared to SRD-fed WT ( $2.1 \pm 0.3$  g, n=73) and KO ( $1.6 \pm 0.2$  g, n=83) mice.

### **Blood glucose and plasma insulin**

Blood samples were collected from the tail vein before and after the diet treatment. Figure 4-3 shows the higher blood glucose observed in both WT and KO mice fed HFD for five weeks compared to concentrations before treatment ( $P<0.001$ ). The blood glucose concentrations of HFD mice were also greater than those of both WT and KO mice fed SRD. Plasma insulin in all groups were similar before treatment, but were increased in WT but not KO mice fed HFD (Figure 4-4,  $P<0.001$ ).



**Figure 4-3:** Ambient blood glucose from all treatment groups. Before starting the diet, all groups had a similar blood glucose concentration. HFD significantly increased blood glucose concentrations (\*\* $P<0.001$ ,  $n\geq 39$ ). HFD mouse glucose concentrations (WF, KF) were also greater than those of both WT and KO mice after feeding SRD for five weeks (WC and KC, ## $P<0.001$ ).



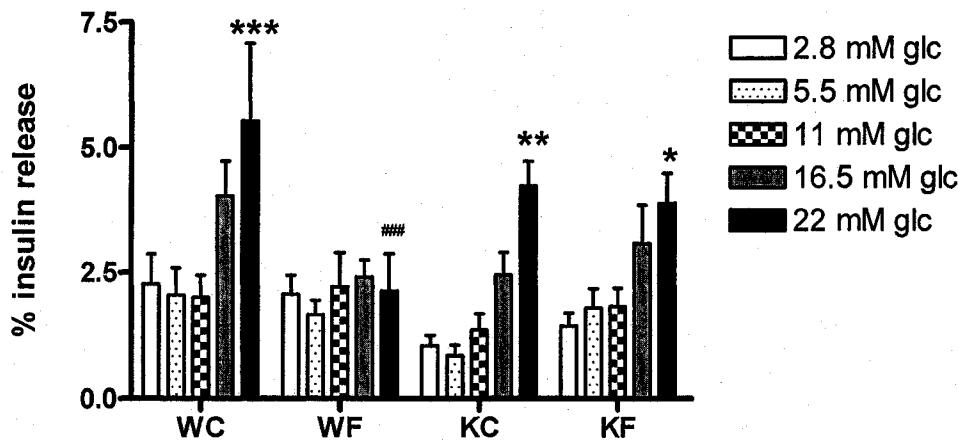
**Figure 4-4:** Ambient plasma insulin of all treatment groups. Plasma insulin concentrations in all groups were similar before treatment, but were increased in WT mice fed with HFD (WF) as compared to pre-diet results (\*\* P<0.01, n ≥ 38).

### ***In vitro* glucose stimulated insulin secretion**

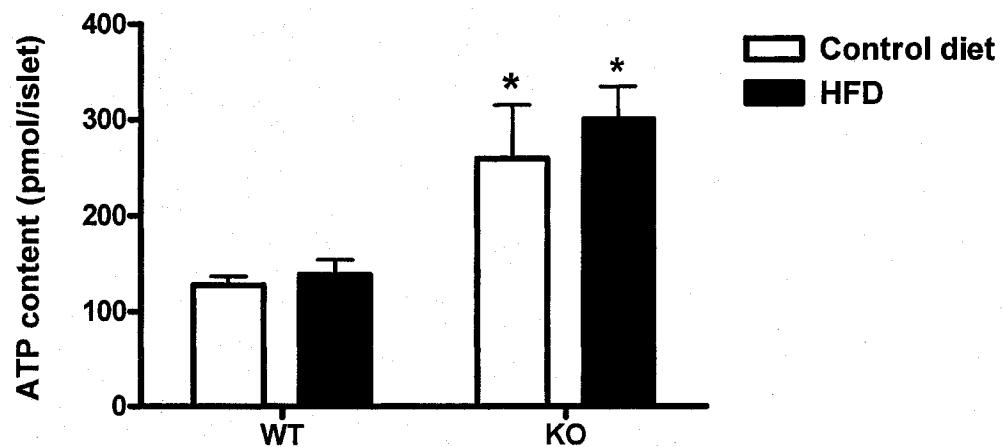
To study the effects of high fat intake on beta cell function, measurements of GSIS were carried out. Isolated islets were incubated in various glucose concentrations for 90 min. Figure 4-5 shows insulin secretion in response to glucose in WT and KO mice either fed with SRD or HFD. In both WT and KO mice fed with SRD, a similar pattern of GSIS was found with glucose concentration dependently increasing insulin secretion. This pattern was lost in WT mice fed a high fat diet. However, increased insulin release in response to a high glucose challenge was still detected in KO mice fed HFD.

### **ATP content and ATP synthase expression**

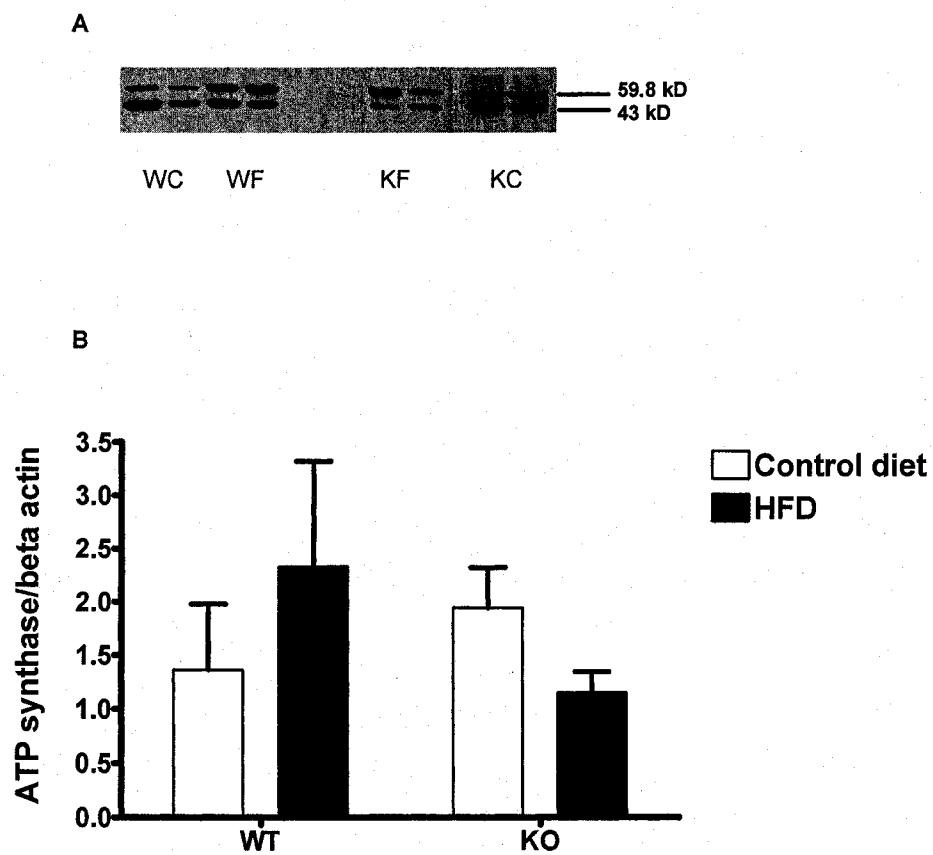
Because GSIS is ATP dependent, we measured the effect of HFD on cellular ATP content. The results are shown in Figure 4-6. The intracellular ATP content was significantly increased ( $P<0.05$ ) in KO mice fed with either SRD or HFD compared to WT mice fed SRD. In order to investigate whether the elevation of intracellular ATP detected in KO mice resulted from an enhanced expression of ATP synthase, western blot analysis for detecting this enzyme was carried out. All treatment groups had similar expression of ATP synthase (Figure 4-7A,B) after normalizing with beta actin ( $p>0.05$ ).



**Figure 4-5:** Effect of HFD on insulin secretion measured acutely over 90 min. Insulin release, expressed as a % of total cell insulin content, was calculated. Effects of both glucose concentration and diet were detected ( $P<0.0001$  for glucose effect,  $P=0.0118$  for diet). In WC, KC and KF mice, % insulin release was significantly higher at 22 than 2.8 mM glucose (\*\* $P< 0.001$ , \*\* $P<0.01$  and \* $P<0.05$  in WC, KC and KF mice, respectively.  $n=8-12$  for all groups). The insulin release in response to glucose was impaired in WT mice fed with HFD (WF, ## $P<0.001$  compared to 22 mM of WC).



**Figure 4-6:** Comparison of intra-islet ATP content in WT and KO mice fed with either SRD or HFD. \* $P<0.05$  for genotype effect,  $n \geq 17$ . There was no effect of diet in either WT or KO mice ( $P>0.05$ ).



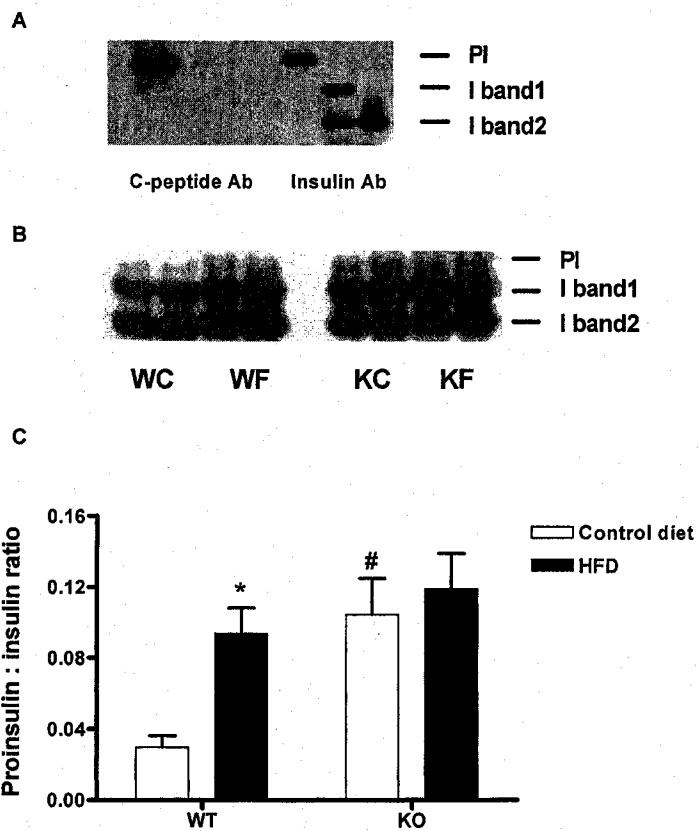
**Figure 4-7:** Effects of HFD on ATP synthase expression. A) Representative blot showing ATP synthase (~60 kD) and beta actin (43 kD) after five weeks on diet. B) The expression of ATP synthase, relative to beta actin, was similar in all treatment groups ( $P>0.05$ ,  $n \geq 10$  for each).

### **Proinsulin processing:**

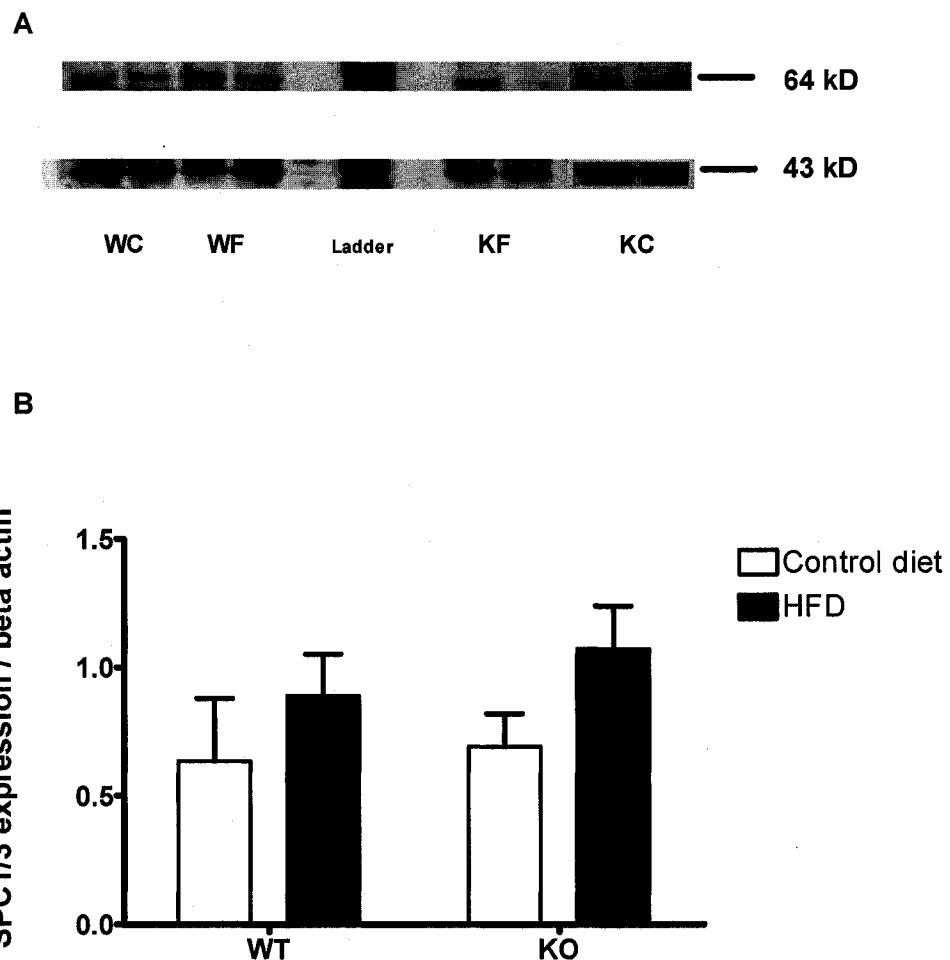
To determine whether proinsulin processing was impaired in HFD mice, western blot analysis for islet proinsulin and insulin was performed after five weeks on diet. In addition to using anti-insulin antibody for detecting both proinsulin and insulin bands, proinsulin bands were differentiated from mature insulin bands using anti-C-peptide antibody (Figure 4-8A). Proinsulin:insulin ratios were calculated after measuring the intensity of proinsulin and insulin bands. Figure 4-8B and C shows that both genotype ( $P=0.0219$ ) and diet ( $P=0.0044$ ) had an effect on the proinsulin:insulin ratio in KO mice ( $0.105 \pm 0.020$  and  $0.119 \pm 0.020$ ,  $n=9$ , SRD and HFD, respectively) compared to WT mice fed SRD ( $0.030 \pm 0.006$ ,  $n=9$ ,  $P<0.05$ ). Moreover, WT mice fed with HFD ( $0.094 \pm 0.014$ ,  $n=8$ ) also had a significantly greater proinsulin:insulin ratio compared to WT mice fed with SRD ( $P<0.05$ ).

### **Prohormone convertase expression**

Proinsulin processing depends on the expression of prohormone convertase (SPC1/3), thus western blot analysis for SPC1/3 was conducted. SPC1/3 expression in all treatment groups, as shown in Figure 4-9, was similar ( $0.63 \pm 0.25$ ,  $0.89 \pm 0.16$ ,  $0.69 \pm 0.13$ ,  $1.08 \pm 0.17$  in WC, WF, KC, KF respectively,  $P>0.05$ ).



**Figure 4-8:** Effect of HFD on the proinsulin:insulin ratio, as an index of processing of hormone to its mature form. A) Representative blot showing control staining that allowed differentiation of proinsulin from insulin on the blots. Proinsulin cross-reacted with both C-peptide (left) and insulin antiserum (right) whereas mature insulin cross-reacted with only insulin antibodies (right). B) Representative blot showing the effects of HFD and genotype. C) Proinsulin:insulin ratios were significantly higher in WT mice fed HFD \* $P<0.05$ , effect of diet). KO mice had a higher proinsulin:insulin ratio independent of diet (n = 9, # $P<0.05$ , effect of genotype).



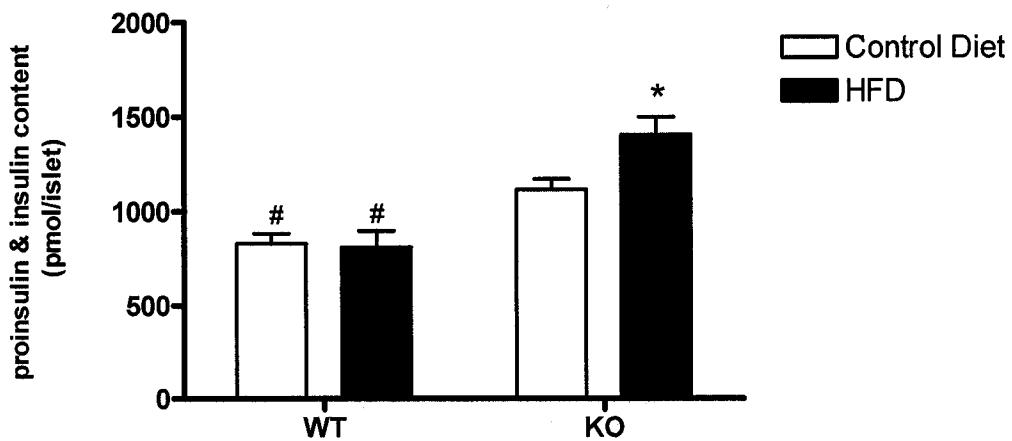
**Figure 4-9:** The effect of HFD on prohormone convertase (SPC1/3) protein expression. A) Representative blot showing the effect of HFD and UCP2 genotype on prohormone convertase (64 kD) relative to beta actin (43 kD). B) All treatment conditions had similar expression of this enzyme ( $p>0.05$ ,  $n \geq 8$  for all groups).

### **Proinsulin and insulin content**

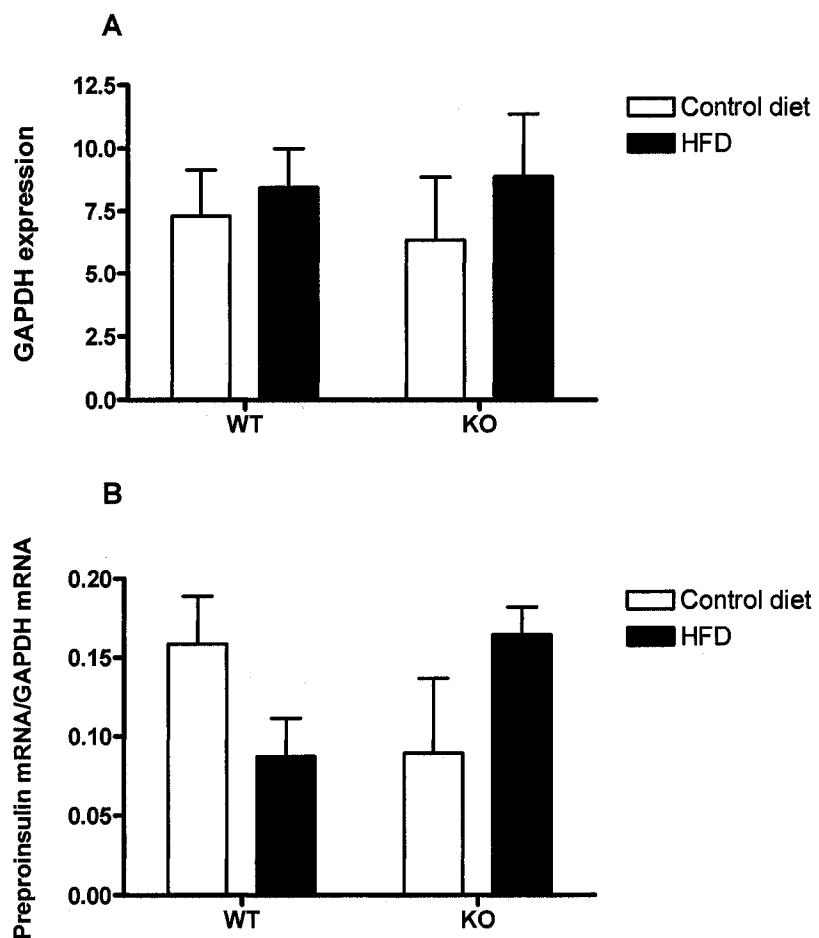
The content of proinsulin and insulin inside the islet was evaluated. Both fractions (release + total) of the samples from insulin secretion experiments were added together. The data are shown in Figure 4-10. Diet had no effect on islet insulin content of WT mice ( $830.3 \pm 53.3$ ,  $810.5 \pm 89.4$  pmol/islet for SRD and HFD, respectively). For KO mice fed HFD, the proinsulin+insulin content was elevated ( $1404.8 \pm 92.1$  pmol/islet,  $P<0.05$ ) compared to SRD ( $1115.8 \pm 57.5$  pmol/islet). In both diet treatments, insulin content of KO mice was higher than in WT mice ( $P<0.05$ ).

### **Quantitative RT-PCR of preproinsulin mRNA**

Quantitative PCR of preproinsulin mRNA was performed and normalized to GAPDH mRNA, the expression of which did not differ between groups (Figure 4-11A). Figure 4-11B shows similar expression of preproinsulin mRNA in all groups ( $P>0.05$ ).



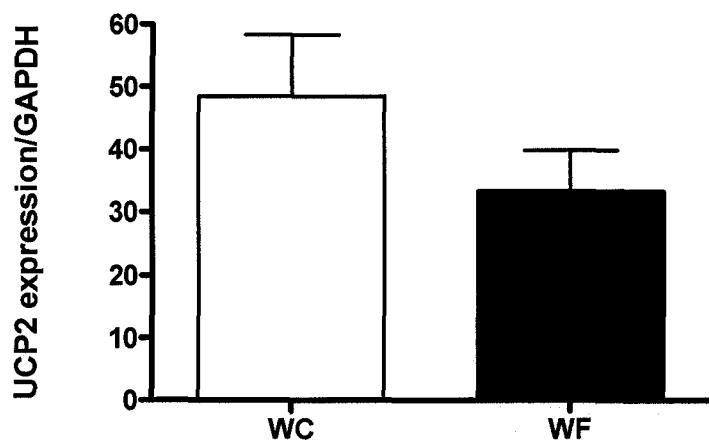
**Figure 4-10:** Effect of HFD and UCP2 genotype on proinsulin and insulin content. KO mice had higher proinsulin and insulin content ( ${}^{\#}P<0.05$ ,  $n=10-12$ ) than WT mice. HFD induced a further increase in islet insulin content in KO mice ( ${}^{*}P<0.05$ ,  $n=10-12$ ) compared to SRD fed mice.



**Figure 4-11:** A) GAPDH expression was similar in all treatment conditions (n=9-10). B) Relative expression of preproinsulin mRNA to GAPDH mRNA ( $P>0.05$ , n=8-9)

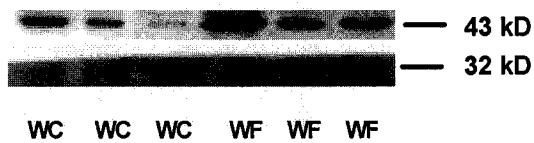
## **Quantitative RT PCR for UCP2 mRNA and western blot analysis for UCP2 protein expression**

To test whether the impaired insulin secretion detected in WT mice fed HFD might be due to induction of UCP2, real time PCR and western blot analysis were performed. UCP2 mRNA expression in isolated islets was compared to GAPDH mRNA in the same sample. Figure 4-12 shows similar expression of UCP2 mRNA in WT mice fed SRD or HFD ( $P>0.05$ ). Likewise, there was no significant difference in UCP2 protein expression between WT mice fed SRD vs HFD (Figure 4-13,  $P>0.05$ ).

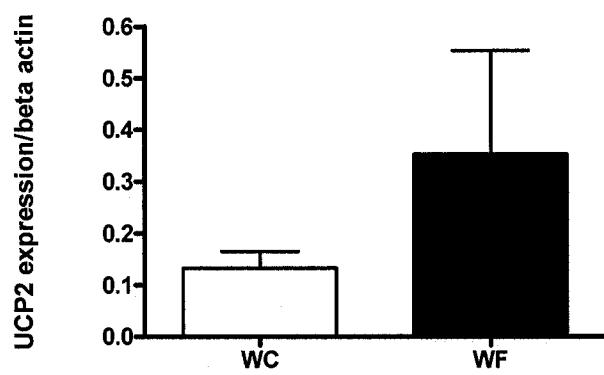


**Figure 4-12:** Effect of HFD on UCP2 mRNA expression. The expression of UCP2 mRNA was normalized to GAPDH ( $P>0.05$ ,  $n=9$ ).

**A**



**B**



**Figure 4-13:** UCP2 protein expression relative to beta actin in WT mice. UCP2 protein (32 kD) normalized with beta actin (43 kD) was similar in both groups ( $P>0.05$ ,  $n=7-8$ ).

## Discussion

The well known effects of high fat intake on pancreatic beta cell function include impaired GSIS and hyperproinsulinaemia. These two features are dependent on ATP. Therefore ATP generation might be impaired under high fat conditions. UCP2, a negative regulator of ATP production, may mediate changes in ATP production. We hypothesized that high fat intake could induce UCP2, in turn reducing ATP, and thereby impairing GSIS and proinsulin synthesis and processing. In the present study, WT and UCP2 KO mice were fed either SRD or HFD for five weeks. Subsequently, islets from all groups were isolated and evaluated for the effects of HFD. Although caloric intake was similar in all groups, both WT and KO mice fed with HFD (WF and KF) had a significant body weight gain compared to genotype-matched controls fed SRD (WC and KC). In addition, a significant elevation of plasma glucose was found in both WF and KF groups but only WF mice had a significant increase in plasma insulin. Intra islet ATP content and proinsulin and insulin content were higher in KC mice, which was further increased in KF mice. As predicted, GSIS was impaired and the proinsulin:insulin ratio was greater in WF mice compared to WC mice. Surprisingly, the proinsulin:insulin ratios were also higher in both KO groups.

These results do not support a role for induction of UCP2 and reduction of cellular ATP being implicated in impairment of proinsulin processing *in vivo*. However, UCP2 KO mice adapted differently to the HFD, providing further evidence that lack of UCP2 can modify beta cell physiology. In the case of WT mice, five weeks of consuming HFD did not induce UCP2 mRNA or protein

expression, nor reduce islet ATP content. Secondly, HFD impaired GSIS in WT mice. This might be partially due to UCP2 activity, independent of an increase in expression, because UCP2 KO mice retained a normal insulin release pattern in response to glucose stimulation even when they were fed with HFD. Thirdly, proinsulin processing was different between KO and WT mice. These differences suggest that UCP2 has a role in the regulation of proinsulin processing.

It is well known that dietary fat can induce body weight gain because it is more energy dense and more palatable than carbohydrate and protein (39). Isocaloric intake of a HFD also induces weight gain (40) because ingestion of dietary fat does not acutely stimulate fatty acid oxidation but rather induces fat storage (41). High fat intake acutely reduces plasma leptin (42), consequently reducing energy expenditure and resulting in positive energy balance. Moreover, glucose uptake is reduced when tissue energy needs are met by FFA oxidation. The oxidation of FFA results in decreased glucose oxidation and an increase in intracellular citrate levels, which decrease glycolysis (43). Therefore, plasma glucose is elevated and this in turn increases plasma insulin, as demonstrated in WF mice. In addition, insulin promotes lipogenesis to further stimulate body weight gain (44). Of note, mice lacking UCP2 that were fed a HFD had a significant increase in plasma glucose without an increase in plasma insulin. Moreover, UCP2 KO mice had a greater islet proinsulin and insulin content than WT controls. Interestingly, when mice lacking UCP2 were fed with a HFD, intraislet proinsulin and insulin content was even greater than in UCP2 KO mice fed a SRD. When we compared KF mice to WF mice, less insulin was secreted

despite similar plasma glucose concentrations. If KF mice have a defect in insulin secretion in response to glucose, we should expect the higher circulating plasma glucose than seen in WF mice. However, KF mice still have normal insulin release in response to glucose *in vitro*. It is therefore unlikely that the lower circulating plasma insulin found in these mice is due to a secretion defect of pancreatic beta cells. One possibility is that insulin sensitivity of target organs in KF mice is retained or improved compared with WF mice. The response of KF mice to insulin was enhanced compared with WF mice after an insulin tolerance test (8). Notably, in those UCP2KO mice fed with high fat diet for 4.5 months, their plasma glucose was normal while plasma insulin was increased. In this present study, a higher plasma glucose but normal plasma insulin were detected in UCP2KO mice after a shorter period of high fat diet consumption. Therefore hyperglycaemic clamp studies need to be done in order to verify differences in insulin sensitivity of KF mice. It is suggested that the hyperglycaemic clamp is the best way to assess both beta cell glucose sensitivity and peripheral tissue insulin sensitivity (45) because this technique requires a fixed glucose infusion at hyperglycaemic levels sufficient enough to evoke insulin secretion. If KF mice had reduced beta cell responsiveness to glucose, a higher rate of glucose infusion would be needed to stimulate insulin secretion. Moreover, since hyperglycaemia was detected in KF mice, organ glucose uptake and hepatic glucose production during hyperglycaemic clamp also need to be investigated to verify the degree of insulin resistance.

The main hypothesis tested in this study was that a consequence of high fat intake would be increased expression of UCP2 in WT mice, thereby lowering

cellular ATP content and contributing to a reduction in proinsulin processing and blunted GSIS. Conversely, mice lacking UCP2 would maintain islet ATP, GSIS and proinsulin processing when they were fed with HFD. However, quantitative PCR and western blot analysis showed that five weeks consumption of 45% fat in the diet did not induce UCP2 in WT mice. Consequently, intra-islet ATP levels were not reduced. In contrast, intra-islet ATP levels of UCP2 KO mice were greater than WT groups, consistent with the role of UCP2 as a negative regulator of ATP production (34). Failure to show induction of UCP2 after high fat diet was unexpected. Others have reported that *in vitro* culturing of islets in high free fatty acid concentrations (2 mM) for 72 h promotes UCP2 protein expression (35). Moreover *in vivo* high fat diet consumption for 3 weeks in rats (34) or >4 months in mice (8) also induces islet UCP2 mRNA expression. Both unsaturated and saturated fatty acids are known to up-regulate UCP2 mRNA transcription in tissue-specific fashion (33). Li and co-workers reported that induction of UCP2 expression by fatty acid parallels their rate of oxidation, not the concentration of fatty acid (46). High concentrations of blood glucose could inhibit fatty acid  $\beta$ -oxidation but promote lipogenesis. When the insulin to glucagon ratio is enhanced, such as under hyperglycaemic conditions, an elevation of malonyl CoA formation occurs. Malonyl CoA is a potent inhibitor of carnitine palmitoyl transferase I (CPT1), the enzyme that catalyzes access of long chain fatty acyl CoA to the mitochondria matrix to initiate fatty acid oxidation. In the present study, elevation of blood glucose, increased plasma insulin as well as the enhanced fat storage (as implied by weight gain) were detected in WF mice. Therefore, fatty acid oxidation

should be reduced and consequently, fatty acid induced UCP2 expression should be suppressed. In contrast, UCP2 protein was increased in mice fed with a diet high in fat for 4.5 months (18) but the same group of investigators did not find an increase in plasma glucose (8). Therefore, it might be implied that the high plasma glucose detected in WF mice inhibited fatty acid oxidation as well as inhibit fatty acid induced UCP2 expression.

Of note, we found that GSIS was impaired and the proinsulin:insulin ratio was increased in WF mice. How can free fatty acid induce beta cell dysfunction? There are several mechanisms proposed, of which two are discussed here. Firstly, saturated free fatty acid could directly activate pancreatic  $K_{ATP}$  channels (12;13). Generally, more ATP produced from nutrient metabolism creates an elevation of the ATP/ADP ratio. The elevated ATP/ADP ratio inhibits the activity of  $K_{ATP}$  channels. As a consequence, the accumulation of  $K^+$  ions causes beta cell depolarization, activates voltage-dependent  $Ca^{2+}$  channels and increases intracellular calcium. The elevation of intracellular  $Ca^{2+}$  stimulates insulin exocytosis (47). Conversely, after high saturated fatty acid exposure, free fatty acids can activate the  $K_{ATP}$  channel. As a result, the channels remain open, beta cells cannot be depolarized and subsequent insulin exocytosis is inhibited. Secondly, free fatty acids can induce reactive oxygen species (ROS) production, thereby increasing the activity of UCP2 (6;48). Mitochondrial membrane potential is increased and proton leak is reduced in thymocytes from mice treated with superoxide dismutase mimetic agents, suggesting that superoxide regulates respiratory coupling. Mitochondrial membrane potential is not changed in

thymocytes from UCP2 KO mice incubated with or without this superoxide scavenger (48). Moreover, addition of exogenous superoxide activates proton leak in WT but not UCP2 KO mitochondria(48). We found impairment of GSIS in WF but not KF mice. If the impairment of GSIS is caused by the direct activation of  $K_{ATP}$  channels by free fatty acid, this impairment should occur in KF mice as well. In contrast, if the impairment of GSIS is caused by an increased activity of UCP2, the ATP level of WF mice should be reduced. Therefore the mechanism of free fatty acid induced impairment of GSIS observed in the current study must not result from either mechanism. If UCP2 activity is increased by free fatty acid stimulation, why isn't ATP reduced? From our previous results (Unpublished data, see chapter 3), the effect of high fat on ATP reduction has a tendency to be dose dependent, since the reduction of ATP levels in the 0.25 mM palmitic acid treated group was not as great as that in 0.4 mM palmitic acid treated group. Moreover, the effect of high fat on UCP2 induction also depends on the concentration of free fatty acid. Plasma free fatty acids were not measured in the present study, therefore plasma concentrations of free fatty in both WF and KF mice might not have been high enough to induce UCP2 and reduce ATP. Also, the *in vivo* environment may be different than *in vitro*. *In vitro*, the cells are exposed continuously to high levels of fatty acids. In the intact mice, free fatty acid levels would fluctuate. Such oscillations might help protect the beta cells.

Despite failure to show a relationship between UCP2, ATP and GSIS in WT mice, the fact remains that UCP2 KO mice retained a normal insulin response to glucose in isolated islets. Because UCP2 KO mice have more intra-islet ATP, it

could be plausible that this greater ATP concentration could overcome the defects induced by HFD. In addition, higher carnitine palmitoyl transferase I mRNA was detected in UCP2 KO than WT mice (8). As a result, more fatty acyl CoAs would enter the  $\beta$ -oxidation cycle, with less fatty acyl CoA accumulating in beta cell cytoplasm. This could lower activation of  $K_{ATP}$  channels. Ultimately, UCP2 KO mice fed with HFD (KF mice) could sustain their insulin release in response to glucose.

It has been suggested that increased mitochondrial  $Ca^{2+}$  loading could induce more GSIS (49). Ordinarily, intracellular  $Ca^{2+}$  enters the mitochondrial matrix via a calcium uniporter and “Rapid Mode” calcium uptake (RaM) (50). Mitochondrial  $Ca^{2+}$  enhances the TCA cycle, thereafter, TCA metabolites are produced. Glutamate, a putative positive regulator of GSIS, is produced from one of TCA metabolites,  $\alpha$ - ketoglutarate (49). As a result, GSIS is potentiated. In contrast, UCP2 reduces  $Ca^{2+}$  accumulation in neuronal mitochondria (51). Moreover, UCP inhibits mitochondrial uptake via RaM (50). In summary, UCP2 inhibits mitochondrial  $Ca^{2+}$  uptake, possibly leading to a reduction in glutamate production and insulin exocytosis. Although this study did not measure intra-islet  $Ca^{2+}$  concentrations, it has been reported that KF mice have more intracellular  $Ca^{2+}$  than WF mice (18). Although the regulatory role of UCP2 on beta cell mitochondrial  $Ca^{2+}$  uptake still needs to be elucidated, it can be surmised that KF mice retain insulin exocytosis because of higher beta cell ATP content, as well as mitochondrial  $Ca^{2+}$  induced glutamate production.

Unexpectedly, the proinsulin:insulin ratio was significantly increased in KC and KF mice compared to WT islets, without any change in SPC1/3 expression and despite higher islet ATP content than WT mice. It is well known that proinsulin conversion to insulin is an ATP-dependent mechanism, therefore it was hypothesized that UCP2 KO mice fed with control diet (KC) should have equal or greater proinsulin conversion compared to WT mice fed with control diet (WC). It has been documented that the synthesis of prohormone convertases, both SPC1/3 and SPC2, is regulated by glucose (52). Moreover, the conversion of proinsulin to mature insulin is also dependent on glucose and the expression of preproinsulin mRNA (53). The plasma glucose and preproinsulin mRNA found in KC mice are similar to those of WC mice. As a consequence, the greater proinsulin:insulin ratio found in KC mice is not likely to be caused by a defect of prohormone convertase. As mentioned above, WF and KF mice have higher plasma glucose compared to genotype controls fed with control diet. If the synthesis of prohormone convertases and the conversion of proinsulin to insulin depends on glucose concentrations, why do WF and KF have a greater proinsulin: insulin ratio and similar level of prohormone convertase expression? The conversion of proinsulin to mature insulin inside the secretory vesicle depends on pH (21) and  $\text{Ca}^{2+}$  concentration (54). As mentioned above, free fatty acids could directly activate  $\text{K}_{\text{ATP}}$  channels (12) thereby suppressing  $\text{Ca}^{2+}$  influx into the pancreatic beta cell. Assuming vesicle  $\text{Ca}^{2+}$  is dependent upon cytosolic concentrations, the suppressed elevation of intra-secretory vesicular  $\text{Ca}^{2+}$  could reduce prohormone convertase activity and cause the increased proinsulin:insulin ratio in WF mice. Moreover, it has been reported

that  $\text{Ca}^{2+}$  release from the endoplasmic reticulum is also suppressed by palmitate (55). Incubating sarcoplasmic reticulum in 50  $\mu\text{M}$  free palmitate (ie., unconjugated to BSA) for 30 min inhibited calcium uptake by 63% (55). Another possible mechanism involves free fatty acids binding to ATPase and altering the interaction between ATPase and membrane phospholipids (56). Since both optimal pH and  $\text{Ca}^{2+}$  maintenance inside insulin vesicles are ATP-dependent, an alteration of ATPase and membrane phospholipid interactions could result in impaired pH and  $\text{Ca}^{2+}$  environment inside the vesicle. As a result of all these functional changes, a sub-optimal environment for prohormone convertase activity may lead to the reduction of proinsulin conversion to insulin. However, these proposed mechanisms can only explain elevated proinsulin:insulin in KF but not KC mice. To better understand the mechanism of increased proinsulin:insulin in KC mice, direct measurement of prohormone convertase activity or the possible differentiation between proinsulin and insulin release could be assessed. It is also possible that factors other than free fatty acids or ATP regulate proinsulin processing. Recently, it has been reported that suppression of the transcription factor PDX-1 reduced proinsulin processing independently of ATP levels (57). PDX-1 expression has not been measured in relation to UCP2 expression. It is of interest to investigate this transcriptional factor to elucidate the role of UCP2 on PDX-1 regulated insulin gene transcription and translation as well as proinsulin synthesis and processing.

Interestingly, the present study found a greater content of proinsulin and insulin in UCP2 KO than WT islets and when UCP2 KO mice were fed with a

HFD, proinsulin and insulin content increased further. Gene transcription and translation are ATP-dependent processes. An important paradigm in transcription regulation is the influence of the chromatin organization and remodelling, which is ATP-dependent (20). Chromatin is remodelled before and during transcription initiation (58). Recently, it has become apparent that the mechanism of nucleosome mobilization by RNA polymerase and ATP-dependent remodelling are very similar (58). Normally, chromatin packages DNA tightly to fit within the nucleus, therefore chromatin must undergo structural reorganisation, including the breaking and reforming of histone-DNA contact, during DNA replication. Chromatin remodelling factors act by reformation of the nucleosome in an ATP dependent manner (59). Additionally, transcription initiation begins with the ATP-dependent phosphorylation of RNA polymerase II by transcription factor for polymerase II H (19). These two steps might be stimulated under conditions of elevated ATP. However, preproinsulin mRNA was not increased in UCP2 KO mice, indicating that the increase in insulin plus proinsulin content was not due to increased synthesis. To prove this point, further studies examining dynamic changes in preproinsulin transcription and insulin biosynthesis would need to be done, for example using pulse-chase methodology (60). Insulin itself is a positive auto-regulator of preproinsulin gene transcription (61). Insulin release from UCP2 KO islets under stimulated conditions was not greater than from WC islets. Therefore it is suggested that a similar level of positive auto-regulation of insulin should be found in UCP2 KO and WC mice.

In summary, the present study showed that five week consumption of HFD did not induce islet UCP2 expression nor reduce ATP generation in WT mice. Therefore, HFD must affect beta cell function, as indicated by blunted GSIS and reduced conversion to insulin of proinsulin by other mechanisms. Because UCP2 KO mice maintained normal GSIS, HFD seems to impair GSIS at least partly by mechanisms involving UCP2 activity without altering ATP synthesis. Moreover, UCP2 KO mice have an increase in the proinsulin:insulin ratio but the mechanism for this observation is unresolved.

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## CHAPTER 5

### GENERAL DISCUSSION

The main objective of this study was to investigate the effect of UCP2 on fatty acid induced impairment of insulin transcription, processing and secretion. To achieve this purpose, three studies were carried out using paradigms predicted to increase UCP2 expression. In the first study, UCP2 cDNA was directly transfected into INS-1 cells. In the second study, INS-1 cells were cultured in the presence of fatty acid (PA). The last study was an *in vivo* study in which WT and UCP2 KO mice were fed with either SRD or HFD for five weeks.

As expected, both UCP2 mRNA and protein expression were increased in UCP2-OE cells. In addition, greater UCP2 mRNA and protein expression was detected in INS-1 cells cultured in 0.4 mM PA, but not in WT mice fed with a HFD (WF mice). ATP content was reduced in UCP2-OE cells and INS-1 cells cultured in 0.25 and 0.4 mM PA, but not in WF mice. Conversely, UCP2 KO mice fed with either SRD or HFD had a greater ATP content than WT mice. The reduction of ATP content was unlikely to be due to ATP synthase because the expression of ATP synthase was similar in all groups, except in INS-1 cells under high potassium condition. Therefore it was confirmed that UCP2 expression is inversely correlated to intracellular ATP content, as shown previously (1). Exposure to elevated fatty acid concentration induced UCP2 expression only under some particular conditions. Failure to induce UCP2 expression in 0.25 mM PA treated cells as well as WF mice was unexpected. Both free unsaturated and saturated fatty acids are

UCP2 up-regulators (2). Culturing islets in high free fatty acid (2 mM) for 72 h induced UCP2 protein expression (3). Moreover, UCP2 protein expression was elevated in islets from mice fed with HFD for four and a half months (4). We speculate that lack of UCP2 induction in WF mice and 0.25 mM PA-cultured INS-1 cells was due to the relatively short duration of the diet in the first case and the low concentration of PA in the second case. Furthermore, UCP2 activity could be increased without an increase in expression. It is well known that free fatty acid induce reactive oxygen species and activate UCP2 (5). Neither reactive species generation nor UCP2 activity was measured in these studies. Alternatively, free fatty acid itself could act as a protonophore uncoupler via ATP/ ADP antiporter (6). Since ATP content was reduced in 0.25 mM PA exposed cells without an increase in UCP2 expression, it suggests that free fatty acid might either act as an uncoupler or induce UCP2 activity in 0.25 mM PA exposed INS-1 cells or WF mouse islets.

Glucose stimulated insulin secretion (GSIS) is one of the markers for beta cell dysfunction. Impaired GSIS was detected in UCP2-OE cells, 0.4 mM PA treated cells and WF mice. Conversely, UCP2 KO mice fed with HFD (KF mice) retained an insulin response to glucose. Superficially, these results are consistent with the hypothesis that increased UCP2 expression leads to reduced intracellular ATP and GSIS. However, beta cell UCP2 expression and ATP content were not altered in WF mice, but an impairment of GSIS was detected in this group. Thus, the effects of fatty acids on GSIS are not solely dependent on total ATP

concentration. Other potential effects of fatty acids on insulin secretion were discussed in Chapters 3 and 4.

Hyperproinsulinaemia is another marker for detecting beta cell dysfunction. The proinsulin:insulin ratio was increased in UCP2-OE cells cultured in high K<sup>+</sup> conditions, in 0.4mM PA treated cells, and in WF mice as well as KO mice fed either with SRD or HFD. Increased proinsulin relative to insulin could occur if proinsulin was being synthesized faster than mature insulin was being secreted. Total proinsulin and insulin content was increased in UCP2-OE cells, particularly those cultured in control medium (regular K<sup>+</sup>), which had low levels of secretion. Proinsulin plus insulin content was also increased in 0.4 mM PA treated cells and KF mice. However, insulin gene transcription was not uniformly elevated under all of these conditions and insulin secretion was normal in KF mice, so it seems unlikely that the above hypothesis applies in all conditions studied. However, because SPC1/3 expression was not decreased in any of these conditions, reduction of processing capacity can be ruled out. Moreover, SPC1/3 was elevated in INS-1 cells supplemented with high K<sup>+</sup>. As mentioned in Chapter 2, under continual depolarization by elevated K<sup>+</sup>, INS-1 cells maintained insulin content and increased expression of SPC1/3, possibly as a compensation for the greater demand for insulin secretion.

A second explanation for an increase in the proinsulin:ratio is disruption of the secretory granule environment to the extent that SPC1/3 (and SPC2) activity is decreased. The main hypothesis of this thesis was that induction of UCP2 and subsequent reduction of intracellular ATP would precipitate such conditions. The

elevation of UCP2 expression and reduction in ATP content found in 0.4 mM PA treated cells could explain why proinsulin:insulin ratio was increased in this group and in UCP2-OE cells cultured in elevated  $K^+$  medium. However, an increased proinsulin:insulin ratio was not found in INS-1 cells cultured in 0.25 mM PA even though ATP was significantly reduced. To increase the complexity, WF mice had increased proinsulin:insulin and normal intracellular ATP. Therefore, no consistent relationship between cellular ATP and proinsulin processing was established in these studies. To better understand this lack of correlation, future experiments aimed at direct measurement of the granule microenvironment under the various conditions could be performed.

Both the 0.4 mM PA treated cells and the UCP2-OE cells cultured in control medium had elevated UCP2 and reduced ATP, but only PA-treated cells had an elevated proinsulin:insulin ratio. We observed that 0.4 mM PA treated cells had higher basal insulin secretion, which might cause beta cell exhaustion. These results are consistent with those from many other laboratories showing that culturing beta cells in high free fatty acid increases basal insulin secretion while suppressing GSIS (7). Culturing INS-1 cells in 0.4 mM PA medium containing 11 mM glucose for 48 h likely stimulated more total insulin secretion compared to culturing UCP2-OE cells in 5 mM glucose. Consistent with the idea that increasing demand in the face of sub-optimal processing conditions could increase proinsulin:insulin, we found that culturing UCP2-OE cells in high  $K^+$  also increased the proinsulin:insulin ratio. In addition, high free fatty acids in 0.4 mM PA treated cells could activate  $K_{ATP}$  channel and alter the interaction between

ATPase and phospholipids, as described in Chapter 4, thereby reducing the activity of SPC1/3 and resulting in elevation of proinsulin: insulin ratio. However, to verify whether the elevation of proinsulin :insulin ratio in 0.4 mM PA treated cells is due to the effect of UCP2 induced by high free fatty acid, the inhibition of UCP2 expression such as using small interference RNA of UCP2 ( siRNA) needs to be conducted.

Insulin gene transcription also depends on ATP availability. Therefore preproinsulin mRNA was measured. Preproinsulin I mRNA was increased in UCP2-OE cells under regular K<sup>+</sup> conditions, but not in UCP2-OE cells under high K<sup>+</sup> conditions. These results suggested that insulin synthesis is normal or higher despite the reduction of ATP content in UCP2-OE cells. There was also an overall increase in preproinsulin mRNA in 0.4 mM PA treated cells whereas preproinsulin mRNA was not changed in islets of any group of the HFD study. These results do not support the hypothesis that UCP2 induction and ATP reduction affect insulin gene transcription.

The present study raises many more questions to answer. Firstly, does elevated K<sup>+</sup> directly regulate gene expression in beta cells? This question arises from the observation that cells cultured in high K<sup>+</sup> had altered ATP synthase, SPC1/3 and insulin gene expression. To address this question, the relationship between K<sup>+</sup>, UCP2 and gene expression need to be examined. Second, we unexpectedly observed that UCP2 KO mice had a greater proinsulin:insulin ratio than WT mice despite a higher islet ATP content. To better understand the mechanism of increased proinsulin:insulin in UCP2 KO mice, direct measurement

of SPC1/3 activity, granule formation and microenvironment, should be assessed. Whether UCP2 KO mice also secrete higher amounts of proinsulin should be measured. Factors unrelated to substrate metabolism and ATP may affect proinsulin processing. Recently, it has been reported that suppression of PDX-1 reduced proinsulin processing (8). In order to explain elevated proinsulin:insulin in KO mice fed with control diet, a comparison of the role of PDX-1 regulated insulin processing in WT and UCP2 KO mice needs to be carried out.

In summary, the present study showed that UCP2 could reduce ATP content without altering ATP synthase, thereby impairing GSIS. UCP2-dependent effects on insulin gene transcription and proinsulin processing could be demonstrated in the UCP2-OE model. Free fatty acid exposure of beta cells also caused impaired insulin processing, but this could not be directly attributed to UCP2 gene expression or correlated with reductions in ATP production. Lastly, HFD for 5 weeks in WT mice was sufficient to impair GSIS and the proinsulin : insulin ratio but HFD did not significantly increase UCP2 expression or reduce intracellular ATP. This suggests that the increase in UCP2 expression after more than 4 months of HFD is a later adaptation to the dietary regime. However elevation of proinsulin: insulin ratio was observed in UCP2 KO mice suggesting that not only HFD but also UCP2 can play a role in the maturation of insulin.

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## APPENDICES

### APPENDIX-A: Western blotting for proinsulin:insulin ratio

#### Separation Gel (15%)

dH <sub>2</sub> O	2.4ml
Buffer (pH8.8)	2.5ml
30%Acrylamide	5.0ml
10%APS	100μl
TEMED	5 μl

#### Stacking gel (4%)

dH <sub>2</sub> O	3.05ml
Buffer (pH6.8)	1.25ml
30%Acrylamide	0.67ml
10%APS	50μl
TEMED	5 μl

#### 10X running buffer&10X Transfer buffer

Tris	30.3g
Glycine	144.0g
dH <sub>2</sub> O	up to 1000ml

working solution : mix 10 ml of 10X running buffer in 90 ml of dH<sub>2</sub>O

working solution fro transfer buffer : mix 10 ml of methanol in 90 ml 1X transfer buffer

#### Stacking buffer pH6.8

0.5 M Tris (Tris 30.25g adjusted pH to 6.8 with HCl; bring up to 500ml)

### **Separating buffer pH 8.8**

1.5M Tris ( Tris 90g adjusted pH to 8.8 with HCl; bring up to 500ml)

### **0.1M PBS**

dibasic sodium phosphate 1.3 g/L

monbasic sodium phosphate 0.23g/L

Sodium chloride 8.8g/L

### **T-PBS**

0.1M PBS 198 ml

Tween 20 2ml

### **2X Sample loading buffer**

100mM Tris-HCL (pH6.8)

2% mercaptoethanol

0.02% bromphenol blue

12% glycerol

### **Stripping solution**

1M glycine( adjusted pH to 2.5 with HCl)

### **Procedure:**

1. Combine in a 15ml tube all the solutions of sparating gel, then swirl gently to avoid the air bubbles.
2. Slowly pour the separating gel into the space between the glass plates. Overlay gel with dH<sub>2</sub>O, allow at least 30 min to polymerized

3. Combine in a 15ml tube all the solutions of stacking gel, then swirl gently to avoid the air bubbles.
4. Slowly pour the separating gel into the space between the glass plates. Carefully insert comb, allow at least 20 min to polymerized
5. After gel is completely polymerized, remove comb and cassette and place the glass plate and gel into electrode assembly.
6. Add Running buffer into the tank on both sides of the chamber
7. Load the samples after mixing with 2X loading sample into well run it at 100 V for 15 min, then increase to 150 V for ~60 min before the dye front run off the gel.
8. Putting gel and nitrocellulose in the transfer assembly and run it at 100 V for 1 h
9. Take nitrocellulose out off the transfer assembly and place it face up in 5% skim milk in T-PBS O/N at 4°C
10. Wash membrane quickly in 2X PBS, then 15 min in PBS, 5 min in PBS and 5 min in T-PBS
11. Dilute primary antibody in T-PBS, incubate membrane in primary antibody at room temperature 1h
12. Wash membrane 2X PBS 15 min and 1X in T-PBS 15 min
13. Dilute secondary antibody in T-PBS, incubate membrane in secondary antibody at room temperature for 1h
14. Wash membrane 2X T-PBS 15 min and 1X in PBS 15 min

15. Developing signal using ECL

16. For stripping procedure:

Incubate membrane 2X 15 min in stripping solution, then 2X  
15 min in PBS and proceed with incubate O/N in 5% skim  
milk

Proceed the regular protocol for incubate in antibody

## **APPENDIX-B: Western blotting for Prohormone convertase, ATP synthase,**

### **Beta actin and UCP2**

#### **Separation Gel (12%)**

dH <sub>2</sub> O	3.3ml
Buffer (pH8.8)	2.5ml
30%Acrylamide	4.0ml
10% SDS	100 $\mu$ l
10%APS	100 $\mu$ l
TEMED	5 $\mu$ l

#### **Stacking gel (4%)**

dH <sub>2</sub> O	3.05ml
Buffer (pH6.8)	1.25ml
30%Acrylamide	0.67ml
10% SDS	100 $\mu$ l
10%APS	50 $\mu$ l
TEMED	5 $\mu$ l

#### **10X running buffer**

Tris	30.3g
Glycine	144.0g
SDS	10.0g
dH <sub>2</sub> O	up to 1000ml

#### **10X Transfer buffer**

Tris	30.3g
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Glycine 144.0g

dH<sub>2</sub>O up to 1000ml

working solution : mix 10 ml of methanol in 90 ml 1X transfer buffer

### **Stacking buffer pH6.8**

0.5 M Tris (Tris 30.25g adjusted pH to 6.8 with HCl; bring up to 500ml)

### **Separating buffer pH8.8**

1.5M Tris (Tris 90g adjusted pH to 8.8 with HCl; bring up to 500ml)

### **0.1M PBS**

dibasic sodium phosphate 1.3 g/L

monbasic sodium phosphate 0.23g/L

Sodium chloride 8.8g/L

### **T-PBS**

0.1M PBS 198 ml

Tween 20 2ml

### **2X Sample loading buffer**

100mM Tris-HCL (pH6.8)

2% mercaptoethanol

0.02% bromphenol blue

3.4% SDS

12% glycerol

### **Stripping solution**

1M glycine( adjusted pH to 2.5 with HCl)

**Procedure:**

1. Combine in a 15ml tube all the solutions of sparating gel, then swirl gently to avoid the air bubbles.
2. Slowly pour the separating gel into the space between the glass plates. Overlay gel with dH<sub>2</sub>O, allow at least 30 min to polymerized
3. Combine in a 15ml tube all the solutions of stacking gel, then swirl gently to avoid the air bubbles.
4. Slowly pour the separating gel into the space between the glass plates. Carefully insert comb, allow at least 20 min to polymerized
5. After gel is completely polymerized, remove comb and cassette and place the glass plate and gel into electrode assembly.
6. Add Running buffer into the tank on both sides of the chamber
7. Load the samples after mixing with 2X loading sample into well run it at 100 V for 15 min, then increase to 150 V for ~60 min before the dye front run off the gel.
8. Putting gel and nitrocellulose in the transfer assembly and run it at 100 V for 1 h
9. Take nitrocellulose out off the transfer assembly and place it face up in 5% skim milk in T-BPS O/N at 4°C
10. Wash membrane quickly in 2X PBS, then 15 min in PBS, 5 min in PBS and 5 min in T-PBS

11. Dilute primary antibody in T-PBS, incubate membrane in primary antibody at room temperature (1h for prohormone convertase- antibody and ATP syntahse- antibody, 45min for beta actin- antibody and 2 h for UCP2-antibody)
12. Wash membrane 2X PBS 15 min and 1X in T-PBS 15 min
13. Dilute secondary antibody in T-PBS, incubate membrane in secondary antibody at room temperature for 1h
14. Wash membrane 2X T-PBS 15 min and 1X in PBS 15 min
15. Developing signal using ECL
16. For stripping procedure:

Incubate membrane 2X 15 min in stripping solution, then 2X 15 min in PBS and proceed with incubate O/N in 5% skim milk. Proceed the regular protocol for incubate in antibody