University of Alberta

Response of Neonatal Porcine Islets to Altered *In Vitro* Conditions

by

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ABSTRACT

Pancreatic islet transplantation as a means to treat diabetes has become a hotbed of research in transplant medicine. However, progress is impeded by a severe shortage of human cadaveric tissue suitable for transplantation. Neonatal porcine islet (NPI) tissue is a potential unlimited alternative tissue source, but many of its qualities remain unknown. The unifying aim of this thesis is to assess and describe characteristics of NPI that render it uniquely suitable for islet transplantation.

Sensitivity to the lipotoxic effect of chronically elevated free fatty acids is a primary concern in the search for an alternative tissue for islet transplantation, as dysfunctional lipid metabolism and hyperlipidemia are commonly present in diabetes. No significant effect on islet survival, insulin content, or glucose responsiveness was observed in NPI incubated with a physiologically representative mixture of fatty acids. However, significant toxic effects, which were not ameliorated by addition of a pan-caspase inhibitor, were noted when NPI were treated with single types of fatty acids. Interestingly, the caspase inhibitor had a beneficial effect when added to NPI in isolation, yielding significantly larger β-cell populations than in control cultures.

The *in vitro* administration of a series of growth factors did not appear to stimulate β-cell proliferation or differentiation of progenitors. A significant increase in insulin biosynthesis was observed, which may prove useful in the transplant setting to reduce islet mass requirements or the time required to correct diabetes.

In summary, the resilience of NPI to toxic surroundings, and their ability to respond positively to external factors, as demonstrated in this thesis, further establish NPI as an advantageous alternative source of transplantable islet tissue.

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List of Abbreviations

ABC	Avidin-Biotin complex
ACC	Acetyl-CoA carboxylase
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
bHLH	Basic helix-loop-helix
BSA	Bovine serum albumin
CK	Cytokeratin
CPT-I	Carnitine palmitoyl transferase I
Ct	Cycle threshold
DAB	3,3 diaminobenzidinetetrahydrochloride
DMEM	Dulbecco's Modified Eagle's Medium
ER	Endoplasmic reticulum
FA	Fatty acid
FFA	Free fatty acid
FITC	Fluoroscein isothiocyanate
Gal-α-gal	Galactose-α(1,3)-galactose
GFP	Green fluorescent protein
GLP-1	Glucagon-like peptide 1
GSIS	Glucose-stimulated insulin secretion
HBSS	Hank's Balanced Salt Solution
IBMIR	Instant blood-mediated inflammatory response
IEQ	Islet equivalent
Ngn3	Neurogenin 3
NOD	Non-obese diabetic
NPI	Neonatal porcine islet
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pdx-1	Pancreatic duodenal homeobox gene-1

PERV	Porcine endogenous retrovirus
PPARα	Peroxisome proliferators-activated receptor α
PUFA	Polyunsaturated fatty acid
SEM	Standard error of the mean
SI	Stimulation index
SFA	Saturated fatty acid
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TG	Triglyceride
TGFβ	Transforming growth factor β
TUNEL	TdT-mediated dUTP nick-end labeling
UCP2	Uncoupling protein 2
UPR	Unfolded protein response

Chapter One
General Introduction

1.1. Diabetes Mellitus

Diabetes mellitus can be defined as a group of chronic and progressive metabolic disorders characterized by hyperglycemia that causes significant macro- and microvascular complications, including retinopathy, neuropathy, nephropathy, and cardiovascular disease. There are two major forms of diabetes mellitus; type 1 (T1DM) and type 2 (T2DM). The two disease forms are unique in that the first is an autoimmune disorder resulting from the immune system-mediated destruction of the insulin-secreting pancreatic β-cells, while in the second form, the β-cells are still intact, but are no longer able to respond adequately to maintain glycemic homeostasis. However, both types of diabetes result in profound metabolic dysfunction, and lead to degradation in both general health and quality of life. The World Health Organization (WHO) estimates that 171 million people worldwide had diabetes in 2000, and that number will nearly double by 2025 (1), with most of the increase concentrated in the developing world (2). Diabetes is the seventh leading cause of death in Canada (3), and it is estimated that the treatment costs of both the disease and its complications in Canada's two million diabetics tops $13.2 billion each year, and is only projected to increase (4).

1.1.1 Type 1 Diabetes Mellitus

Type 1 diabetes mellitus, formerly known as juvenile diabetes, is the far less common form of diabetes, accounting for about 10% of all diabetes diagnoses, and almost half of all type 1 diabetics are diagnosed before the age of 20. While a definitive cause of T1DM remains elusive, diagnoses of T1DM have been associated with variations in a number of genes, including those for the major histocompatibility complex (MHC) and proinsulin (5;6). Environmental factors have not been ruled out as a contributing factor in T1DM.

T1DM is characterized by the presence of a number of anti-islet autoantibodies, followed by the infiltration and ultimate destruction of the pancreatic β-cells by

autoreactive T-lymphocytes. The mechanism that initially draws the cytotoxic T-lymphocytes to the pancreas is incompletely understood (7). During the autoimmune attack, the β-cells exhibit increasing functional impairment and decreasing total mass, eventually reaching a state in which there is no longer sufficient β-cell mass to secrete insulin necessary to maintain metabolic homeostasis, and a state of severe metabolic dysfunction, characterized by hyperglycemia, hypoinsulinemia, and abnormal lipidemia (8) is established.

Current treatment of T1DM involves multiple daily injections of exogenous insulin, lifestyle modification, and intensive monitoring of blood glucose in order to avoid both hypo- and hyperglycemia. However, research is directed into multiple modes of treatment, including "immunotherapy" (9), in which a pharmaceutical form of immunoregulatory intervention seeks to prevent or halt the autoimmune attack on β-cells, and encourage restoration of β-cell mass, as well as islet transplantation, which will be discussed in more detail below.

1.1.2 Type 2 Diabetes Mellitus

Non-insulin dependent, or type 2, diabetes mellitus (T2DM) has no known single cause, but risk factors include age, weight status, and family history. T2DM accounts for approximately 90% of all diabetes diagnoses, and is largely responsible for the rapidly increasing rates of diabetes prevalence. While incidence of T1DM remains relatively stable, T2DM incidence has increased at approximately 6% each year since 1980 in the United States (10), to over 14 million Americans with T2DM in 2005 (11). Given these bleak numbers, and the fact that diabetes occurrence is now increasing faster than 1995 predictions by the WHO (12), largely because of an increasingly older, more obese, and more sedentary population, especially in the Western Hemisphere, it is imperative that research be directed towards the amelioration of diabetics' quality of life, if not curing the disease entirely.

Peripheral insulin resistance, characterized by a diminished insulin responsiveness in peripheral tissues such as skeletal muscle and adipose tissue, is often present years before a T2DM diagnosis (13). For a time, the β-cells are able to compensate for insulin resistance, by way of increased insulin secretion; when β-cells are no longer

able to secrete sufficient insulin to sustain normoglycemia, T2DM results (14;15). A significant decrease in total β-cell mass is also commonly present in type 2 diabetics (16), which exacerbates the pancreas' inability to control hyperglycemia. High serum levels of free fatty acids, or hyperlipidemia, commonly present in individuals with T2DM (17), has been linked to both induction of insulin resistance in peripheral tissues (13) and β-cell dysfunction (17). Hyperlipidemic environments have been observed to induce the accumulation of triglycerides in non-adipocytes, including β-cells (18). The resultant cell dysfunction and cell death, which has been observed in cells as varied as hepatocytes and cardiac myocytes (17;19), has been termed **lipotoxicity**. There is a substantial amount of evidence indicating that hyperlipidemia is necessary to, but not sufficient to induce independently, the pathogenesis of T2DM. The current modes of treatment for T2DM encompass a number of pharmacological agents, including insulin secretagogues, insulin sensitizers, and inhibitors of glucose uptake, often in combination with lifestyle intervention, and in the most serious of cases, administration of exogenous insulin (20).

1.2. A Historical Overview

1.2.1 The Pancreas

Although first described by the Greek anatomist and surgeon Herophilus (335 – 280 B.C.), the pancreas was relatively unknown among ancient physicians and anatomists (21). Indeed, the direct translation of its name, *pan kreas* or "all flesh" makes historical records ambiguous as to whether they are in fact referring to the organ now known as the pancreas.

The pancreas was a recognized organ amongst anatomic researchers of the Renaissance, though the first known picture of the pancreas, produced by the Roman anatomist Bartolomeo Eustachio (1520-1574) was not published until 1714 (21). Despite recognition of its presence, the role of the pancreas remained obscure, and early researchers proposed that it functioned as a cushion for the stomach, or as protection for the splenic vein. It was not until the discovery of the pancreatic duct in

1642 by Johann Georg Würstung that the pancreas' role as a secretory gland was established (21).

Although the first known description of diabetic pathology, "overabundant urine", dates to approximately 1550 B.C., and documentation of a disorder characterized by excretion of large quantities of sweet urine is found across ancient cultures, it was not until the work of Etienne Lanceraux in 1887 that the relationship of the pancreas to diabetes was established (21). This relationship was further verified when JF Mering and Oskar Minkowski observed the development of diabetes in dogs following complete pancreatectomy.

1.2.2 The Islets of Langerhans

Dr. Paul Langerhans, the physician accredited with the initial identification of the endocrine cellular aggregates now known as the islets of Langerhans, was by all accounts a thorough researcher with a keen eye for detail. Born in 1847 in Berlin into a family of physicians, Langerhans began his own medical studies in 1866 in Jena, transferring after one year to Berlin, where he worked under and was heavily influenced by the famed pathologist Rudolf Virchow (21). Demonstrating an aptitude for anatomical investigation early in his career, Langerhans published his first study, an investigation of the nerves in human skin, in 1868.

In 1869, Langerhans published a study on tactile corpuscles in diseases of the nervous system and skin, and delivered his landmark (though not recognized as such at the time) dissertation for a doctor's degree in medicine and surgery at Friedrich Wilhelms University, *Contributions to the Microscopic Anatomy of the Pancreas*[1]. Before this work, the pancreas was largely classified as a salivary gland, thanks in large part to the work of Claude Bernard, who identified the role of pancreatic secretions in the digestions of fats (22). Perhaps foretelling of the mystery that continues to shroud the endocrine cells of the pancreas, Langerhans himself stated,

[1] The German original was entitled *Beiträge zur Mikroskopischen Anatomie der Bauscheicheldrüse.*

> "I can describe, at most, a few isolated observations which suggest a more complicated structure of the organ investigated than hitherto accepted." (23).

Langerhans based the majority of his work on the rabbit pancreas, as its thin, laminated structure was more conducive to anatomical study than the pancreata of guinea pigs or humans. Upon maceration of the pancreatic tissue, Langerhans observed 9 unique cell types, 2 of which had not previously been described; peritoneal and ductal epithelial cells, connective tissue, vascular cells, blood cells, nerve cells, acinar cells, as well as the novel "spindle", or centro-acinar, cells, and *Häuflein*, the small cells "[lying] together generally in considerable numbers scattered diffusely in the parenchyma of the gland". Langerhans could not provide an explanation as to the role of these cellular aggregates, and it was not until 1893, almost 25 years later, that the French histologist Laguesse, assigning these bodies the name *îlots de Langerhans*, suggested they played a role in internal secretion (23). Langerhans continued his investigative work, studying, among other things, the accessory glands of the male genital organs at Freiburg im Breisgau, until poor health forced him to relocate to Madeira, Spain, in 1875 (23). There, Langerhans remained active, performing zoological studies and acting as physician to many German ex-patriots in the area; throughout this time, Langerhans continued to publish papers in *Virchow's Archives* (21). Langerhans' broad and prolific research career was cut tragically short when he died of kidney disease in 1888, just shy of his 41st birthday. Other researchers picked up where Langerhans left off in the study of the cellular bodies named in his honour. However, it was not until 1907, nearly 40 years after Langerhans' original description, that Lane established the heterogeneous cellular composition of islets, based on the differing solubility of secretory granules in individual cells (24). In 1957, Paul E. Lacy, using electron microscopy, described the ultrastructure of the now denoted α- and β-cells in several species, as well as the surrounding capillary and nerve fibres (25). He was also able to establish some of the differentiating characteristics of α- and β-cells, including the smaller Golgi apparatus and reduced endoplasmic reticulum in the α-cells. Notably, 1957 was also the year

when Lacy first demonstrated the ability of fluorescent-labeled antibodies reactive to insulin to label islets in certain species, thus establishing what would become a common method of β-cell identification (26). In 1961, Lacy described the sequence of events involved in the secretion of β-granules, largely believed at that time to be insulin or its precursor, and defined this mechanism as *emiocytosis* (27). Investigation of the cells of the islets of Langerhans continues today, addressing areas as varied as insulin gene regulation (28), the proliferation and redifferentiation potential of adult islets (29), and mechanisms regulating insulin secretion (30).

1.2.3 Insulin

The role of insulin deficiency, both as part of the diabetic pathology and a mode by which to treat diabetes, is a relatively recent concept. Prior to the discovery and isolation of insulin, diabetes treatment consisted of a diet low in carbohydrates, and "opotherapy", or the consumption of raw veal pancreas. However, the discovery of insulin in 1921 by Frederick Banting, Charles Best, James Collip and J.J.R. MacLeod forever changed the face of diabetes treatment.

The complete story of the discovery of insulin, too long to be fully described here, is worthy of a place among the great legends of scientific discovery. Briefly, Banting convinced MacLeod, then a Professor in the Department of Physiology at the University of Toronto, to provide him with lab space, and a student assistant (who was, consequently, Best), with which to conduct his experiments for the eight weeks of summer that MacLeod himself would be on vacation in Scotland. On July 27, 1921, Banting and Best injected an extract isolated from atrophied canine pancreata into a dog previously made diabetic by pancreatectomy (21). Within hours, the diabetic dog's blood glucose levels fell, and the dog recovered from diabetic coma. Elated with their result, Banting and Best repeated their work and convinced MacLeod it was worth pursuing. Collip, a chemist in the department, set to work purifying Banting and Best's extract.

On January 11, 1922, only 6 months after its original discovery, insulin was first used in the treatment of a diabetic patient; in this case a 14-year-old diabetic male (21). The marked success in this, and other, early patients lead to widespread demand for

insulin, soon exceeding the production capabilities of the group at the University of Toronto. Thus, in May 1922, an agreement was reached between the Board of Governors of the University of Toronto and Eli Lilly and Company in which Eli Lilly would produce the pancreatic extract at cost in return for the company's exclusive right for the sale of insulin in US and the Americas (21). Even at this early point, the group seemed cogniscent of the huge impact their discovery would have on the treatment of diabetes worldwide; less than 8 months after their initial observations in the diabetic canines, they wrote in the Canadian Medical Association Journal,

> "These results taken together have been such as to leave no doubt that in these extracts we have a therapeutic measure of unquestionable value in the treatment of certain phases of the disease in man." (31)

In 1923, MacLeod and Banting were awarded the Nobel Prize in Physiology or Medicine for the discovery. The fact that Best was not included in the award was then, and remains, a point of contention. Two other Nobel Prizes have been awarded in the course of the study of insulin; Frederick Sanger won the prize for Chemistry in 1958 for his 1955 report of the structure of insulin, and Rosalyn Yalow won the prize for Medicine or Physiology in 1977 for her development of a method of radioimmunoassay for plasma insulin (21).

1.2.4 Islet Transplantation

Islet transplantation is a form of cellular transplantation, in which a distinct cellular population (in this case, the pancreatic islet), as opposed to complete organ composed of multiple cell types, is transplanted. The modern era of clinical cell transplantation can be traced to 1956, when E. Donnall Thomas performed the first human bone marrow transplant (32). Investigation into clinical islet transplantation progressed slowly, due in part to the fact that a reliable and reproducible method of isolating islet tissue from crude pancreatic tissue had to be developed. The underlying motivation behind islet transplantation was based on the observation that following ligation of the pancreatic duct, the exocrine tissue of the pancreas rapidly atrophied, while the

islet tissue remained functional. Thus, it was thought perhaps exocrine tissue was more antigenic in the transplant setting, and by removing it from the graft, the likelihood of tissue rejection would decrease (21;32). The first successful islet transplant was performed in 1966 at the University of Minnesota by Kelly and associates (21); the recipient achieved insulin independence, but the graft was subsequently removed due to rejection. Dr. Paul E. Lacy, then working at the Washington University School of Medicine, in addition to his previously described work in islet characterization, was a pioneer in the field of islet transplantation; his accomplishments included developing the primary method of processing islets from smalls animals in the 1960s (33), performing the first rodent islet transplant to correct diabetes in 1972 (34), and in 1989, performing the first human islet allograft via intra-portal infusion that resulted in insulin independence (35).

Outside the diabetes research community, islet transplantation remained relatively unknown, largely due to its limited success rate; in 1995, fewer than 10% of all attempted islet transplants had resulted in insulin independence (36), an outcome widely considered the gold standard in terms of the success in islet transplantation. It was not until 2000, when a group of researchers from the University of Alberta in Edmonton, Canada, were able to produce insulin independence in 7 consecutive islet transplant patients following a glucocorticoid-free immunosuppressive regime (37), that the concept of pancreatic islet transplantation caught the public interest. Publication of follow-up studies of the transplants, indicating dramatically improved metabolic control (38;39) fanned the flames of public interest in islet transplants and the Edmonton Protocol. Enthusiasm towards islet transplantation has since cooled, as the long-term outcome of islet transplants is not as positive as it originally seemed; only 10% of recipients remain insulin-independent 5 years after undergoing islet transplantation (40). However, the enhancement of quality of life following islet transplantation, regardless of the status insulin independence, makes islet transplantation a highly attractive therapeutic option for patients with brittle diabetes. Despite recent indications that islet transplantation can be successfully reproduced at multiple centres (41), the path towards islet transplantation becoming common practice is fraught with challenges, foremost of which is the lack of sufficient donor

tissue. Currently, the islet transplant process involves the procurement of cadaveric pancreata, enzymatic digestion of the crude pancreas and density gradient purification, and finally percutaneous intrahepatic infusion of the islets (36). At each step of the process, the yield of islet tissue decreases slightly. The transplant procedure itself may induce the instant blood-mediated inflammatory response (IBMIR), which activates the coagulation and complement cascades, and may in turn lead to a strong immune response and destruction of the transplanted tissue (36). If the tissue is able to engraft, it may be subject to exhaustion in an environment of high metabolic demand in combination with a highly toxic environment of immunosuppressive drugs in the liver. Moreover, there is significant potential for a destructive immune response, in terms of both rejection of the allogeneic transplant, and recurrence of the β-cell based autoimmunity characteristic of T1DM. Current research is directed at all levels of the islet transplant process, from pancreas procurement and islet isolation, to pre-treatment of the islets to enhance immediate post-transplant survival, to development of enhanced immunosuppressive protocols, all with the aim of increasing the level of success observed with islet transplantation. Moreover, there has been some indication that the cellular composition, including both cell type and islet size, significantly affects the long-term outcome of the islet transplant (42;43).

As the procedure currently stands, at least 12,000 islet equivalents per recipient body weight (IEQ/kg) are required to achieve insulin independence, which implies more than one donor is required for each recipient (36). To overcome the shortage of islet tissue suitable for transplant, research is devoted to essentially three alternate sources of islet tissue: xenogeneic, stem cell-derived (both adult and embryonic), and induced regeneration/expansion of mature pancreatic tissue. While limited success has been achieved in the latter two areas, they are beyond the scope of this thesis, and the more relevant topic of xenogeneic islet transplantation will be discussed. Several excellent reviews regarding all three alternate islet sources may be referenced for further information (44-49).

1.2.5 Islet Xenotransplantation

The porcine pancreatic islet is generally accepted as the most plausible source of xenogeneic islets suitable for transplantation. Recently, islets procured from both neonatal (50) and adult (51) pigs have been shown to correct diabetes in non-human primates.

Porcine derived islets pose a desirable source of transplantable tissue for numerous reasons. Porcine insulin is very similar to human, differing by a single amino acid (24), and has been used in the treatment of diabetes in humans. Moreover, porcine islets respond to glucose thresholds similar to humans, ensuring appropriate glycemic control once transplanted. Pigs breed rapidly, have large litters, and through the maintenance of "clean" herds as well as genetic modification, there is potential to eliminate porcine endogenous retrovirus (PERV) and other endogenous porcine viruses, as well as the Gal-α-gal antigen. Gal-α-gal is a carbohydrate surface antigen expressed by porcine endothelial cells in an age-dependent manner to which humans have pre-formed natural antibodies, thus putting porcine islet xenografts at risk for hyperacute rejection (52). Both these challenges demand the exercise of caution during progression toward clinical application of islet xenotransplantation.

Although studies have been performed with pig islets from all stages of development *ie.* fetal, neonatal, and adult, it can be argued that neonatal islets present the ideal compromise between fetal- and adult-derived islets. Neonatal islets possess a moderate degree of both growth potential and glucose-responsiveness, but lack the fragility demonstrated by adult-derived islets (53). Moreover, it has recently been shown that neonatal porcine islets are markedly robust in the typically toxic environments of elevated glucose (54) and hypoxia (55). In 1996, Korbutt *et al.* demonstrated not only the ability to reproducibly isolate and characterize neonatal porcine islets, but the ability of these islets to correct diabetes in nude mice (53). This was followed by their demonstration in 2005 of the correction of pancreatectomy-induced diabetes by allotransplantation of neonatal porcine islets into 2- to 3-month old pigs (56).

There have also been a small number of reports of xenotransplantation of porcine islet tissue into diabetic humans (57-59), but the success of these studies remains limited, even several years later (60-62).

1.3. A Biological Overview

1.3.1 Pancreatic Development

Organogenesis of the pancreas in the developing embryo is an incompletely understood phenomenon, and investigation to develop a coherent picture of the overall process from its earliest stages is ongoing (63;64). Indeed, it is hardly surprising that the development of an organ that contains exocrine, ductal, and endocrine tissue, which itself consists of at least five distinct cells types, is decidedly complex. For a complete description of what is currently known about pancreas organogenesis, the reader is referred to a number of excellent reviews (65-68); this thesis will limit itself to a brief description of pancreas organogenesis.

It is currently believed that pancreatic progenitor cells are multipotent; they are equally capable of ultimately differentiating to endocrine or exocrine cells, and their fate may rely on epithelial-mesenchymal interactions (64). Moreover, pancreatic endocrine cells are remarkably similar to neuronal cells in development and specification, doing so by way of lateral specification mediated by Notch signaling. In pancreatic development, Notch signaling appears to "hold off" the differentiation of progenitor cells, thus maintaining their capacity for proliferation, until some unknown trigger drives differentiation to their final phenotype (64-66), as endocrine, exocrine, or ductal cells.

The embryonic pancreas originates from dorsal and ventral protrusions from the primitive gut endodermal epithelium, appearing at approximately embryonic day 8.5 (E8.5) in mice and E28 in humans. The dorsal pancreatic bud is in direct contact with the notochord, exposing it to notochord-derived factors such as activin-β and fibroblast growth factor (FGF)-2. In sharp contrast, the ventral pancreatic bud is completely independent of the notochord, bringing into question what role, if any, activin-β and FGF-2 have in early pancreatic development (69). Moreover, not only does the mesenchyme surrounding the dorsal bud differ from that surrounding the ventral bud in its expression of *Isl1* (65), but the two buds develop at different rates, with endocrine cells appearing in the dorsal bud more than 24 hours before their

appearance in the ventral bud. Despite these independent development environments and patterns (65), the two distinct buds branch and grow to eventually fuse, forming the pancreas proper. The surrounding mesenchyme supports the proliferation of the epithelial buds via the expression of *Isl1, N-cadherin,* and *Pbx1* (68), as well as the later differentiation of the pancreatic epithelium (65;66).

The homeodomain transcription factor *Pdx1* is expressed by all pancreatic progenitor cells prior to and during the budding process, though the development of pancreatic buds in *Pdx1* mutants indicates the involvement of other factors upstream of *Pdx1* in pancreatic precursor specification (68). The transcription factor *Ptf1a* is also expressed throughout the early pancreas. Both *Pdx1* and *Ptf1a* expression subside in undifferentiated precursor cells during the period of marked bud growth, only to reappear during the "secondary transition", during which endocrine precursors are driven towards a β-cell fate. The basic helix-loop-helix (bHLH) transcription factor neurogenin 3 (*Ngn3*) is expressed transiently by all endocrine precursors, and though necessary for islet cell development, is downregulated during differentiation of endocrine precursor cells (64), at which time the cells begin to organize into islet-like aggregates. The newly differentiated endocrine cells are non-mitotic, but resume proliferative activity near birth (64;70), contributing to a four-fold increase in β-cell mass in the first two weeks after birth (71;72). Pancreatic α-cells typically appear several days before β-cells, but do not appear to be an intermediate stage in β-cell formation (68). β-cell fate may be driven by a number of factors, including *Pax4*, the NK-homeodomain genes *Nkx2.2* and *Nkx6.1*, and the *Ngn3*-related transcription factor *NeuroD*, all present downstream of *Ngn3*; however, the subsequent differentiation among islet cell sub-types remains ill-defined (73).

1.3.2 The Mature Pancreas

The adult pancreas is a heterogeneous organ, consisting of three cell types: exocrine acinar cells responsible for the production and secretion of digestion enzymes; epithelial ductal cells; and endocrine cells, whose secretions are responsible for maintaining glycemic homeostasis. Acinar cells make up the vast majority of the pancreas, and convey their secretions via a highly branched ductal tree structure that

collectively terminates in the common bile duct, which opens directly to the intestine. The endocrine tissue, which makes up approximately 2% of the total pancreas mass, is distributed throughout the organ in globular cellular aggregates, the previously described islets of Langerhans. The endocrine tissue is further sub-divided into five distinct cell types: glucagon-producing α-cells (15-20% of total islet mass); insulin-producing β-cells (60-80% of total islet mass); somatostatin-producing δ-cells (5-10% of total islet mass), pancreatic polypeptide-producing PP cells (<2% of total islet mass), and the recently described ghrelin-producing ε-cells (74;75). Glucagon and insulin work antagonistically to maintain normoglycemia, while somatostatin and pancreatic polypeptide work both locally to inhibit pancreatic secretions, as well as globally, with neuromodulatory and satiety-inducing effects, respectively. Ghrelin is also a metabolic regulatory peptide, and appears to promote food intake. In humans, β-cells are typically found near the core of the islet, while the other cell types populate the periphery.

Adult pancreatic mass is largely static, with mitosis and apoptosis under homeostatic regulation (70;76-78), though β-cell mass is known to increase during pregnancy (79-81) and in the presence of obesity (82;83). Studies in mutant murine models illustrate the reliance of the mitotic/apoptotic balance to sustain post-natal β-cell populations. For example, mice lacking cyclin-dependent kinase 4 (Cdk4) exhibited a normal β-cell population at birth, only to post-natally develop hyperglycemia and exhibit decreased β-cell mass (84). In contrast, mice carrying a gain-of-function allele for Cdk4, again exhibiting normal β-cell population at birth, rapidly developed β-cell hyperplasia. Interestingly, the exocrine fraction of the pancreas appeared unaffected in both mutant strains. Experiments examining disruptions in receptor signaling in β-cells (85) and genetic-lineage tracing of β-cells both in maturing and partially-pancreatectomized mice (86) support the mitotic mechanism of adult β-cell population maintenance, with no evidence in either case of adult stem cell-derived β-cells. However, there is a large body of research that supports the potential for β-cell neogenesis, or the appearance of new β-cells resulting from the differentiation of progenitor cells, including preliminary identification of β-cell progenitor markers (87;88).

There is some evidence that pancreatic acinar tissue may be capable of trans-differentiation to endocrine tissue (89;90). The most recent work in this area (89) implies that exocrine cells, when placed in culture, de-differentiate and lose some of their exocrine characteristics. When these de-differentiated cells are exposed to certain growth factors, they assume a β-cell-like phenotype, and appear capable of correcting hyperglycemia in alloxan-treated nude mice.

The pancreatic ductal cell population has also been suggested as a source of β-cell progenitors (44;91), based on the post-pancreatectomy observations of simultaneous endocrine regeneration and ductal cell proliferation (92-94). Subsequent analysis of ductal tissue both *in vitro* (95;96) and post-transplantation in diabetic animal models further supports the potential for pancreatic ductal cells to trans-differentiate to an endocrine fate (97;98). It remains unclear what factors trigger the proliferation and subsequent differentiation of islet progenitor cells; as described above, tissue damage following pancreatectomy seems sufficient, and endocrine hormone-positive proliferating ductal cells are observed in cases of chronic pancreatitis, but the induction of β-cell tolerance in NOD mice via anti-CD3 administration, thus stemming the autoimmune attack on pancreatic tissue, was insufficient to induce proliferation in ductal cells (99). Moreover, the findings regarding the ability of acinar or ductal cells to trans-differentiate to β-cells will remain inconclusive until it can be demonstrated in real-time, *in situ*, with the decisive elimination of contaminating endocrine tissue (100). The current body of research is based on histological snapshots, in which co-localization of β-cell and acinar or ductal markers is the evidence investigators use to support their hypothesis that trans-differentiation is occurring, but the actual phenomenon of trans-differentiation has not been observed.

1.3.3 Insulin

Insulin is a hormone polypeptide, composed of a 21-amino acid A-chain linked to a 30-amino acid B-chain by two disulphide bridges. It was the first polypeptide for which the primary amino acid sequence was determined, by Ryle and associates in the early 1950's (24).

Insulin is transcribed in proinsulin form starting from the amino-terminus of the B-chain, terminating at the carboxy-terminus of the A-chain. It undergoes conversion from proinsulin to insulin in the ER cisternae, at which time the connecting peptide, or C-peptide, between the A- and B-chains is excised. Because C-peptide is produced in equimolar amounts to insulin, it is commonly used in experimental assays to assess the ability of the tissue in question to synthesize insulin. Simply measuring insulin content of tissue is not always sufficient, as recently shown in studies where cells mistakenly attributed with insulin synthesis capability were actually the result of insulin uptake from the medium (101). Approximately 95% of proinsulin is converted to the active hormone and C-peptide, which are then both released into the bloodstream. The β-cell of the pancreas is the only physiologically significant site of insulin synthesis (102;103), and the average insulin content of the pancreas is 40-80 μg insulin/g pancreas, depending on species and the metabolic state of the specimen. Secretion of insulin by the pancreatic β-cells in response to glucose is a biphasic mechanism, consisting of an ATP-sensitive potassium (K_{ATP}) channel-dependent and a K_{ATP} channel-independent pathway. In high glucose environments, the oxidative activity of the β-cell increases, increasing the cellular ATP/ADP ratio, thus triggering closure of the K_{ATP} channels. The subsequent depolarization of the plasma membrane causes an influx of calcium ions (Ca^{2+}) through L-type voltage-gated calcium channels, and the increase in intracellular Ca^{2+} concentrations facilitates the exocytosis of insulin granules. This K_{ATP} channel-dependent mechanism is responsible for the first phase of insulin secretion, and is proposed to exhaust the β-cell's "immediately releasable" insulin stores (104). The second phase of insulin secretion depends upon the K_{ATP} channel-independent pathway and an undefined signaling molecule(s), and is likely limited by the rate of conversion of the cells' "readily releasable" insulin granules to their "immediately releasable" form (104).

1.4. The Physiological Niche of Lipids

The foundation of most modern lipid metabolic research is the so-called "Randle Cycle", first postulated by Randle *et al.* in 1963 (105). Randle observed the

inhibition of glucose utilization in perfused heart tissue by acetoacetate and palmitate, leading him to develop the concept of a glucose/fatty acid cycle, whose essential elements included:

> "(a) the relationship between glucose and fatty acid metabolism is reciprocal and not dependent; (b) *in vivo*, the oxidation of fatty acids and ketone bodies released into the circulation in starvation or diabetes may inhibit the catabolism of glucose in the muscle; (c) *in vitro*, the oxidation of fatty acids released from muscle triacylglycerol plays a similar role; (d) effects of fatty acid and ketone body oxidation are mediated by the inhibition of phosphofructokinase-1, hexokinase and the pyruvate dehydrogenase (PDH) complex; (e) the essential mechanism is an increase in the mitochondrial ratio of [acetyl-CoA]/[CoA], which inhibits the PDH complex and which leads to the inhibition of phosphofructokinase-1 by citrate and of hexokinase by glucose 6-phosphate; (f) oxidation of fatty acids or ketone bodies inhibits the effect of physiological concentrations of insulin to accelerate sugar transport in rat heart." (106)

Randle's basic theory, that free fatty acids (FFA) promote fatty acid oxidation and inhibit glucose oxidation, has held for the past 40 years, though it is evident that there is more at work than simply oxidative substrate competition. Fatty acids have been observed to affect the metabolic processes of glucose uptake and oxidation, glycogen synthesis, and gluconeogenesis. Additionally, there is increasing data on the role of fatty acids in insulin secretion. This section will address the known effects of fatty acids on metabolic enzymes and molecules, β-cell lipid composition, β-cell response, and certain cell proteins, as well as a general discussion of fatty acid metabolism and their physiological effect.

1.4.1 Lipid Metabolism

The ability of the human body to meet its metabolic energy needs requires the interaction of numerous biochemical pathways and substrates. Glucose levels are manipulated via glycolysis, gluconeogenesis, and glycogenolysis; lipid levels through

β-oxidation, lipogenesis, and biological molecule (*e.g.* cholesterol, phospholipid) production; amino acids are metabolized via the urea cycle. The interplay of these mechanisms is largely regulated by two metabolic hormones: insulin and glucagon. Insulin and glucagon, both peptide hormones, work in an antagonistic fashion. Insulin promotes the storage of excess glucose, stimulating glycogen synthesis, protein synthesis, and lipogenesis. In contrast, glucagon stimulates glucose release through the processes of glycogenolysis and lipolysis.

1.4.1.1 Lipid Absorption and Digestion

Approximately 90% of both dietary lipid and lipid metabolic energy storage is as triacylglycerols, or triglycerides (TG) (107). Triglycerides are glycerol triesters, and in human adipose tissue tend to have palmitate at carbon-1 and oleate at carbon-2 (107). Given the non-polar nature of most lipids and the water-soluble enzymes responsible for their digestion, lipid digestion occurs at lipid-water interfaces, and is catalyzed by pancreatic lipase, yielding either mono- or diglycerides. The mixture of glycerides and fatty acids is absorbed by the intestinal mucosa, aided by bile acids, or detergent molecules synthesized by the liver and secreted via the gall bladder. Upon entering the intestinal cells, the fatty acids complex with intestinal fatty acid-binding protein (I-FABP), thus enhancing their solubility. These fatty acids are consequently converted back to TG, packaged into chylomicrons, and released into the blood stream for delivery to the tissues. A parallel process occurs in the liver, where endogenously synthesized TG are packaged into very low density lipoproteins (VLDL) prior to release.

The packaged TG are again hydrolyzed to free fatty acids and glycerol by lipoprotein lipase in the adipose tissue and skeletal muscle. The free fatty acids are taken up by the tissues while glycerol returns to the liver or kidneys.

Lipids are efficiently stored as a potential energy source as TG in adipose tissue. Indeed, given their lower oxidative and anhydrous state, fats are able to provide six times the metabolic energy as an equal weight of hydrated glycogen (107). Even in terms of dry weight, fats have twice the specific enthalpy of carbohydrates or protein. When fat-derived energy is required, hormone-sensitive triacylglycerol lipase, itself

controlled by AMP levels, stimulates lipolysis. The resultant fatty acids bind to albumin in the bloodstream, facilitating delivery to the tissues.

1.4.1.2 Fatty Acid Oxidation

The ultimate energy "currency" of the cell, ATP, results from the metabolism of fatty acids just as it does from glucose. However, the steps leading up to ATP generation are markedly different.

Before they can be oxidized, the fatty acids must be "activated", or complexed with acetyl-CoA to yield fatty acyl-CoA. This ATP-dependent reaction is catalyzed by fatty acyl-CoA synthetase, or FACS, and occurs in the cytosol. Because fatty acid-, or β-, oxidation occurs in the mitochondrion, but long chain fatty acyl-CoA are unable to cross the inner mitochondrial membrane, a specific mechanism is required to transport fatty acyl-CoA into the mitochondrion. First, the acyl group of the fatty acyl-CoA is transferred to carnitine, and CoA released to the cytosol. Carnitine palmitoyl transferase I (CPT-I), located on the outer mitochondrial membrane, transports the acyl-carnitine into the mitochondrial matrix. The acyl group is then transferred to a CoA molecule present in the mitochondrion, reforming fatty acyl-CoA, and carnitine returns to the cytosol.

Once in the mitochondrion, oxidation of the fatty acyl-CoA is able to proceed. β-oxidation is a cycle of four distinct reactions, and each cycle forms acetyl-CoA and a new acyl-CoA, which is two carbons shorter than the acyl-CoA that began the cycle, as well as one $FADH_2$ and one NADH. These entities are reoxidized by the mitochondrial electron-transfer chain, leading to ATP generation, while the acetyl-CoA enters the citric acid cycle and the acyl-CoA undergoes another round of β-oxidation. The oxidative cycle terminates upon the generation of two acetyl-CoA, or in the less common case of fatty acids with odd numbers of carbon atoms, acetyl-CoA and propionyl-CoA. The acetyl-CoA enters the citric acid cycle as usual, while propionyl-CoA is converted to succinyl-CoA, facilitating its entry to the citric acid cycle. Given the large number electrons transferred to the electron-transfer system during β-oxidation from the cyclic generation of $FADH_2$, NADH and acetyl-CoA, fatty acid oxidation is highly exergonic. Both saturated and unsaturated fatty acids

are oxidized in this cyclic mechanism, but isomerase and/or reductase must be used to alter the unsaturated acyl-CoA substrate into a form that is recognized by the oxidative enzymes.

Fatty acids can also be endogenously produced, in a mechanism that largely resembles the reverse of β-oxidation. The fatty acid is generated in two-carbon units, with malonyl-CoA as the C_2 unit donor and acyl-carrier protein (ACP) taking the place of CoA. Two key enzymes in this endergonic reaction are acetyl-CoA carboxylase and fatty acid synthase.

1.4.1.3 Fatty Acid Metabolic Regulation

The overall scheme of hormonal regulation of fatty acid metabolism is relatively simple; insulin secreted by the pancreatic β-cells stimulates glucose uptake and glycolysis, yielding decreased cellular AMP levels. This, in turn, inhibits the activity of AMP-activated protein kinase (AMPK). AMPK is then unable to phosphorylate and inactivate acetyl-CoA carboxylase (ACC). Thus, ACC can catalyze the first step of fatty acid synthesis, the production on malonyl-CoA (108) (**Figure 1.1**). Simultaneous to enhanced glycolysis, insulin inhibits hormone-sensitive lipase-mediated TG hydrolysis in adipocytes. The ultimate response to insulin is glycogen and TG formation, or promotion of energy "deposition". In contrast, glucagon and epinephrine stimulate fatty acid oxidation and inhibit fatty acid synthesis, promoting energy "withdrawal". Thus, the balance of insulin and glucagon plays a key role in determining the direction and rate of fatty acid metabolism.

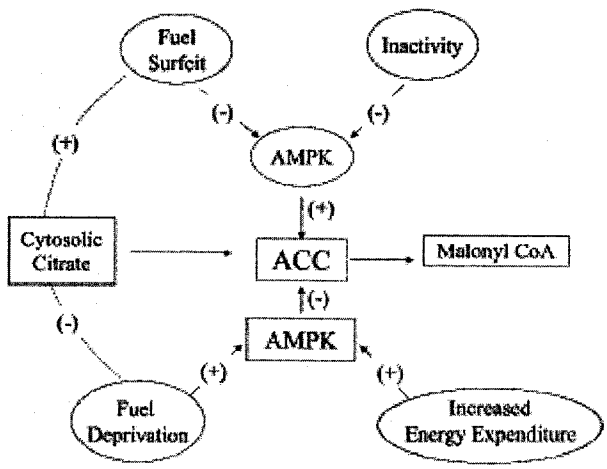

Figure 1.1. Positive and negative regulation of ACC in skeletal muscle. Changes in fuel availability and energy expenditure in the muscle cell lead to alterations in the cytosolic concentration of citrate, an allosteric activator of ACC, and the activity of AMPK, which phosphorylates and inhibits ACC. This in turn alters the concentration of malonyl CoA and the rate of fatty acid oxidation. Ruderman *et al.* (2003) (108).

However, the picture becomes more complicated when internal feedback loops and the crossover of negative feedback products between glucose and fatty acid metabolism are considered. Carnitine palmitoyltransferase-I (CPT-I) is responsible for the transport of long chain fatty acyl moieties from the cytosol to the mitochondrion, and is thus a rate-determining factor in FA oxidation (19). There are primarily two modes of CPT-I regulation; inhibition of acyl-CoA transport by malonyl-CoA, and the dependence upon the cytosolic concentration of carnitine (109). As previously mentioned, malonyl-CoA is regulated indirectly by glucose levels, via the activation state of AMPK and ACC. However, it has been demonstrated that increased cytosolic citrate, itself a result of high glycolytic flux, is an allosteric activator of ACC (108;109). Both these mechanisms suggest that increased glucose metabolism simultaneously inhibits FA oxidation and promotes FA storage as triglyceride. Carnitine is also a substrate of carnitine acetyltransferase (CAT), which buffers high levels of mitochondrial acetyl-CoA during rapid glycolytic flux by transporting excess acetyl-CoA from the mitochondrion to the cytosol (109). Carnitine in the CAT complex is unable to take part in acyl-CoA transport, thus further exacerbating the decline in FA oxidation during glycolysis. Additionally, malonyl-CoA not used for FA synthesis may be degraded by malonyl-CoA decarboxylase (MCD), which is activated by AMPK (108). Finally, there is some evidence that increased free calcium (*i.e.* during muscle contraction) may prevent the synthesis or enhance the degradation of malonyl-CoA (110).

Other negative feedback pathways may also influence FA metabolism. For example, an inverse relation between AMPK and two enzymes essential to the synthesis of triglycerides, mitochondrial glycerol 3-phosphate acyltransferase (mGPAT) and acyl CoA:diacylglycerol transferase (DGAT), has been observed (108).

It is becoming increasingly evident that hormones other than insulin and glucagon play a role in the regulation of FA metabolism. Leptin, a protein hormone secreted by adipocytes, is believed to prevent lipid accumulation in nonadipose tissues, thus

suppressing a potential source of lipotoxicity (108). Adiponectin, another adipoctye-derived hormone, has been inversely related to a number of metabolic abnormalities, including insulin resistance and T2DM (108). Further studies have observed that both leptin and adiponectin activate AMPK, and adiponectin may reduce ACC activity (108).

1.4.2 β-Cell Lipid Composition

The fact that a cell's lipid environment plays a key role in its lipid composition is intuitively obvious, and certainly holds true for the β-cell. Although lipids may take various cellular forms, two of the most prominent are triglyceride and membrane lipids.

1.4.2.1 Triglycerides

As previously described, triglycerides (TG), the major physiological energy storage form, are triesters of glycerol-3-phosphate and 3 fatty acyl-CoA. Glycerol-3-phosphate is derived glycolytically from glucose, as adipocytes lack the kinase required to phosphorylate endogenous glycerol. If glycerol-3-phosphate is unavailable due to low glucose uptake by adipocytes, fatty acids remain unesterified and are released back into the bloodstream. Hence, glucose uptake plays a key role in fatty acid storage capability. TG biosynthesis occurs in a series of NADPH-dependent reactions in the endoplasmic reticulum by acyltransferases. Acyltransferases are not specific to particular acyl-CoAs, so a variety of TG can be generated endogenously.

Upon stimulation by epinephrine and/or glucagon, hormone-senstive triacyl-glyercol lipase stimulates the hydrolysis of TG in adipose tissue. The resultant FFA are released into the bloodstream and taken up by muscles and the liver for β-oxidation.

1.4.2.2 Membrane Lipids

Glycerophospholipids are the primary lipid component of biological membranes. Their basic structure consists of a glycerol-3-phosphate backbone esterifed at C_1 and C_2 to fatty acids, and at the phosphoryl group to a variable molecule, which

determines the role of the lipid. Typically, saturated C_{16} and C_{18} fatty acids are bound at C_1 (*i.e.* palmitic and stearic acids), while their unsaturated counterparts are found at C_2 (*i.e.* palmitoleic and oleic acids). The resulting molecules are amphiphilic, with hydrophobic hydrocarbon tails, and hydrophilic phosphoryl heads. This family of lipids includes lecithins (phosphatidylcholine) and cardiolipins (diphosphatidylglycerol).

Sphingolipids are another major membrane lipid. They are derived from ceramides, which are themselves derived from palmitoyl-CoA and serine. Variations in the component bound to the head group of ceramide can result in sphingomyelins, cerebrosides, and gangliosides. Gangliosides in particular are of key physiological importance; their large carbohydrate head groups may play a role in cell recognition, and thus differentiation, and are known to be receptors for pituitary hormones, thus influencing a number of physiological processes.

Membrane lipid composition within the cell is not uniform; the outer leaflet of the plasma membrane contains high levels of sphingomyelin, resulting in a highly ordered membrane, while the endoplasmic reticulum (ER) has low cholesterol content and a higher proportion of unsaturated fatty acids, resulting in a more disordered membrane structure (111). It is not clear why membrane compositions vary throughout the cell, but is assumed to be related to the specific organelle function. For example, the higher level of disorganization of the ER membrane may facilitate more rapid diffusion of protein products through the membrane. Lipid organization can also vary within a single lipid bilayer, resulting in small domains with different degrees of lipid organization (111). Different membrane proteins may preferentially associate with a certain type of lipid organization, resulting in the physical segregation of different proteins within a single membrane, referred to as "rafts" (111).

There are numerous ways in which cellular membrane composition could affect cell function and viability. As previously noted, some membrane proteins prefer certain lipid environments, so changes in membrane composition may interfere with expression of these surface proteins (111). Membranes with higher levels of organization (*i.e.* greater fractions of saturated FA) tend to have decreased membrane

fluidity and decreased insulin receptor affinity (112). Additionally, changes in membrane lipid composition may affect processes as varied as signal transduction, glucose transport, enzyme activity, and substrate availability.

1.4.2.3 Relation of Lipid Composition to Pathophysiological Symptoms

The direct association of dietary lipid consumption with physiological lipid content is difficult to make, given the endogenous processes of both *de novo* lipogenesis and desaturation of exogenous FA (113). Indeed, it appears that while plasma levels of saturated FA and polyunsaturated FA correlate well to dietary consumption, monounsaturated FA do not, likely due to high levels of endogenous synthesis (112). Additionally, the argument can be made that the same genetic factors that predispose one to metabolic syndrome, and T2DM in particular, may affect lipid processing and metabolism; abnormal lipid profiles may be an additional symptom of metabolic syndrome, rather than a cause. Thus, it is imperative to examine the effect of lipid composition as it exists endogenously before trying to extrapolate this information to assess the affect of dietary lipids.

There is a lack of consensus regarding whether increased deposition of TG in pancreatic β-cells, commonly observed in the progression of T2DM, may serve as a protective or pathogenic mechanism in the context of lipotoxicity and β-cell dysfunction (114-116). The highly abnormal insulin secretion observed in Zucker Diabetic Fatty (ZDF) rats is largely attributed to a 50-fold increase in intra-islet TG relative to nondiabetic controls (117). Cnop *et al.* (114) observed that palmitate and oleate were able to induce apoptosis in both β-cells and non-β-cells in Wistar rat islets, and neither elevation of glucose levels nor inhibition of CPT-I affected the degree of apoptosis. They also determined that the degree of apoptosis varied inversely with cellular TG content. Thus, they proposed that the ability to store FA as cytoplasmic TG may alleviate the lipotoxic effect. Listenberger *et al.* (115) demonstrated that the presence of unsaturated FA promoted TG formation, and even in combination with excess saturated FA, reduced apoptosis in Chinese hamster ovary (CHO) cells. Additionally, given the inability to form TG (*i.e.* *Dgat1*$^{-/-}$ cells), lipotoxic apoptosis occurred despite the presence of unsaturated FA. This further

supports the notion that TG formation is a buffering mechanism during chronic FA exposure. Moffitt *et al.* (116) similarly observed greater saturated FA uptake and storage as TG in the presence of unsaturated FA in INS1 cells, and this storage coincided with decreased apoptosis. However, they also noted the presence of vacuoles of solid tripalmitin in the cytosol, determined to be dilated endoplasmic reticulum, and thus proposed that palmitic acid-induced apoptosis is the result of solid TG formation in the endoplasmic reticulum, thus disrupting cell integrity and eliciting an ER stress response. They concluded that prevention of lipotoxicity was not directly related to TG formation, but the composition of TG formed, and that incubation with a single FA in isolation produced results unrepresentative of the physiological situation. They also observed, but failed to explain: (i) the degree of TG accumulation in the cells was proportional to media glucose concentrations; (ii) exposure to both saturated and unsaturated fatty acids resulted in decreased glucose stimulated insulin secretion (GSIS), indicative of β-cell dysfunction.

There is evidence that intracellular lipid accumulation may contribute to the systemic pathogenesis of T2DM, which raises the question of how intracellular lipid could take on a protective role in β-cells, but detrimental in other tissues. However, in support of this proposition, there is significant evidence to suggest that fat accumulation in skeletal muscles, the primary sites of glucose uptake, induces insulin resistance (19). One proposed mechanism for the development of myocyte insulin resistance is mitochondrial oxidative damage; excess FA accumulate near the mitochondrial matrix and become substrates for oxidation, producing cytotoxic and reactive lipid peroxides. The peroxides damage the mitochondria, reducing its overall oxidative capacity (19).

There is also increasing evidence of a relationship between membrane lipid composition and the pathogenesis of T2DM (111-113;118). While few studies examine the membrane lipid profiles of β-cells in particular, findings in other cell types may pertain to the islet. In a study of middle-aged adults, a high proportion of saturated FA in both phospholipids and cholesterol esters was positively associated with the development of T2DM, and this relationship was maintained even after adjustment for other diabetes risk factors (112). A similar study of overweight

adolescents found the proportion of polyunsaturated FA in overweight individuals was inversely related to symptoms of metabolic syndrome and inflammation (113). Interestingly, Klein-Platat *et al.* (113) found no association between FA composition and insulin resistance in these adolescents. Tang *et al.* (119) observed that membrane lipid composition influenced the activity of plasma membrane Ca^{2+}-ATPase (PMCA); PMCA exhibited higher activity when the surrounding membrane composition contained a high degree of order. This supports the premise that membrane composition may indirectly affect overall β-cell function, given the dependence of insulin secretion on intracellular Ca^{2+} concentrations (120).

1.4.3 Lipid Signaling Molecules and Metabolic Enzymes

Finally, this general overview of the physiological role of lipids will examine a few of the relationships between lipid environment, signaling molecules, and enzyme expression currently under investigation. As the description of lipid metabolism shows, the possible subjects of lipid influence are virtually endless, so here the focus shall be on some of the most recent studies.

Gremlich *et al.* (17) observed that 10 h incubation of Sprague-Dawley rat islets with 0.6 mM palmitic acid induced a significant decrease in expression of islet/duodenum homeobox-1 (IDX-1, also known as Pdx-1), a transactivator of the insulin, somatostatin, *Glut2*, and glucokinase genes. This effect required the presence of glucose levels at or greater than 5.6 mM, and functioning CPT-I. The subsequent decrease in GLUT2 and glucokinase protein expression, key positive regulators of glycolysis, demonstrated a possible mechanism by which chronic FA exposure shifts the oxidative balance in favour of FA, rather than glucose, metabolism. Interestingly, they also noted enhanced glucagon release, which may serve to further inhibit glycolysis.

As the onset of T2DM coincides with the inability of β-cells to compensate for peripheral insulin resistance, much research has focused on potential regulators of β-cell mitogenesis and initiators of apoptosis. Because physiological levels of glucose have been observed to increase β-cell mitogenesis, Cousin *et al.* (14) examined whether alternative fuel sources *i.e.* long chain fatty acids, had a similar effect. They

reported the ability of 0.2 mM palmitate to inhibit glucose/insulin-like growth factor I (IGF-I) induced β-cell mitogenesis in INS-1 cells, in a mechanism independent of ceramide, and likely mediated by inhibition of PKB and/or activation of PKCζ. PKC is a serine kinase that inhibits kinase activity of the insulin receptor and insulin receptor substrate (IRS-1); PKB is stimulated by insulin to enhance glucose uptake (**Figure 1.2**) (121), and is believed to have an anti-apoptotic role (15). Wrede *et al.*, (15) upon examination of ceramide-independent, FFA-induced apoptosis in INS-1 cells, determined that by expressing constitutively active (adenovirally mediated) PKB, FFA-induced apoptosis was almost completely prevented. They propose that, rather than stimulating mitogenesis, PKB prolongs β-cell survival. Boden and Shulman (122) also propose elevated circulating FFAs alter cellular function by activating PKC, which may ultimately decrease GLUT-4 translocation, blocking glucose transport into the cell. They also note that overexpression of lipoprotein lipase (LPL) results in significantly increased intracellular TG, and decreased insulin-stimulated glucose transport.

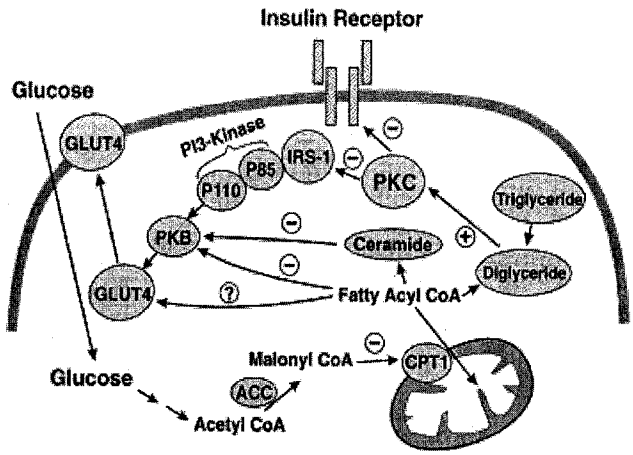

Figure 1.2. Potential interactions between lipids and insulin signaling. -, Potential inhibitors; +, potential activators. ACC, acetyl-CoA carboxylase; PKB, protein kinase B. Kelley and Mandarino (2000) (121).

Eto *et al.* (18) performed a number of adenoviral infections of β-cells to mimic dysfunctional metabolism. Upon adenoviral infection of INS-1 cells with constitutively active sterol regulatory element binding protein (SREBP), they observed significant increase in numerous lipogenic enzymes, such as fatty acid synthase, but no change in oxidative enzyme levels. Additionally, they observed enhanced basal insulin secretion, but impaired GSIS, which mimics the effect of

chronic FFA exposure on β-cells. Overexpression of AMPK in INS-1 cells enhanced FFA oxidation and suppressed lipogenesis, resulting in significantly reduced cellular TG. The simultaneous reduction in glucose oxidation, insulin content, and basal insulin secretion underlines AMPK's role of fuel source regulation during starvation. Hormone sensitive lipase (HSL), an acylglyerol lipase expressed in β-cells, may be another factor of the cellular dysfunction exhibited in T2DM. Transgenic mice overexpressing β-cell-specific HSL fed a high-fat diet had lower islet TG accumulation than their wild type, high-fat fed counterparts (123). In addition, the transgenic mice exhibited impaired GSIS, and increased PPARα and UCP2 expression. The resulting hypothesis was that FA or a derivative thereof, when mobilized from TG by HSL, or when TG storage capacity is exceeded *i.e.* during hyperlipidemia, upregulate the expression of UCP2 via a PPARα-regulated mechanism, ultimately impairing GSIS. This hypothesis is strongly supported by the previous data suggesting the diversion of FFA to intracellular TG decreases or prevents the lipotoxic effect (114-116).

Islets from Sprague-Dawley rats, cultured in elevated glucose or FFA for up to 8 days exhibited significantly enhanced levels of two repressive forms of cAMP response element modulator, CREM (124). The increased expression of these two transcriptional repressors occurred in parallel to suppression of GSIS, and adenovirus-mediated overexpression of the repressors in β-cells significantly reduced islet insulin protein and mRNA content.

1.5. Thesis Objectives and Outline

The aim of this thesis is to further characterize the neonatal porcine islet, particularly in terms of its potential use as a source of transplantable tissue for the treatment and correction of diabetes in humans. The ability of this tissue to ameliorate and correct the diabetic phenotype has been demonstrated in a significant number of previous works, including allo- and xenogeneic animal transplant models, and most recently, in non-human primates. However, the characteristics of neonatal porcine tissue and the unique way in which it responds to the stresses of an environment similar to what

would be found in a diabetic islet transplant recipient remain largely unknown. The physiological conditions first encountered by transplanted islets, hyperglycemia and dysfunctional lipid metabolism, are highly toxic to human islets, and likely impose a severe limitation on the ability of human islets to correct diabetes following transplantation. Thus, it is of extraordinary interest to determine if neonatal porcine islets are more robust in these conditions, and hence more likely to succeed in the correction of diabetes in the recipient.

Chapter Two examines the ability of neonatal porcine islets to survive and maintain appropriate metabolic function, specifically in terms of insulin secretory response to glucose, in an environment of chronically elevated free fatty acids. The driving hypothesis of this study was, in line with recent findings in settings of elevated glucose and hypoxia with reoxygenation injury, neonatal porcine islets would prove resistant to the lipotoxic effect of elevated free fatty acids, thus establishing NPI as a potential source of transplantable islet tissue with numerous merits. There is also a preliminary investigation into the mechanism by which chronically elevated levels of free fatty acids induce β-cell death, as this phenomenon is well-documented in numerous cell types, but a coherent explanation of how and why it occurs remains elusive.

Chapter Three is concerned with the potential for β-cell progenitor expansion and differentiation, as well as proliferation of existing, mature β-cells in neonatal porcine islet tissue. NPI is an immature tissue, but the extent to which the immature cells within the islets can be externally influenced is incompletely understood. The study was based upon the hypothesis that a prescribed regimen of growth factors, administered *in vitro,* would result in increased functional islet yields from a single piglet donor. Increased functional islet yields, whether resulting from mitosis of existing β-cells or maturation of progenitor cells present in the neonatal tissue, serve to decrease the donor to recipient transplant ratio, and increase the degree to which a tissue source may be considered unlimited. Given the current dearth of tissue suitable for islet transplantation, any manner by which functional islet populations can be increased is of great importance.

The unifying theme of this thesis was to investigate and demonstrate characteristics of the neonatal porcine islet which make it an advantageous, if not ideal, source of tissue for islet transplantation in the treatment of diabetes. The ability of islets to withstand the numerous stresses, including hyperlipidemia, encountered in the diabetic recipient is likely of paramount importance to the long-term survival of the islet graft and its ability to correct diabetes. Moreover, a reliable method by which to ensure an adequate supply of transplantable islets is required before islet transplantation can be considered a viable therapy. Thus, this thesis, in its examination of the ability of NPI to withstand elevated levels of free fatty acids and undergo expansion *in vitro*, acts to further reinforce the credentials of NPI as a plausible source of tissue for islet transplantation.

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Chapter Two

The Effect of Chronically Elevated Free Fatty Acids on Neonatal Porcine Islets *In Vitro*

2.1. Introduction

The prevalence of diabetes has increased at unprecedented rates, due primarily to increased incidence (1). While a portion of these diagnoses are of the insulin-dependent autoimmune form of the disease, over 90% of diabetes diagnoses are of non-insulin dependent diabetes mellitus (T2DM) (2). T2DM is characterized by severe metabolic dysfunction, primarily hyperinsulinemic hyperglycemia and hyperlipidemia (3-7). Chronic exposure to elevated levels of free fatty acids (FFA), as typically exhibited by type 2 diabetics, is known to induce both pancreatic β-cell dysfunction (7) and death (3), in a phenomenon termed lipotoxicity.

The mechanism by which lipotoxicity proceeds remains unclear; recent work suggests FFA affect β-cell function and mass independently (8-10). Potential factors implicated in the lipotoxic effect of FFA on β-cells include oxidative stress and accumulation of reactive oxygen species (11;12), ER stress and the unfolded protein response (13-15), nitric oxide-induced damage of mitochondrial DNA (16), and ceramide signaling (17;18).

The near-pandemic occurrence of T2DM (19) has encouraged increasingly innovative investigations into potential treatments. The definitive, though limited, success of clinical islet transplantation (20-22), nourishes the hope that islet transplantation may someday be used in the treatment of T2DM. Before this can happen however, numerous obstacles must be overcome, including the acute shortage of suitable donor pancreatic tissue for transplantation (23;24).

Neonatal porcine islets (NPI) have long been an area of intense interest in diabetes research (25), and are becoming increasingly plausible as an alternate source of islets for clinical transplantation (24;26-28). Not only are NPI less fragile than adult porcine islets and more responsive to glucose than fetal porcine islets (23;29), they

have recently been shown to exhibit enhanced resistance to hypoxia- (30) and glucose-induced apoptosis (31).

The aim of this study was to determine if NPI possess similar resistance to the lipotoxic effects of chronic exposure to both single FFA and a physiologically relevant combination treatment. Additionally, by pharmaceutically blocking one arm of the pro-apoptotic signaling cascade in a lipid-rich environment, a further refinement of the mechanism(s) by which FFA act upon β-cells could be made.

2.2. Materials and Methods

2.2.1 Neonatal pig pancreatic islet isolation and culture

Islets were isolated from male Duroc neonatal pigs (Swine Research and Technology Centre, University of Alberta) aged 1-2 days old (1.4 – 1.9 kg body weight) as previously described (25). Piglets were anaesthetized under halothane and subjected to laparotomy and completely exsanguinated. The pancreases were removed and placed in cooled HBSS supplemented with 0.25% w/v BSA, then minced, digested in 1.0 mg/ml collagenase XI (Sigma-Aldrich, Oakville, ON, Canada), and filtered through a 500 μm nylon screen. The filtrate was then cultured in Hams F10 medium (Invitrogen, Burlington, ON, Canada) containing 10 mM glucose, supplemented with 10 mM nicotinamide (BDH Biochemical, Poole, Dorset, UK), 2 mM L-glutamine, 0.5% w/v BSA (fraction V) (Sigma), 50 μM IBMX (ICN Biomedicals, Montreal, QC, Canada), 100 U/ml penicillin and 100 μg/ml streptomycin (Cambrex Bio Science, East Rutherford, NJ, USA) for 5 days at 37°C in humidified 5% CO_2/95% O_2, with media changes every 2 days. After 5 days in culture, the islets were divided into 4 groups and cultured for 4 additional days in the fatty acid-enriched or control treatments.

2.2.2 Fatty acid-enriched media preparation

50 mM fatty acid stock was made by adding palmitic and/or oleic acid (Nu-Chek Prep, Inc., Elysian, MN, USA) to hot 40% v/v ethanol supplemented with 0.1 N NaOH, and stirring until dissolved. Stock was then added to 37°C Hams F10 media

containing 10 mM glucose and 0.75% w/v BSA (fraction V) for a final concentration of 0.5 mM, vortexed for 10 seconds, and incubated for 30 minutes at 37°C. z-VAD-fmk (R&D Systems, Minneapolis, MN, USA) was dissolved in DMSO as per manufacturer's instructions, then added directly to culture media.

2.2.3 Fatty acid analysis

Cell sample aliquots were washed with PBS and stored as dry cell pellets at -80°C. At time of analysis, whole cell lipids were extracted using a modified Folch procedure (32). The total lipid extract was separated into lipid fractions on thin layer chromatography plates (G-25 20 x 20 cm, Agilent Technologies, Santa Clara, CA, USA) using a solvent system of petroleum ether, diethyl ether, and acetic acid in a ratio of 80:20:1 by volume. Separated total phospholipids and triglycerides were visualized with 8-anilino-1-napthalne-sulfonic acid (Sigma Chemical) and identified under ultraviolet light with appropriate standards (Sigma Chemical). Phospholipid and triglyceride methyl esters were prepared from the scraped silica bands using boron trifluoride methanol (14%) (Sigma Chemical) and hexane (Fisher Scientific), and separated and identified by gas chromatography (Varian 3800, Varian Instruments, Mississauga, ON, Canada) using fused silica CP-SIL 88 column (100 m x 0.25 mm internal diameter X 0.2, Varian) as previously described (33). Peak identification was quantified using Chemstation V10.02 software (Agilent Chromatography). The analytical conditions allowed the separation of saturated, mono-, di-, and polyunsaturated fatty acids of 14 to 24 carbon chain length.

2.2.4 Cellular DNA and insulin content

PicoGreen, a fluorescent nucleic acid stain for double stranded DNA, was used to assess cellular DNA content, as per the manufacturer's instructions (Invitrogen). Duplicate aliquots were washed in citrate buffer (150 mM NaCl, 15 mM citrate, 3 mM EDTA, pH 7.4) and stored as dry cell pellets at -20°C. At time of analysis, pellets were resuspended in lysis buffer (10 mM Tris, 1mM EDTA, 0.5% Triton-X100, 4°C, pH 7.5), sonicated, vortexed, and analyzed (25).

Duplicate aliquots were also collected to determine cellular insulin content by RIA (Diagnostic Products Corp., Los Angeles, CA, USA). Samples were collected in 2 mM acetic acid (0.25% BSA), sonicated, and centrifuged (800 *g*, 10 minutes, 4°C). The supernatants were collected and stored at -20°C until time of analysis (25).

2.2.5 Glucose-stimulated insulin secretion

Islet glucose responsiveness was assessed using a static incubation assay (25). Islets were gently washed three times with Hams F10 (0.5% BSA, 10 mM glucose) and sampled for cellular insulin content. Islets were then incubated for 120 minutes at 37°C in 24 well plates with Hams F10 (0.5% BSA) containing either 2.8 or 20 mM glucose. Following incubation, supernatant was collected and insulin release measured by RIA. Islet glucose responsiveness was calculated by dividing the amount of insulin released by cellular insulin content (percent secretion). Stimulation index (SI) was determined as the ratio of percent secretion at 20 mM to 2.8 mM glucose.

2.2.6 Immunohistochemistry

To assess insulin- and glucagon-positive fractions of the islet cell population, samples were collected in calcium-free HBSS supplemented with 1 mM EGTA and 0.5% BSA. Islets were then mechanically dissociated with siliconized glass pipettes at 37°C for 7 minutes. 0.4 mg/ml DNAse and 1.0 mg/ml trypsin were added (Boehringer Mannheim, Laval, QC, Canada), and mechanical dissociation continued for a further 4 minutes. Cells were washed and resuspended in PBS, allowed to adhere to Histobond slides (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany), fixed in Bouin's fixative without picric acid, and stored in 70% ethanol at 4°C until analysis.

The avidin-biotin complex (ABC, Vector Laboratories, Inc., Burlingame, CA, USA) method with diaminobenzidine (DAB) chromagen (Signet Laboratories) was used to quantify insulin- and glucagon-positive cell fractions. Slides were rehydrated and endogenous peroxidases quenched with 10% v/v H_2O_2 in methanol, then blocked for 20 minutes with 20% v/v normal goat serum (Jackson ImmunoResearch Laboratories,

West Grove, PA, USA). Primary antibodies (1:1000 guinea pig anti-porcine insulin and 1:5000 mouse anti-porcine glucagon, DAKO, Mississauga, ON, Canada) were added for 30 minutes at room temperature in a humidified chamber. Slides were then washed twice in PBS before addition of secondary antibody (1:200 biotinylated goat anti-guinea pig and 1:200 biotinylated goat anti-mouse, Jackson) for 30 minutes. Slides were incubated with ABC for 40 minutes before addition of DAB. Single cell counts were performed on a minimum of 500 cells, and positively stained cells expressed as a percentage of total cell number. The total number of α- and β-cells was determined using the total islet cellular DNA content and the percentage of hormone-positive cells (25):

$$\frac{\text{Total DNA content}}{7.1\frac{pg\,\text{DNA}}{\text{cell}}} \times \frac{\text{Insulin - positive cells}}{100} = \text{number}\,\beta - \text{cells}$$

TUNEL staining was used to assess the degree of apoptosis occurring in islets. Whole islets were washed in PBS and stored overnight in Z-fix (Anatech Ltd., Battle Creek, MI, USA) at 4°C. Islets were then suspended in 2% low-melting point agarose (Sigma-Aldrich), cooled at 4° C until the agar had solidified, embedded in paraffin, and sectioned. Slides were rehydrated and antigen retrieval performed by microwave in sodium citrate (10 mM, pH 6.0). Slides were incubated with DNA labeling solution (TdT enzyme and BrdUTP, Invitrogen) for 60 minutes in a humidified chamber at 37°C. Slides were then labeled with BrdU Alexa 488 (Invitrogen) for 30 minutes at room temperature. After 3 washes in PBS, slides were blocked 20 minutes with 5% normal goat serum, insulin primary antibody was added for 30 minutes at room temperature, then secondary antibody, 1:200 anti-guinea pig Alexa 594 (Invitrogen), was added for 30 minutes following 3 washes in PBS. Slides were coverslipped with ProLong Gold antifade reagent with DAPI (Invitrogen) and stored at 4°C until viewed using a Zeiss Axioscope II fluorescent microscope with an Axiocam MRc camera and HBS 100W Atto Arc light source.

2.2.7 Statistical analysis

Results are presented as mean ± standard error of three independent experiments performed on islets from at least 4 pancreata. Statistical comparisons were conducted with single-factor and 2-factor ANOVA, as appropriate, using SPSS statistical software 13.0 for Windows (Chicago, IL, USA). Fisher's LSD method was used as a post-hoc test to determine which means were significantly different from each other. A P-value of less than 0.05 was considered significant. Analysis for block effect was calculated manually.

2.3. Results

2.3.1 Incorporation of fatty acids in islet cell lipids

To assess if the NPI were incorporating FFA from their surroundings, fatty acid composition of whole cell phospholipids and triglycerides was assessed using gas chromatography. As shown in **Table 2.1**, all fatty acid treatments induced significant changes in phospholipid composition. After 4 days, the percentage of oleic acid in the overall phospholipid composition in oleic acid-treated islets was three times the percentage observed in control islets ($P<0.001$). The significant increase oleic acid was countered by moderate decreases in all other fatty acids.

In contrast, 4 days of culture in elevated palmitic acid significantly increased the percentage of palmitic acid in the total composition to 34%, compared to 21% in control islets ($P<0.001$). However, unlike oleic acid, the increase in palmitic acid was reciprocated only by a decrease in the percentage of total polyunsaturated fatty acids (PUFA) as compared to control islets ($P<0.05$ *vs* control).

Treatment with the combination of fatty acids appeared to have a more marked effect on the contribution of oleic, rather than palmitic, acid to the overall phospholipid composition. Oleic acid increased to 29% in the combination-treated islets, compared to 17% in control islets ($P<0.002$), while palmitic acid did not significantly increase in the combination-treated islets. The increase in oleic acid was countered by a significant decrease in stearic acid.

None of the fatty acid treatments had a significant effect on the fatty acid composition of islet triglycerides (data not shown).

Table 2.1. Whole cell phospholipid fatty acid composition of neonatal porcine islets after 4 days in elevated free fatty acid (0.5 mM) conditions. Values are means ± S.E.M. from 1 experiment, for a total n=4. Values are expressed as percentage contribution to total phospholipids composition. Only major fatty acids are reported. **a** P<0.05 *vs* control; **b** P<0.05 *vs* oleic treatment; **c** P<0.05 *vs* palmitic treatment.

	Treatment			
Fatty Acid	Control	Oleic Acid	Palmitic Acid	2:1 Oleic and Palmitic Acid
16:0	20.8 ± 1.2	12.0 ± 0.6 [a, c]	34.0 ± 2.8 [a, b]	23.0 ± 1.2 [b, c]
16:1	2.8 ± 0.4	2.7 ± 0.3	5.9 ± 1.0	2.8 ± 0.3
18:0	23.1 ± 1.0	12.7 ± 0.7 [a, c]	22.1 ± 2.1 [b]	16.7 ± 1.2 [a, c]
18:1n9	17.4 ± 0.3	52.1 ± 1.2 [a, c]	11.6 ± 0.2 [b]	29.3 ± 4.0 [a, b, c]
18:1c11	5.3 ± 0.2	3.7 ± 0.2 [a]	2.9 ± 0.2 [a]	4.1 ± 0.5 [a, c]
Σ PUFA	30.8 ± 1.8	17.0 ± 0.8 [a]	23.6 ± 1.8 [a]	24.2 ± 3.6 [b]
SFA:PUFA	1.4	1.5	2.4	1.6

2.3.2 Exposure to saturated fatty acid reduces islet cell populations

Culture of neonatal pig pancreatic islets in elevated levels of a single FFA resulted in reduced cellular DNA content, indicative of a decrease in the number of islet cells (25). Incubation in palmitic acid (0.5 mM), a saturated fatty acid, had the most dramatic effect; cellular DNA content fell to 39% of the original amount after 2 days in culture, and further decreased to 24% of original after 4 days (**Figure 2.1.A**). This decrease was significantly greater than that observed in control tissue ($P<0.05$) over the same time period. Incubation in unsaturated oleic acid (0.5 mM) had a modest effect on cellular DNA content, dropping to only slightly below control values (57% of day zero content, *vs* 68% for control islets) after 4 days in culture. Interestingly, treatment with a combination palmitic and oleic acids appeared to prevent the detrimental effect on islet cell population incurred by culture in single fatty acid; DNA content of islets cultured in a mixture of oleic and palmitic acids in a 2:1 ratio was not significantly different from control islets, and was significantly greater than DNA content of palmitic acid-treated islets.

2.3.3 FFA in isolation alter islet cell composition

To determine if FFA were selectively toxic to a specific islet cell sub-population, islets were mechanically dissociated, adhered to microscope slides, and subject to analysis by immunohistochemistry. Insulin-positive cells were classified as β-cells, and glucagon-positive cells were classified as α-cells. After 4 days in culture, the control population consisted of approximately 150,000 α-cells (15% of the total islet cell population, data not shown) and 180,000 β-cells (18% of total population). Endocrine cell populations fell precipitously after 4 days in palmitic acid; only 48,000 α-cells ($P<0.05$ *vs* control) (6% of total population) and 61,000 β-cells ($P<0.05$ *vs* control) (9% of total population) remained (**Table 2.2**).

Oleic acid appeared to have an effect similar to that of palmitic acid; the α-cell population fell to 64,000 and β-cells decreased to 92,000 ($P<0.05$ *vs* control).

It was interesting to observe that the fatty acid combination treatment did not cause a significant decrease in either α- (67,000) or β-cell (110,000) populations.

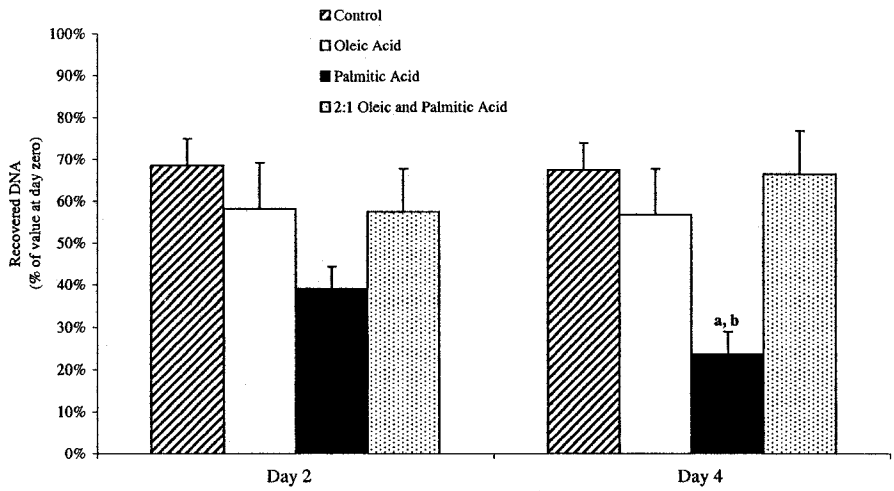

Figure 2.1.A. Cellular DNA content assessed in control and fatty acid-treated (0.5 mM) neonatal porcine pancreatic cells after 2 and 4 days in culture. Palmitic acid treatment significantly reduced the cellular DNA content at day 4. Combination treatment with 2:1 oleic and palmitic acid had no effect on cellular DNA content. Values expressed as mean percentage of content at day zero ± S.E.M., from 3 independent experiments for a total n=5. **a** $P<0.05$ *vs* same-day control; **b** $P<0.05$ *vs* same-day combination treatment.

Table 2.2. Cellular composition of neonatal porcine islets after 4 days in elevated free fatty acid (0.5 mM) conditions. Values are means ± S.E.M., from 3 independent experiments, for a total n=5. Cell composition was determined by calculating the product of the total number of cells in the preparation as determined from cellular DNA content and the percent glucagon- and insulin-containing cells as determined by immunohistochemistry, using the equation described in Methods. **a** $P<0.05$ *vs* control.

	Number of Cells (x 10^5)	
Treatment	α-cells	β-cells
Control	1.50 ± 0.7	1.83 ± 0.6
Oleic Acid	0.64 ± 0.2	0.92 ± 0.2 [a]
Palmitic Acid	0.48 ± 0.3 [a]	0.61 ± 0.4 [a]
2:1 Oleic and Palmitic Acid	0.67 ± 0.3	1.10 ± 0.5

2.3.4 FFA in isolation reduce cellular insulin content

Cellular insulin content was measured to assess the degree to which exposure to elevated FFA influenced insulin production in β-cells. Palmitic acid induced the greatest change, with cellular insulin content falling to 35% and 23% of its original value, after 2 and 4 days in culture, respectively (**Figure 2.1.B**). This represents approximately 50% less insulin than that in same-day controls at the same time-points ($P<0.05$ *vs* same-day control).

After 4 days in culture, oleic acid-treated islets maintained only 48% of their original insulin content, a significant decrease ($P<0.05$) compared to control islets, which maintained 89% of their insulin content from day zero.

Unlike the single FFA treatments, the combination treatment had no significant effect on cellular insulin content compared to the controls after 4 days in culture, and exhibited significantly greater insulin content than palmitic-acid treated islets.

2.3.5 FFA in isolation reduce the secretory insulin response

The ability of islets to respond to stimulatory levels of glucose was determined by measuring insulin secretion following a 2-hour incubation in either 2.8 mM or 20 mM glucose in the absence of FFA, and expressing this value as a percentage of cellular insulin content. Four days of culture in palmitic acid significantly impaired the secretory response; stimulation index (SI) (ratio of amount of insulin secreted at 20 mM glucose to 2.8 mM glucose) fell to 1.74, nearly one third the stimulation index exhibited by control islets ($P<0.02$) (**Table 2.3**). The dramatic change in SI was attributed to moderate hypersecretion of insulin in low glucose conditions, and marked hyposecretion in high glucose conditions.

Islets treated with oleic acid also exhibited a serious impairment in glucose response, manifested primarily as hypersecretion of insulin in low glucose conditions. As a consequence, islets treated with oleic acid exhibited a depressed SI of 2.45, compared to an SI of 5.02 in control islets ($P<0.05$ oleic-treated *vs* control).

As with insulin content measurements, glucose response in islets treated with the combination of FFA was not significantly different from control islets after 4 days in culture. These islets maintained their capacity to respond appropriately to

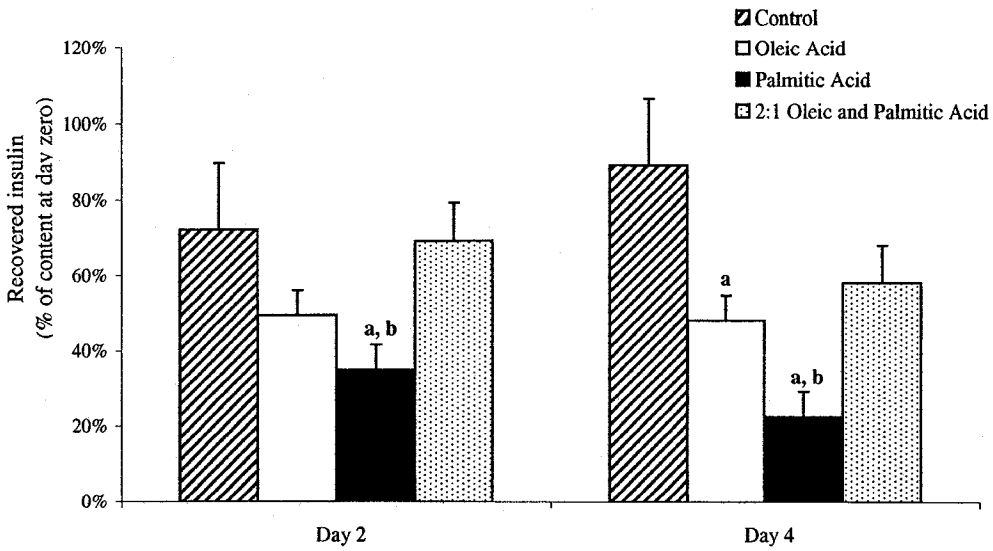

Figure 2.1.B. Cellular insulin content assessed in control and fatty acid-treated (0.5 mM) neonatal porcine pancreatic islets after 2 and 4 days in culture. Palmitic acid significantly reduced cellular insulin content by day 2. Values expressed as mean percentage of content at day zero ± S.E.M., from 3 independent experiments for a total n=5.
a $P<0.05$ *vs* same-day control, **b** $P<0.05$ *vs* same-day combination treatment.

Table 2.3. Effect of elevated levels of free fatty acids on insulin secretory capacity of neonatal porcine islets after 4 days in culture. Values are means ± S.E.M. from 2 independent experiments, for a total n=4 except for palmitic acid treatment, where n=3. NPI were cultured for 4 days in oleic acid, palmitic acid, or a combination of the two; total fatty acid concentration for all treatments was 0.5 mM. Stimulation indices were calculated by dividing the amount of insulin released at high (20 mM) glucose by that at low (2.8 mM) glucose. **a** $P<0.02$ *vs* control; **b** $P<0.05$ *vs* control.

	Insulin secretion (% of total insulin content)		
Treatment	2.8 mM glucose	20 mM glucose	Stimulation index
Control	1.59 ± 0.4	7.82 ± 1.7	5.02 ± 0.6
Oleic Acid	4.12 ± 1.7	6.71 ± 1.5	2.45 ± 0.8 [b]
Palmitic Acid	2.87 ± 0.8	3.86 ± 0.8	1.74 ± 0.7 [a]
2:1 Oleic and Palmitic Acid	2.09 ± 0.6	7.53 ± 1.8	3.99 ± 0.7

both high and low glucose conditions, exhibiting a SI not significantly different from the control islets.

2.3.6 Caspase inhibitor does not prevent palmitic acid-induced islet cell death

It is widely held that FFA exert their cytotoxic effect through an apoptotic mechanism. Thus, it was proposed that blocking one arm of the apoptotic pathway could prevent FFA-induced cell death. To investigate this possibility, we incubated neonatal pig pancreatic islets in the caspase inhibitor z-VAD-fmk (11.1 μM) (34-36) for 90 minutes, followed by 4 days culture in palmitic acid (0.5 mM).

As previously described, incubation with palmitic acid caused a significant decrease in cellular DNA content, indicating a marked decrease in the number of cells. As shown in **Figure 2.2**, DNA content of islets pre-incubated with z-VAD-fmk before palmitic acid treatment was not significantly different from those islets treated with palmitic acid alone, indicating that z-VAD-fmk was unable to prevent palmitic acid-induced cell death. Interestingly, treatment with z-VAD-fmk alone markedly increased DNA content to a value almost 50% higher than in the control islets ($P=0.11$, control *vs* z-VAD-fmk), significantly greater than the cellular DNA content of both the FFA treatments ($P<0.05$).

Analysis of islet cell composition by immunohistochemistry yielded results reminiscent of those observed with DNA content. Again, pre-incubation of islets with z-VAD-fmk provided no protection from the toxic effects of palmitic acid on the α- and β-cell populations (**Table 2.4**). However, pre-incubation with z-VAD-fmk and no subsequent FFA treatment yielded significantly larger α- and β-cell populations compared to FFA-treated islets ($P<0.05$), and more interestingly, yielded a significantly larger β-cell populations than control islets ($P<0.05$).

To ensure palmitic acid's toxic effect on islets was indeed manifested through apoptosis, islets were embedded in agar, thus ensuring islet integrity, and sections were assessed for TUNEL-positive cells. Control islets exhibited low levels of TUNEL-positive cells throughout the islets, and a marked increase in TUNEL-positive cells was observed upon addition of palmitic acid alone (**Figure 2.3**). Pre-incubation in z-VAD-fmk did not visibly decrease the degree of TUNEL-positive

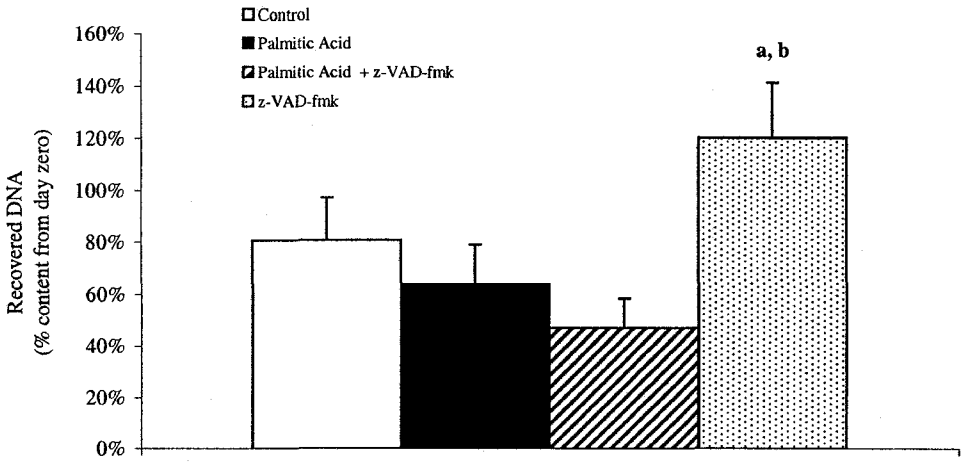

Figure 2.2. Cellular DNA content assessed in control, fatty acid (0.5 mM), and caspase 3 inhibitor (11.1 μM) treated neonatal porcine pancreatic islets after 4 days in culture. z-VAD-fmk alone significantly increased cellular DNA content compared with palmitic acid-treatedcells, both with and without z-VAD-fmk pre-treatment. Values expressed as mean percentage of content at day zero ± S.E.M., from 2 independent experiments for a total n=4. **a** $P<0.05$ *vs* palmitic acid; **b** $P<0.01$ *vs* palmitic acid with z-VAD-fmk pre-treatment.

Table 2.4. Cellular composition of neonatal porcine islets after 4 days in elevated free fatty acid (0.5 mM) conditions, with or without pre-incubation in z-VAD-fmk (11.1 μM), a pan-caspase inhibitor. Values are means ± S.E.M., from 2 independent experiments, for a total n=4. Cell composition was determined by calculating the product of the total number of cells in the preparation as determined from cellular DNA content and the percent glucagon- and insulin-containing cells as determined by immunohistochemistry, using the equation described in Methods. **a** $P<0.05$ *vs* pre-incubation with z-VAD-fmk only.

	Number of Cells (x 10^5)	
Treatment	α-cells	β-cells
Control	2.21 ± 1.03	2.15 ± 0.83 [a]
Palmitic Acid	1.57 ± 0.67 [a]	2.17 ± 1.03 [a]
Palmitic Acid + z-VAD-fmk	1.09 ± 0.47 [a]	1.45 ± 0.58 [a]
z-VAD-fmk	3.15 ± 1.22	3.74 ± 1.38

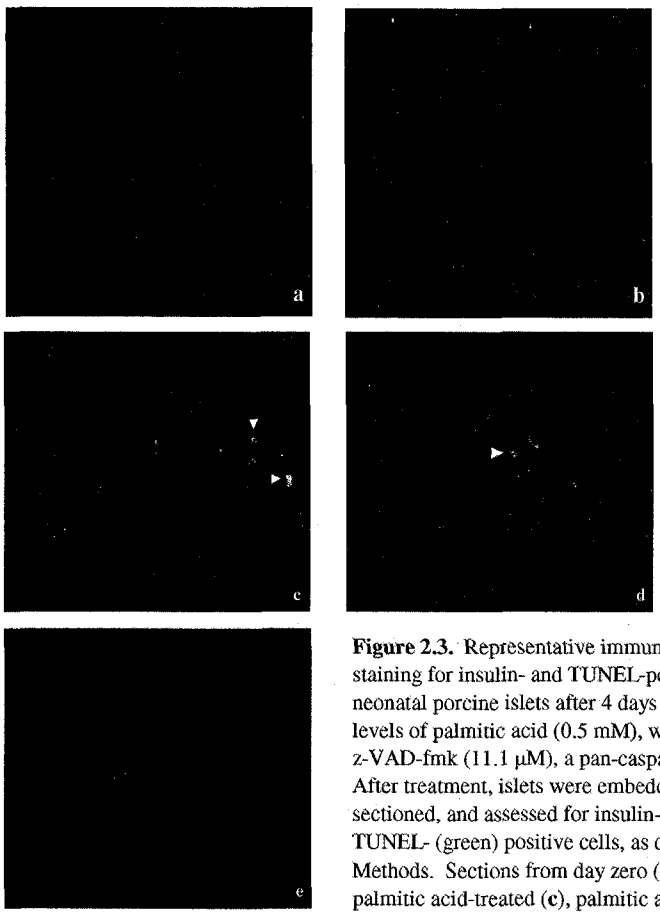

Figure 2.3. Representative immunohistochemical staining for insulin- and TUNEL-positive cells in neonatal porcine islets after 4 days in elevated levels of palmitic acid (0.5 mM), with and without z-VAD-fmk (11.1 μM), a pan-caspase inhibitor. After treatment, islets were embedded in agar, sectioned, and assessed for insulin- (red) and TUNEL- (green) positive cells, as described in Methods. Sections from day zero (**a**), control (**b**), palmitic acid-treated (**c**), palmitic acid + z-VAD-fmk-treated (**d**), z-VAD-fmk-treated (**e**) neonatal pig pancreatic islets were assessed. Low levels of TUNEL-positive cells were present in the absence of palmitic acid (**a, b**), while a marked increase in TUNEL-positive cells was observed upon the addition of palmitic acid, both with (**d**) and without (**c**) pre-incubation in z-VAD-fmk, including some cells staining positive for both insulin and TUNEL (►). Addition of z-VAD-fmk markedly reduced the low-level of TUNEL-positive cells observed in control islets (**e**).

staining in islets cultured in palmitic acid. In contrast, islets pre-incubated in z-VAD-fmk without subsequent fatty acid treatment displayed a marked absence of TUNEL-positive staining.

2.4. Discussion

Chronic exposure to elevated levels of free fatty acids can induce cell death (14;15;17;18;37) and dysfunction (38;39) in both pancreatic islets and pancreatic β-cell-derived clonal lines. In this study, we sought to determine if neonatal pig islets (NPI), recently shown to have enhanced resilience in hypoxic (30) and high glucose surroundings (31), would be similarly robust in a lipid-rich setting. The results presented here suggest that NPI are sensitive only to the cytotoxic effects of specific FFA treatments. The FFA cytotoxic mechanism appears to be two-pronged, both inducing apoptosis in the general islet cell population, and severely impairing the function of the β-cell population in particular.

Palmitic acid (0.5 mM), a saturated fatty acid, induced a marked decrease in cellular DNA content in less than 2 days. In contrast, both oleic acid (0.5 mM), an unsaturated fatty acid of similar chain length, and a combination of both oleic and palmitic acids in a 2:1 ratio for a total concentration of 0.5 mM, failed to induce a significant loss of cells after even 96 hours in culture. These results are similar to those found in both human (17) and rat (40) islets, where unsaturated fatty acid alone or in combination with saturated fatty acid failed to induce cell death. However, other work has shown unsaturated acids are capable of initiating apoptosis, both alone (15;18) and in combination with saturated fatty acid (41). A modest decrease in NPI DNA content after 4 days in culture with oleic acid suggests that oleic acid has the potential to induce pancreatic islet cell death, and that this effect manifests over a longer time course than investigated in this study. Indeed, a varying time-scale for the induction of death, depending on cell type and fatty acid used, could account for the previously noted contradictory studies (14;15;17;40;41).

Immunohistochemical analysis of islet protein expression supports this hypothesis, as islets treated with palmitic and oleic acids, both alone and in combination, exhibited

differing degrees of decreased α- and β-cell sub-populations among the total islet cell population. Palmitic acid treatment induced a far more profound decrease in α- and β-cell populations than in oleic acid-treated islets, further supporting the notion that while both FFA induce cell death, the saturated fatty acid was able to do so in an accelerated manner compared to unsaturate. Moreover, the fact that the percentage of insulin- and glucagon- positive cells decreased independently of the decrease on total islet cell number suggests that FFA preferentially target endocrine cells for cell death.

Our data indicate that FFA are acutely able to cause functional failure of NPI. After 4 days in culture, insulin content of both palmitic and oleic acid-treated islets was significantly lower than control values. This is likely due in large part to the hypersecretion of insulin observed in non-stimulatory glucose conditions. Unregulated insulin hypersecretion in low glucose surroundings may exhaust islet insulin stores, leaving islets unable to adequately respond to elevated glucose levels, explaining the markedly lower levels of insulin secretion observed in response to 20 mM glucose by oleic- and palmitic-treated islets and the correspondingly depressed stimulation indices. What remains unresolved is how islets treated with the combination of fatty acids, which had a total fatty acid concentration equal to the single fatty acid treatments, caused neither a significant decrease in cellular insulin content nor a deterioration in the islets' ability to respond appropriately to stimulatory and non-stimulatory glucose concentrations. One possible explanation is that islets possess an enhanced capacity to metabolize a mixture of fatty acids, as compared to single fatty acids, into the triglyceride storage form. Previous studies have correlated cellular triglyceride accumulation to decreased fatty acid cytotoxicity in β-cells (10;37).

Perhaps one of the most interesting findings was the significant change in total cell phospholipid composition in fatty acid-treated islets. The significant increase in contribution to phospholipid composition by the predominant fatty acid in which they were cultured unequivocally shows the islets were taking up and incorporating the fatty acids in their structural lipids. Phospholipids are key structural components of cellular membranes, including those of the ER and mitochondria, and phospholipid

composition can significantly affect protein release from the ER, protein segregation within lipid rafts of the membrane, signal transduction, and glucose transport (42;43). As an endocrine cell of high levels of insulin protein synthesis and secretion, even a modest change in phospholipid composition may severely impact the β-cell's ability to function. Moreover, changes in membrane composition have been associated with the clinical etiology of diabetes (44). A more focused investigation must now examine the phospholipid composition of individual organelles in fatty acid treated β-cells, to determine if the effect is sequestered to only one area of the cell, and how that may impact function.

It is surprising that, despite the drastic changes in phospholipid composition, exposure to elevated fatty acids had no effect on triglyceride composition. This does not, however, exclude the possibility that the total amount of triglyceride in the islets has changed; it is merely that the composition of these triglycerides is fixed. Moffitt *et al.* (10) did observe changes in both phospholipid and triglyceride composition in their study of lipotoxicity, but the clonal β-cell line used in that study likely has differing fatty acid processing capabilities from primary β-cells.

While it is clear that treatments of single FFA are cytotoxic to pancreatic islets, the pathway by which apoptosis proceeds remains unclear. Various groups have suggested the ER stress response (14;15;45), mitochondrial DNA damage resulting from oxidative stress and nitric oxide production (16;46), and induction of apoptosis by elevated endogenous ceramide production (17;18;40;41). To determine if the caspase cascade was a relevant factor in FFA-induced β-cell apoptosis, we pre-incubated islets in z-VAD-fmk, a pan-caspase inhibitor tripeptide (34;36;47), then proceeded with palmitic acid treatment as before. The caspase inhibitor was unable to prevent palmitic acid-induced decreases in islet DNA content and β-cell populations, indicating that lipotoxic induction of apoptosis proceeds through a caspase-independent mechanism. Alternatively, the moderate concentration of caspase inhibitor used in these experiments may have simply been insufficient to completely prevent FFA-mediated apoptosis. Perhaps the most interesting results from the caspase inhibitor experiments were those from the islets treated with z-VAD-fmk alone. Not only did islets pre-incubated with z-VAD-fmk with no

subsequent FFA treatment exhibit significantly greater DNA content than their FFA-treated counterparts, but their DNA content was nearly 50% higher than that of control islets. Moreover, at the end of the culture period, islets pre-incubated with z-VAD-fmk had significantly more β-cells than all other treatments, including control islets. These findings clearly suggest that while z-VAD-fmk was unable to prevent FFA-induced cell death, it was able to reduce basal apoptosis, resulting in a larger β-cell population at the end of a 4 day culture period than that found in control islets. Moreover, the inability of z-VAD-fmk to prevent apoptosis in islets treated with palmitic acid suggests ER stress is not the sole mechanism of β-cell apoptosis in the lipotoxic environment, in contrast with two recent studies that strongly implicated FFA-induced cell death with ER stress (14;45). ER stress, which may result from elevated secretory protein synthesis, glucose deprivation, perturbed calcium homeostasis, or a number of other factors, ultimately leads to accumulation of misfolded proteins in the ER lumen (48). This in turn elicits the unfolded protein response (UPR), a 4-stage stress response terminating in apoptosis. β-cells, whose primary function is the biosynthesis of insulin, are particularly susceptible to ER stress. The signaling molecules directly associated with initiating apoptosis during the UPR are DNA-damage inducible transcript 3 (DDIT3, previously known as CHOP), mitogen-activated protein kinase 8 (MAPK8, previously known as JNK1), and caspase 12 (45). MAPK8 initiates mitochondria-dependent apoptosis, while the other two molecules require caspase signaling to trigger the apoptosis (48). Thus, in the presence of z-VAD-fmk, only the MAPK8 or some other caspase-independent apoptotic pathway would be free to proceed. Whether this single signaling mechanism is sufficient to induce the degree of dell death observed in islets treated with palmitic acid is unclear. However, this finding does allow that other, mitochondria-driven apoptosis signaling pathways, such as oxidative stress, may play a role in lipotoxicity.

Given the disproportional effect of FFA on cell death and cell function (*eg.* after 4 days in culture, islets treated with oleic acid exhibited significantly depressed stimulation indices, but cellular DNA content was not significantly different from that of control islets); it seems possible that FFA are responsible for the lipotoxic

phenomena of islet cell death and dysfunction via a sequential pathway. Additionally, the decreasing fraction of β-cells within a total islet cell population that is itself decreasing suggests that there are in fact two pro-apoptotic mechanisms at play. The first is likely the ubiquitous pro-apoptotic effect that FFA possess on multiple cell types, including cardiomyocytes, pre-adipocytes, and breast cancer cells (49-52). The second is a result of a sequential lipotoxic effect specific to β-cells. FFA increasingly interfere with β-cell function, finally reaching a level of dysfunction at which the β-cell initiates apoptosis in response. The data suggests FFA indirectly drive β-cells to cellular suicide by preventing normal function. When FFA are present as a mixture of various chain lengths and degrees of saturation, their detrimental effect on β-cell function is somehow ameliorated or delayed, thus preventing the β-cell dysfunction and apoptosis, and potentially accounting for the unaffected DNA content and β-cell function observed in NPI treated with a combination of fatty acids in this study.

The described sequential scheme of lipotoxicity in pancreatic islets becomes infinitely more complex when confronted with the innumerable permutations of FFA environments. In the present study, it appears that palmitic acid almost immediately impaired β-cell function, triggering a significant degree of β-cell apoptosis within 48 hours of exposure. Oleic acid required more time to impair β-cell function to a degree sufficient to trigger apoptosis, as after 4 days of exposure, while signs of β-cell dysfunction were observed, the β-cell population had decreased less substantially than in islets treated with palmitic acid. Islets treated with the combination of fatty acids may have delayed dysfunction and death by re-directing excess FFA into triglyceride stores of fixed composition, a response facilitated by the mixture of fatty acids present. The diversion of excess FFA into storage form may have slowed the β-cell specific cytotoxic effect, enabling islets to retain function for a longer period of time. It remains to be seen whether extended exposure to even the combination of fatty acids would ultimately result in β-cell dysfunction and death.

The greatest weakness of current research into the *in vitro* mechanisms of lipotoxicity is the short length of exposure utilized (generally less than 96 hours), which is not representative of chronic exposure *in vivo*. Moreover, the tendency to expose islets

only to single fatty acids puts the physiological relevance of results obtained by such experiments into question. Perhaps, then, the most effective path on which to proceed in the investigation of lipotoxicity in an *in vitro* setting is to expose islets to fatty acid treatments that more accurately represent the physiological situation, as the 2:1 combination of oleic and palmitic acids did in this study, over a greater time period, thus enabling investigation of the mechanism by which fatty acids impair islet function prior to the induction of apoptosis. Deceleration of the lipotoxic cascade will provide a more accurate depiction of the *in vivo* reality of fatty acid exposure and will therefore help to illuminate critical steps in fatty acid-induction of islet cell dysfunction, potentially revealing novel modes prevent β-cell failure and death in the face of hyperlipidemia.

In conclusion, it is clear that while neonatal pig pancreatic islets are not adversely affected by a physiologically representative combination of FFA, they are unequivocally sensitive to the lipotoxic effects of single FFA environments. This suggests that for clinical transplantation, these islets may demonstrate enhanced resistance to the abnormal fatty acid dynamics exhibited in type 1 diabetics (53;54) and hyperlipidemia of type 2 diabetics (4;5;55;56). Combined with their previously demonstrated resistance to glucotoxicity (31) and hypoxia with subsequent reperfusion injury (30), neonatal porcine islets possess significant advantages as a source of tissue for islet transplantation. Moreover, it appears that by inhibiting one arm of the apoptotic cascade, it is possible to significantly increase NPI β-cell populations *in vitro*, thus partially addressing the requirement that any tissue used for islet transplantation be essentially unlimited in supply.

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Chapter Three

Exposure to Growth Factors Increases Insulin Biosynthesis in Neonatal Porcine Islets

3.1. Introduction

The initial publication of the Edmonton Protocol (1), and its subsequent successes, both in terms of patient outcomes and reproducibility (2-4), are fundamental motivations for the current plethora of research devoted to establishing islet transplantation as a feasible mode of treatment for diabetes mellitus. Before islet transplantation can become standard treatment for the hundreds of millions diabetics worldwide, numerous obstacles must be overcome, including the acute shortage of tissue available for transplantation (5;6). Alternative islet sources include adult and embryonic stem cells, induced replication of mature human β-cells *in vitro*, and xenogeneic sources (7). The neonatal pig in particular is a suitable xenogeneic source of tissue, and has recently been shown to correct diabetes in non-human primates (8). Despite the relative ease with which neonatal porcine islet (NPI) tissue can be isolated and processed into a form suitable for transplantation (9), the ability to manipulate this tissue *in vitro* to a state in which it may more efficiently correct the diabetic phenotype, whether by inducing β-cell replication or β-cell progenitor maturation to increase total β-cell mass, or by stimulating existent β-cells to a higher functional state, is an area of interest and importance. This study was undertaken with the aim of examining the effect of an established differentiation protocol on NPI, in terms of induction of replication, differentiation, and improvement in function. Neonatal porcine islets are an ideal model in which to investigate differentiation potential; isolated from 1- to 2-day old piglets, the pancreata contain both actively proliferating and not yet fully mature islets (9-12). Thus, the population of cells receptive to imposed differentiation is maximized, as opposed the pancreata from mature specimens, where the plastic cell population is limited by the highly differentiated state of the organ (13). Moreover, because of their immaturity, NPI may hold the potential for expansion without loss of phenotype.

The differentiation protocol used in this study was originally designed with the aim of inducing the trans-differentiation of mesenchymal tissue to β-cells, but the growth factors used are typical of β-cell progenitor treatments. The overall regime, depicted in **Figure 3.1**, consists of the sequential addition of growth factors to neonatal porcine islets cultured *in vitro* over a period of 10 days. Prior to initiation of the differentiation protocol, islets are cultured 5 days in serum-free media to facilitate the death of contaminating exocrine tissue. A portion of the progenitor population may die during this period, but previous work in our lab has shown significant histological staining for CK-7, a ductal cell marker recognized as representative of the precursor cell population (14-17), at this time-point (5;10;18). Thus, when the differentiation treatment in this study is initiated, a large number of these potential precursor cells are present.

For the first 72 hours of treatment, the islets under the differentiation protocol are cultured in unsupplemented high glucose (25 mM) media. A parallel set of islets is cultured in low-glucose, unsupplemented media for the duration of the study act as controls. While chronic exposure to elevated glucose has been associated with islet cell death (19-21), short-term exposure to high levels of glucose are known to promote islet cell replication (22-24). Thus, the high glucose treatment is intended to stimulate both β-cell and progenitor replication. There has also been some suggestion in transplant models that hyperglycemic environments may encourage progenitor differentiation to β-cells (15;25;26).

The treated islets are then cultured in low glucose (5.6 mM) media supplemented with 10 mM nicotinamide for the remainder of the treatment period. Nicotinamide has been associated with increased β-cell populations, the fall-out of both increased total number of islet cells and increased frequency of β-cells within the islet population in both human and mouse preparations (27-29). Nicotinamide is also commonly used to induce islet-specific gene expression in embryonic stem cell-derived insulin-expressing cells (30-32).

48 hours after transfer to a low glucose surroundings, the media is further supplemented with 10 nM exendin-4. Exendin-4 is a 39 amino acid polypeptide isolated from venom of *Heloderma suspectum*, or Gila monster (33), and bears a 53%

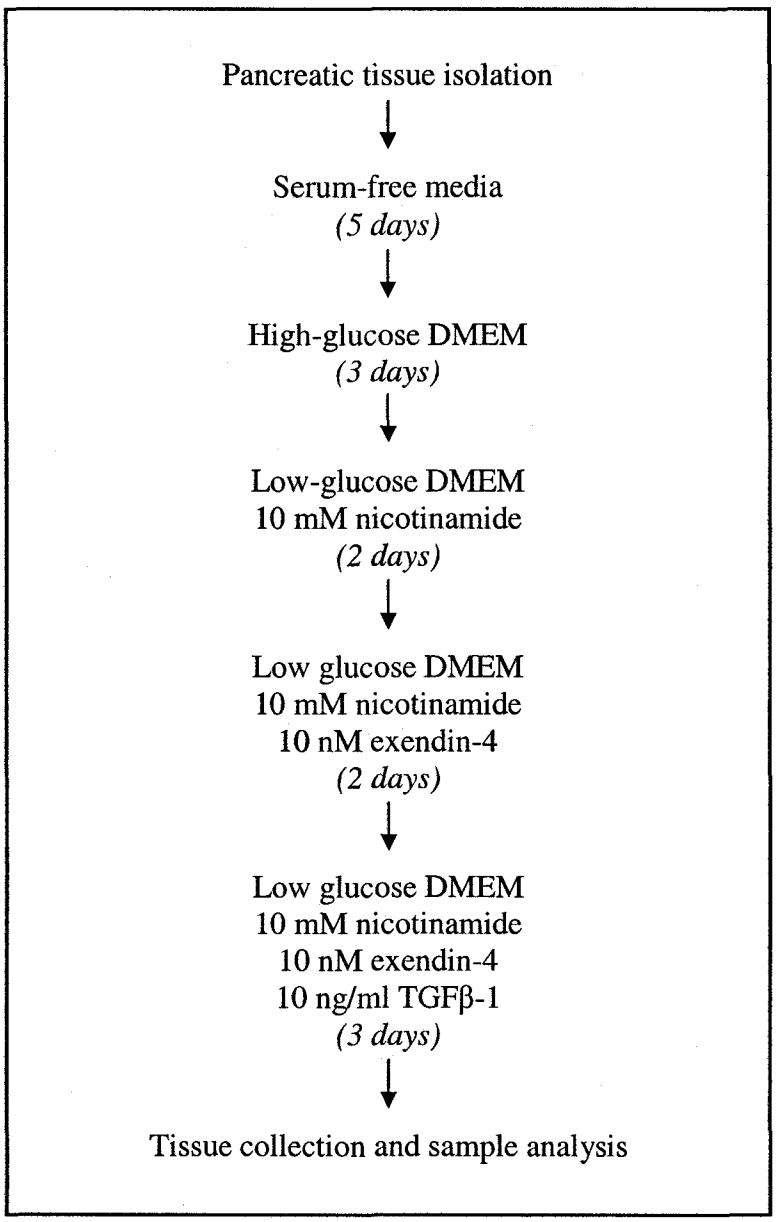

Figure 3.1. Growth factor delineation for culture of neonatal porcine islets during differentiation protocol experiments. Islets from wild-type 1- to 2-day old piglets were isolated and sequentially exposed *in vitro* to mitogenic and differentiation-inducing growth factors as described in Methods, in 2 independent experiments, for a total n=4.

homology to glucagon-like peptide (GLP) 1 (34). GLP-1 is an incretin, released by the L-cells of the intestine in response to nutrient ingestion (35). It is a gluco-regulatory hormone, primarily associated with slowed gastric emptying, reduced food intake, and enhanced insulin sensitivity, but increasing evidence suggests it may also stimulate islet cell proliferation and prevent islet cell apoptosis (35-37). Exendin-4 is favoured over GLP-1 for potential therapeutic applications, as its resistance to degradation by dipeptidyl peptidase-IV provides it with a significantly longer half-life *in vivo* (36;37). Exendin-4 treatment has been associated with differentiation of new β-cells from rat and human ductal cell precursors (38), enhanced maturation of fetal human (39) and porcine (40) pancreatic tissue to a β-cell fate, and increased insulin biosynthesis and glucose-stimulated secretion in rat islets (41). Recent work has described the mechanism by which GLP-1, or a GLP-1 receptor agonists like exendin-4, may potentiate glucose-stimulated insulin secretion (42).

The differentiation protocol concludes with the addition of TGFβ-1 (10 ng/ml) to the media, to act in synergy with the nicotinamide and exendin-4. TGFβ-1 has been shown to not only increase insulin positive cell populations when added in isolation (43), but to enhance β-cell population and insulin expression in exendin-4 treated mouse and human tissue (44;45). After 72 h exposure to the triad of growth factors, the tissue is collected and analyzed for DNA and insulin content, as well as mRNA and histological analysis.

3.2. Materials and Methods

3.2.1 Neonatal pig pancreatic islet isolation and culture

Islets were isolated from male Duroc neonatal pigs (Swine Research and Technology Centre, University of Alberta) aged 1-2 days old (1.4 – 1.9 kg body weight) as previously described (9). Piglets were anaesthetized under halothane and subjected to laparotomy and completely exsanguinated. The pancreata were removed and placed in cooled HBSS supplemented with 0.25% w/v BSA, then minced, digested in 1.0 mg/ml collagenase XI (Sigma-Aldrich, Oakville, ON, Canada), and filtered through a

500 μm nylon screen. The filtrate was cultured in Hams F10 medium (Invitrogen, Burlington, ON, Canada) containing 10 mM glucose, supplemented with 10 mM nicotinamide (BDH Biochemical, Poole, Dorset, UK), 2 mM L-glutamine, 0.5% w/v BSA (fraction V) (Sigma), 50 μM IBMX (ICN Biomedicals, Montreal, QC, Canada), 100 U/ml penicillin and 100 μg/ml streptomycin (Cambrex Bio Science, East Rutherford, NJ, USA) for 5 days at 37°C in humidified 5% CO_2/95% O_2, with media changes every 2 days. After 5 days in culture, the islets were divided equally into fractions that would be subject to control or differentiation treatment conditions.

3.2.2 Differentiation media preparation

High- and low-glucose DMEM (GIBCO) were supplemented with 0.5% w/v BSA (fraction V), 1% ITS (insulin, transferrin, selenous acid) + Premix (VWR International, Mississauga, ON, Canada), 100 U/ml penicillin and 100 μg/ml streptomycin. Stock media containing growth factors (10 mM nicotinamide, 10 nM exendin-4 [Sigma], and 10 ng/ml TGFβ-1 [Chemicon, Millipore, Billerica, MA, USA]) was mixed, filtered, and used for media changes of the tissue at the appropriate time points. Control islets were cultured in low-glucose DMEM for the duration of the experiment.

3.2.3 Cellular DNA and insulin content

PicoGreen, a fluorescent nucleic acid stain for double stranded DNA, was used to assess cellular DNA content, as per the manufacturer's instructions (Invitrogen). Duplicate aliquots were washed in citrate buffer (150 mM NaCl, 15 mM citrate, 3 mM EDTA, pH 7.4) and stored as dry cell pellets at -20°C. At time of analysis, pellets were resuspended in lysis buffer (10 mM Tris, 1mM EDTA, 0.5% Triton-X100, 4°C, pH 7.5), sonicated, vortexed, and analyzed (9).

Duplicate aliquots were also collected to determine cellular insulin content by RIA (Diagnostic Products Corp., Los Angeles, CA, USA). Samples were collected in 2 mM acetic acid (0.25% w/v BSA), sonicated, and centrifuged (800 *g*, 10 minutes, 4°C). The supernatants were collected and stored at -20°C until time of analysis (9).

3.2.4 Immunohistochemistry

To assess insulin-, glucagon-, and CK-7 positive fractions of the islet cell population, samples were collected in calcium-free HBSS supplemented with 1 mM EGTA and 0.5% w/v BSA. Islets were then mechanically dissociated with siliconized glass pipettes at 37°C for 7 minutes. 0.4 mg/ml DNAse and 1.0 mg/ml trypsin were added (Boehringer Mannheim, Laval, QC, Canada), and mechanical dissociation continued for a further 4 minutes. Cells were washed and resuspended in PBS, allowed to adhere to Histobond slides (Paul Marienfeld, Lauda-Königshofen, Germany), fixed in Bouin's fixative without picric acid, and stored in 70% v/v ethanol at 4°C until analysis.

The avidin-biotin complex (ABC, Vector Laboratories, Inc., Burlingame, CA, USA) method with diaminobenzidine (DAB) chromagen (Signet Laboratories) was used to quantify insulin-, glucagon-, and CK-7 positive cell fractions. Slides were rehydrated and endogenous peroxidases quenched with 10% H_2O_2 in methanol, then blocked for 20 minutes with 20% normal goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Antigen retrieval for CK-7 staining consisted of microwaving rehydrated slides in sodium citrate (10 mM, pH 6.0) for 6 intervals of 5 seconds, then allowing the slides to cool completely before proceeding with quenching and blocking. Primary antibodies (1:1000 guinea pig anti-porcine insulin, 1:5000 mouse anti-porcine glucagon, and 1:50 mouse anti-human CK-7, DAKO, Mississauga, ON, Canada) were added for 30 minutes at room temperature in a humidified chamber. Slides were then washed twice in PBS before addition of secondary antibody (1:200 biotinylated goat anti-guinea pig and 1:200 biotinylated goat anti-mouse, Jackson) for 30 minutes. Slides were incubated with ABC for 40 minutes before addition of DAB. Single cell counts were performed on a minimum of 500 cells, and positively stained cells expressed as a percentage of total cell number. Cells that stained positively for glucagon or insulin were categorized as α- or β-cells, respectively. The total number of α-, β-, and CK-7-positive cells was determined using the total islet cellular DNA content and the percentage of protein-positive cells (9):

$$\frac{\text{Total DNA content}}{7.1\frac{pg\,\text{DNA}}{\text{cell}}} \times \frac{\text{Protein - positive cells}}{100} = \text{number of sub - population cells}$$

3.2.5 RNA Isolation and Polymerase Chain Reaction

At both the outset and conclusion of the differentiation experiment, islet cell samples were collected, dissolved in 1 ml Trizol (Invitrogen) and RNA extracted as per manufacturer's instructions. cDNA was synthesized from 1 μg RNA together with TaqMan Reverse Transcription Reagents (Applied BioSystems, Foster City, CA, USA) as per manufacturer's instructions, and stored at -20°C until analysis.

Each cDNA sample was analyzed in triplicate for each gene of interest in individual PCR reactions in MicroAmp optical 96-well plates as per the TaqMan Universal PCR Mastermix protocol. 1 μl cDNA was combined with 12.5 μl Master Mix, 0.75 μl (10μM) of both forward and reverse primers, 0.38 μl (10 μM) probe, and 9.7 μl Sigma water, for a final volume of 25 μl. Master Mix buffer and probes were ordered from Applied BioSystems. Primers were contructed using known GenBank sequences (University of Alberta DNA Core Lab).

Samples were amplified over an initial 2 minute incubation at 50°C, 10 minutes at 95°C, then 40 cycles of 95°C for 15 seconds followed by 60°C for 1 minute, and measured using ABI Prism 7000. Threshold cycle, or the cycle at which the probe-generated fluorescent signal from the gene of interest appears above the background, and is normalized to the threshold cycle of both a housekeeping gene (in this study, porcine GAPDH was used) and the endogenous control (here, the level of transcript expression at day zero), was used as the basis of measurement.

3.2.6 Statistical analysis

Results are presented as mean ± standard error of two independent experiments performed on islets from at least 2 pancreata. Statistical comparisons were conducted using SPSS statistical software 13.0 for Windows (Chicago, IL, USA) with a student's t-test to determine significance. A P-value of less than 0.05 was considered significant. Analysis for block effect was calculated manually.

3.3. Results

3.3.1 Differentiation treatment significantly increases cellular insulin, but not DNA content

As shown in **Figure 3.2,** after 10 days in culture, the total cellular insulin content of treated NPI was significantly greater than in the control NPI ($P<0.02$), increasing to 180% of insulin content of the islets of day zero, compared to the control islets, which merely maintained their insulin content from day zero. The increase in insulin content was not due to proliferation of insulin-positive cells, as there was no significant difference in cellular DNA content between control and treated NPI, implying an equal number of cells present; both treated and control islets retained approximately 75% of their DNA content from day zero.

3.3.2 Differentiation treatment alters gene expression

Treated NPI exhibited over twice the level of insulin mRNA transcripts as control islets ($P<0.05$), as depicted in **Figure 3.3**. Interestingly, insulin mRNA expression in treated islets was essentially equal to the level of expression in islets at day zero, though, as previously described, cellular insulin content after 10 days of treatment was over 1.8 times the levels observed in islets at day zero, indicating a marked increase in translational activity. Glucagon mRNA expression also modestly increased in treated islets as compared to control islets, though this trend did not reach significance.

There was no significant difference in Pdx-1 mRNA expression between control and treated cells after 10 days of culture.

3.3.3 Culture duration, but not differentiation treatment, affects protein expression

To assess the treatment's level of influence on differentiation to, or expansion of, specific islet cell sub-populations, islets were mechanically dissociated, adhered to microscope slides, and subject to analysis by immunohistochemistry. Insulin-positive cells were classified as β-cells, and glucagon-positive cells were classified as α-cells.

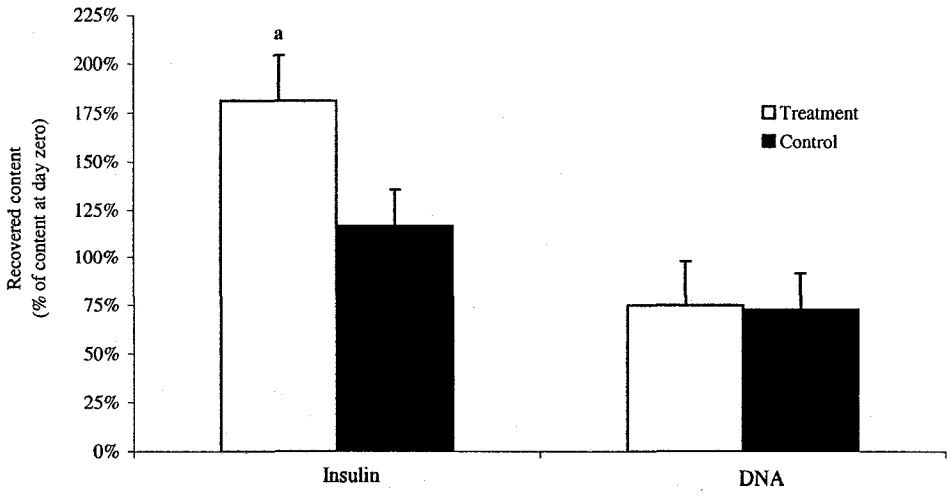

Figure 3.2. Cellular insulin and DNA content in control and differentiation protocol-treated neonatal porcine pancreatic islets after 10 days in culture. The differentiation protocol had no effect on cellular DNA content, but significantly increased cellular insulin content. Values expressed as mean percentage of content at day zero ± S.E.M., for a total n=4. **a** $P<0.02$ *vs* control.

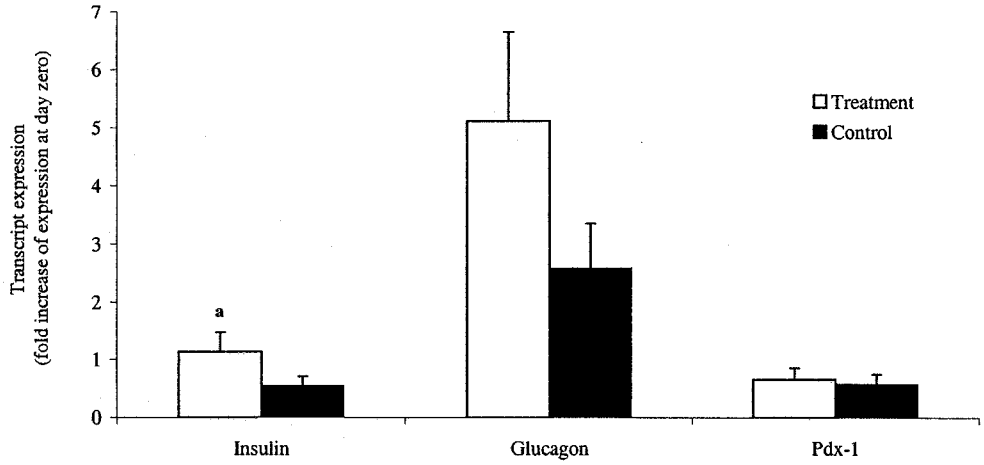

Figure 3.3. Cellular mRNA content in control and differentiation protocol-treated neonatal porcine pancreatic islets after 10 days in culture. The differentiation protocol significantly increased cellular insulin mRNA expression. Values expressed as mean fold change in mRNA expression from day zero ± S.E.M., for a total n=4, and standardized with GAPDH levels. **a** $P<0.05$ *vs* same gene control.

CK-7 was used as a marker of pancreatic epithelial ductal cells, a cell population proposed to be a source of β-cell progenitors (14-17). At the beginning of the treatment period, the islet tissue preparation contained approximately 230,000 α-cells; 290,000 β-cells; and 480,000 ductal cells. As shown in **Table 3.1**, after 10 days in culture, the number of CK-7-positive cells dropped significantly in both treated and control islets ($P<0.025$ *vs* day zero), to less than a quarter of the original value. However, at the end of the culture period, there was no significant difference in the number of CK-7 positive cells between treated and control islets.

There was no difference in the number of α- or β-cells in the control or treated islets after the 10 day treatment period. Indeed, the total number of α- and β-cells in both control and treated islets was unchanged from the populations observed at day zero. The absence of an increase in endocrine cell population reciprocal to the decrease in CK-7-positive cells indicates that the CK-7-positive cells are not differentiating into hormone-producing endocrine cells.

Table 3.1. Cellular composition of neonatal porcine pancreatic islets before and after 10 days of growth factor treatment. Values are means ± S.E.M., from 2 independent experiments for a total n=4. Cell composition was determined by calculating the product of the total number of cells in the preparation as determined from cellular DNA content and the percent glucagon-, insulin-, and CK7-positive cells as determined by immunohistochemistry, using the equation described in Methods. **a** $P<0.025$ *vs* day zero; **b** $P<0.01$ *vs* day zero.

	Number of cells (x 10^5)		
	α-cells	β-cells	CK-7 positive cells
Day zero	2.34 ± .48	2.90 ± 0.55	4.84 ± 1.47
Treatment, day 10	2.70 ± .34	3.24 ± 0.71	0.71 ± 0.31 [b]
Control, day 10	2.95 ± .45	2.54 ± 0.78	0.86 ± 0.25 [a]

3.4. Discussion

The ability to create a pool of β-cells that is capable of controlled expansion while maintaining a functional phenotype, complete with glucose responsiveness, is considered a Holy Grail among pancreatic islet researchers. In the physiological state, unless presented with the metabolic challenge of pregnancy (46-48) or obesity (49;50), total β-cell mass is static, exhibiting basal mitotic activity. When isolated and expanded *in vitro*, β-cells tend to lose their differentiated phenotype, and no longer respond adequately to metabolic stimulation (49;51-53). To circumvent the difficulties posed by expansion of mature β-cells, much attention has been focused towards the targeted maturation of endogenous β-cell progenitors. Both the acinar (54;55) and epithelial ductal (16;17;55;56) cells of the pancreas have been proposed as potential sources of β-cell progenitors. At present, the spectrum of protocols and growth factor treatments under investigation for their potential to induce differentiation of β-cell progenitors is wide-reaching and diverse, and beyond either coherent summary or analysis. It was the aim of this study to examine the effect of a single specific growth factor protocol on neonatal porcine islets (NPI).

Despite the mitogenic effect often observed with both acute elevated glucose and nicotinamide treatment, there appeared to be no stimulation of islet cell replication in the treated tissue; cellular DNA content in control and treated islets was essentially identical. However, it must be noted the cited studies that examined these mitogenic factors were carried out in rodent or human tissue, not NPI. Indeed, another study that specifically examined fetal and neonatal porcine pancreatic tissue reported no beneficial effect of nicotinamide on DNA content alone (26). Moreover, the inability of elevated glucose culture conditions to induce β-cell replication in neonatal porcine tissue *in vitro* has previously been observed in our lab (18). Thus, the lack of effect on DNA recovery following treatment should not be completely unexpected, and further supports the hypothesis that the ability to stimulate replication is species-dependent.

Similarly, the treatment did not appear to induce the maturation of β-cell progenitors, as the total number of β-cells did not change significantly over the culture period,

regardless of whether growth factors were administered. Previous studies in fetal and neonatal porcine islets have indicated the ability of nicotinamide and GLP-1 to increase the insulin-positive cell population (25;40;57). However, it is worth noting that in this study, as previously described, the isolated tissue was cultured for 5 days in serum-free media to facilitate the death of contaminating exocrine tissue prior to administration of the differentiation protocol. It may be that upon removal from their physiological environment, progenitor cells have a limited period of time during which they can be externally influenced, after which they move towards a pre-determined fate, and that in this experiment, the crucial time-point at which progenitor cells may be driven by external factors towards a β-cell fate had already passed at the initiation of the differentiation treatment.

The ultimate fate of the approximately 400,000 CK-7-positive cells lost over the 10 day culture period in both the control and treatment islet preparation remains unclear. Undoubtedly, a portion of this loss is due to cell death, as both control and treatment preparations lost approximately 25% of their original DNA content over the 10 day *in vitro* period. Similarly, a small fraction of the CK-7-positive cells may have differentiated to α- and β-cells, to replace endocrine cells that underwent apoptosis over the 10 day period, thus maintaining the total number of α- and β-cells. However, without *in situ* analysis of the CK-7-positive population, it is not possible to define the mechanism by which the loss of these cells occurred.

TaqMan analysis was used to qualitatively analyze changes in mRNA expression. Changes in expression of transcription factors associated with β-cell maturation could indicate the stimulated maturation of progenitors within the total population. In this study, no significant difference in the expression of pancreatic duodenal homeobox gene-1 (Pdx-1) between treated and control islets was observed. Pdx-1 is a master regulator of pancreas and β-cell development; it is expressed both early in endocrine cell development and during islet cell differentiation in the developing embryo (58;59). It is also expressed in the mature β-cell, and is necessary for maintenance of the β-cell phenotype (58;59). Thus, it is expected that if progenitors were differentiating to a mature β-cell fate, total expression of Pdx-1 would increase. The fact that Pdx-1 expression in both the control and treated islets was essentially

unchanged from expression levels at day zero, along with the unchanged number of β-cells over the course of the experiment, further supports the conclusion that no differentiation of progenitors to mature β-cells occurred.

In contrast with other findings in this study, insulin transcript expression was significantly influenced by the differentiation treatment, increasing to more than double the level observed in the control islets. Moreover, the increase in insulin gene expression had a downstream effect on protein expression, as treated islets exhibited significantly greater insulin content than control islets. It is clear, then, that the differentiation protocol stimulated biosynthesis of insulin protein. This is consistent with other studies that have reported increased insulin transcript and protein levels following treatment with exendin-4 and nicotinamide (40;41). mRNA transcript analysis also suggested that the differentiation protocol may stimulate glucagon gene expression, but the increase in transcript level in the treated islets was not significantly greater than in the controls, and no measurement of glucagon protein content was conducted.

When taken together, it is clear what this differentiation protocol can, and cannot, induce when administered to neonatal porcine islets. The results of immunohistological and mRNA analysis, as well as DNA content, clearly indicate that the treatment is neither inducing β-cell replication, nor is it stimulating the differentiation of β-cell progenitors into mature, functional β-cells. However, it is capable of significantly upregulating insulin synthesis in existing β-cells, which is manifested as significantly increased insulin transcript and protein content.

These results underscore the fact that findings in one species are not necessarily universal, most clearly illustrated by the complete absence of induction of β-cell replication by nicotinamide, an acknowledged phenomenon in pancreatic tissue of other species (24;27;28). They also raise the suggestion that there is a "best before" date on progenitor cells, and that time itself, both before and during *in vitro* manipulation, has a significant impact on cell fate. Lastly, these results raise an interesting question: how would these treated islets perform upon transplantation into a diabetic recipient, as compared to untreated islets? Would islets that are more functionally active in terms of insulin production correct the diabetic phenotype more

rapidly than control islets? There is no clear consensus regarding the improved capacity of islet tissue cultured *in vitro* with growth factors to correct hyperglycemia following transplantation (27;60), and the suggestion has been made that the more important factor is the developmental age of the islets and the physiological environment into which they are transplanted (26).

If, in fact, neonatal porcine islets cultured under this differentiation protocol are able to more rapidly correct hyperglycemia, or are able to do so with fewer islets than are traditionally needed, then this protocol holds great promise as an additional tool with which to push xenotransplantation of neonatal porcine islets to the clinical stage. Indeed, findings that further characterize the neonatal porcine islet will allow the medical research community to firmly establish the safety and feasibility of pig-to-human xentransplantation as a realistic, and much-needed, treatment for diabetes.

3.5. References

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Chapter Four
General Discussion and Conclusions

4.1. Discussion and Conclusions

Diabetes mellitus is a disease of extraordinary burdens, in terms of the physical symptoms and complications experienced by those who suffer from it, the responsibility and worry shouldered by their loved ones, the lost productivity in the workforce, and the healthcare costs necessary to manage the disease and treat its complications. It is no wonder, then, that finding a feasible and effective treatment for the over 171 million diabetics worldwide is an area of intense medical research. Perhaps most prominent among the avenues of diabetes research is that of islet transplantation. It remained an area of minimal success until the development of the Edmonton Protocol in 2000 (1). That study, along with its subsequent follow-up publications (2-4), re-ignited the flame of interest in islet transplantation as a means to treat diabetes. Seven years later, there remain several obstacles that prevent islet transplantation from becoming standard treatment for diabetes. Foremost among these is the significant decline in the islet graft's ability to correct diabetes; 1 year post-transplant, 75% of allogeneic islet transplant recipients were insulin-independent (2), but 5 years post-transplant, only 10% remained so (5). This issue is compounded by the need to develop immunosuppressive regimens less toxic to recipients, and to guarantee against the recurrence of autoimmune attack of transplanted islets in those with type 1 diabetes.

If these challenges are overcome, widespread use of allogeneic islet transplantation as a means to treat diabetes is further impeded by the severe paucity of pancreatic tissue suitable for transplantation (6;7). Currently, all tissue used in islet transplantation is harvested from donor cadavers. The ideal solution to this tissue shortage is to develop an unlimited supply of insulin-producing, metabolically responsive cells.

Investigation into this unlimited supply is focused in three areas: i) differentiation of embryonic or adult stem cells, ii) expansion of mature β-cell populations *in vitro*, and iii) xenogeneic islet sources. Embryonic stem cells pose obstacles both in terms of

the social and cultural attitudes towards stem cell research in general, and, more technically, the ability to render these cells capable of both producing insulin and secreting it in a physiologically relevant fashion. Though the differentiation of insulin-producing human embryonic stem cells has been reported, the functional yield of these cells following the differentiation process remains low (8;9). Differentiation of adult stem cells to insulin-producing, glucose-responsive cells has also experienced limited success (10;11). Expansion of mature β-cells carries fewer societal implications, but the maintenance of β-cell function over long-term culture has proven difficult (12-15).

Xenogeneic islets, and particularly the use of porcine islet tissue, is arguably the closest of any of these approaches towards clinical trials. Indeed, two recent landmark studies have demonstrated the ability of neonatal (16) and adult (17) porcine islets to correct diabetes in non-human primates under novel immunosuppressive protocols. Despite these remarkable successes, the safety of islet xenotransplantation must be established before it can be taken to the clinical stage. The potential for transmittance of porcine endogenous retrovirus (PERV) to the human genome, characterization of the human immune response to xenogeneic tissue, including the potential for a hyperacute humoral response to the porcine cell surface antigen Gal-α-gal, and the widespread social acceptance of the use of animal organs for transplantation into humans must all be resolved (18;19).

Once these preliminary hurdles are overcome, numerous questions regarding the transplanted islets' ability to survive, as well as establish and maintain metabolic homeostasis in transplant recipients, remain unanswered. The immediate post-transplant period is extremely stressful on the transplanted islets; they may be subject to hypoxia and reoxygenation injury (20), immune-mediated attack initiated by the instant blood-mediated inflammatory reaction (IBMIR) (6), and functional exhaustion when suddenly put in an environment of high metabolic demands, particularly the hyperglycemia (6) and abnormal lipid dynamics (21) present in diabetic recipients. It is of great interest then, to characterize how the various alternate sources of islets react and respond to these stressful environments; to determine if any alternate islet

source is more robust in the presence of these stresses, and thus holds greater potential to successfully correct the diabetic phenotype in the long-term.

With this in mind, the aim of the study described in Chapter Two was to investigate the effect of elevated levels of free fatty acids (FFA) on neonatal porcine islets. Elevated plasma FFA are commonly exhibited by both type 1 and type 2 diabetics (22;23), and are believed to exert a lipotoxic effect on pancreatic β-cells, inhibiting β-cell function and inducing β-cell apoptosis (24-27). However, neonatal porcine islets (NPI) have previously been shown to exhibit enhanced survival in the typically stressful environments of chronically elevated glucose (28) and hypoxia/reoxygenation (20). Thus, this study sought to determine if these islets were similarly robust during chronic exposure to elevated FFA. NPI were found to be susceptible to the toxic effects of elevated FFA only when incubated in single FFA, exhibiting cell death, particularly among the β-cell population, and significant impairment of β-cell function, in terms of insulin content and responsiveness to glucose, in both palmitic and oleic acids. Oleic acid, an unsaturated fatty acid, had a less significant toxic effect than palmitic acid, a saturated fatty acid, resulting in less dramatic decreases in both cell population and β-cell function. Importantly, islets treated with a physiologically representative combination of oleic and palmitic acids exhibited no significant effect on either cell death or β-cell dysfunction, suggesting that NPI are resilient in an environment that more accurately depicts what would be encountered upon transplantation. This enhanced survival and maintenance of function in a mixed FFA environment may be the result of enhanced triglyceride synthesis by the β-cells, but this was not measured in this study.

Chapter Two also investigated the potential for a pan-caspase inhibitor peptide to prevent cell death in islets treated with palmitic acid. While the caspase inhibitor could prevent neither the decrease in cellular DNA content nor the fall in the number of β-cells in islets treated with palmitic acid, it demonstrated a beneficial effect when added in isolation to islets. Islets incubated with the caspase inhibitor had significantly more β-cells, as well as greater cellular DNA content than control islets. This finding implies that the caspase inhibitor inhibited basal apoptosis in the cultured islets, resulting in a larger β-cell population than would normally be obtained at the

end of the culture period. Hence, these findings demonstrate an efficient way by which β-cell mass can be expanded *in vitro*. This study also suggests that FFA likely induce numerous pro-apoptotic pathways, at least one of these being caspase-independent.

The aim of Chapter Three was to investigate the potential for an established differentiation protocol to induce the expansion of existing β-cells, and/or the proliferation and subsequent differentiation of β-cell progenitors in neonatal porcine islet tissue *in vitro*. The differentiation protocol in question had been previously developed with the aim of trans-differentiating mesenchymal tissue to metabolically active, insulin-producing tissue. Porcine pancreata from 1-2 day old piglets contains a significant population of immature cells (29-32) that may be receptive to exogenous induction or re-direction of differentiation to a mature β-cell fate, resulting in a larger β-cell population than would be obtained during unmanipulated culture *in vitro*. From the clinical standpoint, any manipulation of donor tissue *in vitro* that will reduce the donor to recipient ratio, and thus decrease the number of islet donors needed to correct diabetes in a single recipient is highly desirable. This study was undertaken with the aim of examining the proliferation and differentiation potential of neonatal porcine islets. Interestingly, we found no evidence indicating induction of β-cell progenitor differentiation with the modified protocol. Indeed, the number of β-cells was static in both control and treated populations over the course of the experiment. However, treated cells displayed significantly increased insulin biosynthesis, suggesting that the differentiation protocol stimulated existing β-cells into a heightened state of protein transcription and translation. The question remains of whether the enhanced insulin production activity of these β-cells would translate into more rapid correction of hyperglycemia upon transplantation into a diabetic recipient.

While distinct in exact focus, both these studies act to further validate NPI as a source of transplantable tissue in the drive to establish islet transplantation as a feasible mode by which to treat diabetes. The findings of Chapter Two suggest that NPI will ably withstand hyperlipidemia and abnormal lipid dynamics as they are presented in the diabetic patient. This work also gave insight into a method that may be used to

expand β-cells *in vitro*, thus providing a reliable tissue source in a field that is currently plagued by a lack of transplantable islets. Chapter Three describes an *in vitro* procedure that dramatically increases the level of insulin production in NPI. The repercussions of this finding in terms of what it may mean for the amount of islet tissue required for the correction of diabetes, and the length of time required for this corrections, are exciting and worthy of investigation. Taken as a whole, this thesis adds to the already sizable body of knowledge describing NPI as a highly attractive and advantageous source of tissue for islet transplantation.

4.2. Future Directions

Islet xenotransplantation has taken a large step forward with the recent publication of two landmark studies, both demonstrating the ability of porcine islets to engraft and reverse diabetes in non-human primates (16;17). Furthermore, both studies demonstrated that porcine islets are not subject to Gal-specific antibody-mediated rejection, and that immunosuppression of the cell-mediated immune response protected the islet grafts from rejection. No evidence of PERV transmission to the recipient was observed following xenotransplantation of neonatal porcine islets. Thus, these studies break down some of the barriers that have prevented islet xenotransplantation from reaching the clinical stage. However, significant obstacles remain, particularly the morbidity associated with the immunosuppressive therapies used. Opportunistic infections and thromboembolism were among the outcomes reported in these studies. Modification of the immunosuppressive protocol to reduce or eliminate these side effects, while maintaining complete protection of the xenograft, is required before this combination of xenotransplantation and immunotherapy may be used in humans; definitive improvement of quality of life, rather than the exchange one set a of physiological frailties for another, is the only setting in which islet transplantation is an acceptable mode of treatment.
Under current isolation procedures in our laboratory, a single piglet yields 40,000 to 45,000 islet equivalents (IEQ) (unpublished observation). Human allogeneic transplants typically require at least 12,000 IEQ/kg to restore glucose control and

establish insulin independence (6). This means that under current methods, a 60 kg human receiving an islet xenograft would require tissue from a minimum of 16 piglets. This significant tissue requirement combined with the labour intensive nature of the current method of neonatal porcine islet isolation renders islet xenotransplantation as treatment for diabetes untenable. Only a future in which islet tissue destined for xenografts can be stably expanded *in vitro*, a method for the reliable, large-scale isolation of neonatal porcine islets is established, and/or a means by which current IEQ tissue requirements for reversal of diabetes in humans is decreased, will islet xenotransplantation be a feasible mode of treatment for diabetes. In conclusion, islet xenotransplantation has crossed the threshold, and is no longer the stuff of science fiction novels. Moreover, as this thesis clearly demonstrates, neonatal porcine islets possess numerous qualities and abilities that would significantly contribute in their ability to successfully correct diabetes. However, the journey towards making islet xenotransplantation a reality is far from over, and only by the combined efforts of individuals from fields as varied as immunology, transplantation medicine and public health policy will it become a truly feasible and sustainable treatment for diabetes.

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