CHARACTERIZATION AND PREVENTION OF CELL DEATH IN ISOLATED ISLETS OF LANGERHANS

by

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ABSTRACT

A major limitation to the success of islet cell transplantation as a therapy for type I diabetes is the cell loss induced by the islet isolation procedure. The aim of this thesis was to elucidate signal transduction events and related intracellular activities that are implicated in islet cell survival/death following islet isolation in order to develop therapeutic interventions to promote islet survival.

The isolation of pancreatic islets imposes considerable stress on these cells, resulting in significant levels of cell death following isolation which was associated with activation of the stress-activated c-jun NH_2-terminal kinase (JNK). However, within 24 hours in culture, JNK activation was greatly reduced concomitant with an increase in AKT activation. Inhibition of phosphatidylinositol 3-kinase (PI3K)/AKT signalling resulted in sustained JNK phosphorylation, while activators of AKT suppressed JNK phosphorylation, indicating that the rise in AKT activity during islet culture suppresses JNK. One of the stimulus of the PI3K/AKT pathway was found to be insulin secreted by the islets themselves, acting in an autocrine manner. The result of this autocrine activation of the pro-survival AKT pathway, and subsequent suppression of JNK, was a decrease in the appearance of apoptotic cells in islets after 72 hours in culture. Caspase inhibition alone was unable to prevent cell death in isolated islets. In addition, the amount of mitochondrial depolarization occurring in isolated islets was unaffected by caspase inhibition, leading to the notion that the commitment to islet cell death could be occurring at the level of, or upstream of, mitochondrial dysfunction. Indeed, inhibition of BAX translocation to the mitochondria, a critical event

mediating mitochondrial permeabilization, prevented islet cell death. Inhibition of JNK also prevented mitochondrial permeabilization and cell death.

The current results demonstrate that insulin can act as an autocrine survival signal in isolated human islets. These findings also reveal the interdependence of necrosis and apoptosis in isolated islets, suggesting therapeutic strategies which target early events in cell death signalling in order to prevent multiple forms of islet cell death.

RÉSUMÉ

Une limitation importante au succès des transplantations de cellules d'îlot pancréatique comme thérapie pour le diabète de type I est la perte de cellules induite par l'isolement des îlots. Cette thèse a pour but d'élucider les événements de transduction de signal et leurs activités intracellulaires relatives impliqués dans la survie ou la mort des cellules suivant l'isolement des îlots pour développer des interventions thérapeutiques maximisant la survie des îlots.

L'isolement des îlots pancréatiques impose un stress considérable aux cellules, résultant en un niveau significatif de mortalité cellulaire associée à l'activation de la SAP (stress-activated protein) kinase JNK (c-jun NH2-terminal kinase). Cependant, après 24 heures en culture, l'activation de JNK diminue considérablement avec une activation concomitante d'AKT. L'inhibition de la phosphatidylinositol-3-kinase (PI3K) et d'AKT a comme conséquence la phosphorylation soutenue de JNK, alors que les activateurs d'AKT suppriment cette même phosphorylation, indiquant que l'élévation de l'activité d'AKT pendant la culture des îlots supprime JNK. Le stimulus de la voie de signalisation PI3K/AKT s'avère être l'insuline sécrétée par les îlots eux-mêmes, agissant de manière autocrine. Cette activation autocrine des signaux AKT pro-survie, et la suppression ultérieure de JNK, résultent en une diminution de l'apparition de cellules apoptotiques dans les îlots après 72 heures en culture. L'inhibition seule des caspases n'empêche pas la mort des cellules d'îlot isolés. De plus, la quantité de dépolarisation mitochondrique se produisant dans les îlots isolés est inchangée par l'inhibition des caspases, indiquant que l'engagement à la mort

des cellules d'îlot se produirait au niveau (ou en amont) du dysfonctionnement mitochondrique. En effet, l'inhibition de la translocation de BAX aux mitochondries, un événement critique régissant la perméabilisation mitochondrique, empêche la mort des cellules d'îlot. L'inhibition de JNK proscrit également la perméabilisation mitochondrique et la mort des cellules.

Les résultats présentés démontrent que l'insuline agit de manière autocrine comme signal de survie dans les îlots humains isolés. Ces résultats indiquent également l'interdépendance nécrose/apoptose chez les îlots isolés, suggérant des stratégies thérapeutiques qui ciblent des événements tôt dans la voie de signalisation de la mort cellulaire dans le but d'empêcher plusieurs types de mortalité chez les cellules d'îlot.

To Jane,

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LIST OF FIGURES

ABBREVIATIONS

AP-1	activator protein-1
APAF-1	apoptosis-protease activating factor-1
ASK1	apoptosis signal-regulating kinase 1
ATF2	activating transcription factor 2
ATP	adenosine triphosphate
BH	Bcl-2 homology
BIP	Bax-inhibiting peptide
cAMP	cyclic adenosine monophosphate
CARD	caspase recruitment domain
CBP	CREB binding protein
CHAPS	3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate
COX4	cytochrome c oxidase subunit IV
CREB	cAMP-response element binding protein
CsA	cyclosporine A
DED	death effector domains
DMSO	dimethyl sulfoxide
DN	dominant negative
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EDTA	ethylenediaminotetraacetic acid
ER	endoplasmic reticulum
ERK	extracellular-signal regulated kinase
FADD	Fas-associated death domain
FAM	carboxyfluorescein
FBS	fetal bovine serum
FDA	fluorescein diacetate
FKHRL1	Forkhead (Drosophila) homolog rhabdomyosarcoma-like 1
FLIP	FADD-like IL-1β converting enzyme inhibitory protein
GRPP	glicentin-related pancreatic peptide
HBSS	Hanks' balanced salt solution
HIF-1	Hypoxia-inducible factor-1
HNMPA-$(AM)_3$	hydroxy-2-naphthalenylmethylphosphonic acid trisacetoxymethyl ester
IBMIR	instant blood-mediated inflammatory reaction
IBMX	3-isobutyl-1-methylxanthine
IEQ	islet equivalent
IGF	insulin like growth factor
IKK	IκB kinase
IL-1β	interleukin 1-beta
INF-γ	interferon gamma
iNOS	inducible nitric oxide synthase
IR	insulin receptor
IRS-1	insulin receptor substrate-1
JIP1	JNK-interacting protein 1

JNK	c-jun NH_2-terminal kinase
LPS	lipopolysaccharide
LVA	low voltage-activated
MAPK	mitogen-activated protein kinases
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
MDR-1	multidrug resistance transporter-1
MEKK	mitogen-activated protein/ERK kinase kinase
MKP-1	mitogen-activated protein kinase phosphatase-1
MLK	mixed-lineage protein kinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAD	nicotinamide adenine dinucleotide
NG	newport green
NOD	non-obese diabetic
NOS	nitric oxide synthase
PARP	poly (ADP-ribose) polymerase
PCAF	p300/CBP-associated factor
PDK1/2	phosphoinositide-dependent kinase 1 and 2
PH	pleckstrin homology
PI	propidium iodide
PI3K	phosphatidylinositol 3-kinase
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PTD	protein transduction domain
PTP	permeability transition pore
RNA	ribonucleic acid
ROS	reactive oxygen species
SERCA	sarcoplasmic endoplasmic reticulum Ca^{2+} ATPase
SIRT1	silent mating type information regulation 2 homolog 1
SOD	superoxide dismutase
STAT	signal transducer and activator of transcription
STZ	streptozotocin
TAK1	transforming growth factor β-activated kinase 1
TF	tissue factor
TG	thapsigargin
TLM	two-layer method
TLR4	Toll-like receptor 4
TNF-α	tumor necrosis factor alpha
TRAF	TNF-R-associated factor
UV	ultraviolet
UW	University of Wisconsin

GUIDELINES FOR THESIS PREPARATION

Manuscript-based thesis

"As an alternative to the traditional thesis format, the dissertation can consist of a collection of papers of which the student is an author or co-author. These papers must have a cohesive, unitary character making them a report of a single program of research. The structure for the manuscript-based thesis must conform to the following: 1. Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis.) 2. The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges preceding and following each manuscript are mandatory. 3. The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts. The thesis must include the following: a) a table of contents; b) a brief abstract in both English and French; c) an introduction which clearly states the rational and objectives of the research; d) a comprehensive review of the literature (in addition to that covered in the introduction to each paper); e) a final conclusion and summary; f) a thorough bibliography; g) Appendix containing an ethics certificate in the case of research involving human or animal subjects, microorganisms, living cells, other biohazards and/or radioactive material. "

CONTRIBUTIONS OF AUTHORS

All experiments were performed by the candidate except:

The insulin ELISA in Figure 4.2A was performed by Jie-Ping Ding.

The experiments in Figure 5.1C were performed by Steven Paraskevas.

PREFACE

The aim of this thesis was to elucidate signal transduction events and related intracellular activities that are implicated in islet cell survival/death following islet isolation in order to develop therapeutic interventions to promote islet survival. This thesis is composed of 7 chapters. In the first chapter, the introduction, a general introduction to diabetes and islet transplantation is provided, which highlights the problem of islet cell death following isolation from the pancreas. Chapter 2 examines the role of the PI3K/AKT pathway in regulating islet survival and is published in *Biochemical and Biophysical Research Communications* (277:455-461, 2000). Chapter 3 explores the relationship between the PI3K/AKT pathway and JNK signalling, and demonstrated that JNK inhibition can improved survival following isolation. This work was published in *Endocrinology* (145:4522-4531, 2004). In chapter 4, we demonstrate that autocrine insulin action activates the AKT pathway in isolated human islets. This work has been submitted for publication. The 5th chapter demonstrates that particular caspase inhibitors do not prevent islet cell death and suggests that mitochondrial dysfunction may lead to a necrotic-like form of cell death in the absence of caspase activity. The work in chapter 5 was published in the *Journal of Molecular Medicine* (82:389-397, 2004). Chapter 6 assesses the ability of a Bax-inhibitory peptide (V5) to prevent islet cell death following isolation. This work has been submitted for publication. Chapter 7 contains a general discussion of the results presented and how these may impact the field of islet transplantation.

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

1.1. DIABETES MELLITUS

Diabetes mellitus comprises a series of heterogeneous metabolic disorders caused by defects in insulin production and/or insulin action. Diabetes is the most common metabolic disease in the world and afflicts approximately 6% of the population of developed countries (1;2). It is estimated that 150 million individuals worldwide have diabetes and that this number will more than double by 2025 (3). Diabetes is the leading cause of blindness as well as end-stage renal failure, and is the fourth leading cause of death in North America (2;4). The World Health Organization (WHO) has labelled diabetes as one of the most substantial threats to human health in the 21st century.

Type 1 diabetes accounts for approximately 10% of adult patients with diabetes and results from autoimmune-mediated destruction of the insulin producing β-cells of the pancreas (5). Although the underlying aetiology of type 1 diabetes remains poorly understood, both genetic and environmental factors are involved (6). In the absence of endogenous insulin production, these patients are dependent on insulin injections in order to maintain glucose homeostasis. However, clinical studies have demonstrated that even strict blood glucose control by intensified insulin treatment can delay, but not prevent, the development of secondary complications such as neuropathy, retinopathy, and nephropathy (7;8). These findings suggest that exogenous insulin therapy is unable to provide adequate regulation of blood glucose levels.

Type 2 diabetes occurs when the β-cells fail to secrete sufficient insulin to manage the body's metabolic demands (9). The disease is almost always

associated with an increase in insulin requirements due to peripheral insulin resistance, a condition where the liver and skeletal muscle display defective insulin responsiveness (10). Diabetes develops when insufficient insulin is produced because of an insulin secretory defect and/or decreased β-cell mass (9;11). The fact that most type 2 diabetic patients display a net decrease in β-cell mass indicates that type 2 diabetes is ultimately a disease of insulin insufficiency (9;12).

1.2. ANATOMY OF THE ISLETS OF LANGERHANS

The endocrine cells of the pancreas are organized into dense clusters known as the islets of Langerhans, which are scattered throughout the exocrine parenchyma (Figure 1.1A). The islets are highly innervated and vascularized structures, receiving roughly 20% of the pancreatic blood supply despite representing only 3% of its total mass (13). Although most endocrine cells are organized as islets, single endocrine cells can be observed (14). In addition, islet size can vary considerable from a few cells up to 400 μm in diameter. It is estimated that a normal human pancreas contains roughly 1.5 million islets greater than 23 μm in diameter (15). Importantly, islets greater than 100 μm in diameter only constitute 20% of the total number of islets, but make up almost 80% of the total islet volume in the pancreas (16).

The islets are made up of four principle cell types, the glucagon producing α-cells, the insulin producing β-cells, the somatostatin producing δ-cells, and the pancreatic polypeptide producing PP-cells (Figure 1.1B). The relative

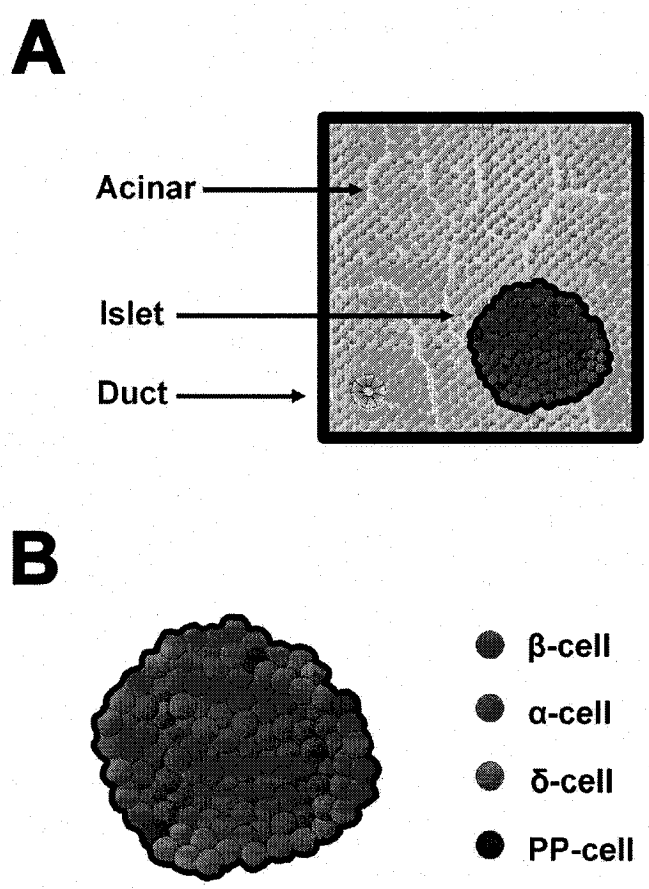


Figure 1.1. Islet structure. (**A**) Distribution of acinar, islets, and duct cells within the pancreas. (**B**) Typical cell type distribution within the islet.

percentages of the different islet cell types is roughly 68:20:10:2 (for β:α:δ:PP) (14), with the β-cells forming the islet core (medulla) and the other cell types distributed mainly in the surrounding mantle of the islet. However, recent findings suggest that the cell-type distribution in human islets can be quite variable, with α,β, and δ-cells being observed throughout the islets (17).

1.2.1. Cell types of the islet

1.2.1.1. β-cells

The β-cells secrete insulin in response to elevated blood glucose levels. Proinsulin is synthesized in the endoplasmic reticulum (ER) and transported to the Golgi, from which immature proinsulin containing secretory granules are formed. Mature secretory granules are formed once proinsulin is cleaved into insulin, a dimer consisting of an A-chain (21 amino acids) and a B-chain (30 amino acids) connected by two sets of disulfide bonds, and C-peptide (18). Increased blood glucose levels stimulate changes in β-cell metabolism that trigger the exocytosis of insulin-containing granules. Briefly, glucose equilibrates across the β-cell plasma membrane and is phosphorylated by glucokinase to generate glucose-6-phosphate, which is converted to pyruvate during glycolysis. Compared with other cell types, a high proportion of the pyruvate generated in β-cells is transported to the mitochondria and enters the TCA cycle due to low lactate dehydrogenase levels (19;20). The resulting generation of ATP increases the ATP:ADP ratio in the cytosol, leading to closure of the ATP-sensitive K^+ channels and membrane depolarization (21). Depolarization causes opening of

voltage-dependent Ca^{2+} channels leading to an increase in cytosolic Ca^{2+} which triggers exocytosis. Alternative mechanisms of insulin secretion also exist, which allow regulation of insulin secretion in response to metabolic and hormonal cues (22;23). Secreted insulin stimulates glucose uptake into tissues such as the liver and skeletal muscles, thereby reducing blood glucose levels.

In addition to the ability to secrete insulin in response to increased blood glucose levels, pancreatic β-cells have other unique properties. In order to secrete large amounts of insulin, β-cells are adorned with highly developed endoplasmic reticulum (ER) (24). The high levels of protein translation may render islets more susceptible to ER stress (24), during which cell death can be triggered by the accumulation of misfolded proteins in the ER (25). In addition, β-cells express low levels of endogenous antioxidant proteins and are thus are particularly sensitive to oxidative stress (26;27). β-cells also have very low rates of replication (12), which makes it difficult to expand these cells in vitro. As stated earlier, β-cells are excitable cells capable of action potentials and they have well developed Ca^{2+} handling machinery that can play a role in mediating β-cells death (28-32).

1.2.1.2. α-cells

The α-cells are located in the islet mantle and they are more numerous in islets of the tail and body region of the pancreas when compared to islets in the head of the pancreas. Processing of preproglucagon in α-cells yields glucagon, glicentin, glicentin-related pancreatic peptide (GRPP), and trace amounts of

glucagon-related peptide-1 (GLP-1); all of which are secreted by α-cells (33). Glucagon is secreted in response to decreased blood glucose or decreased amino acid levels and induces the breakdown of glycogen (glycogenolysis), the synthesis of glucose (gluconeogenesis), and the synthesis of ketones, resulting in increased blood glucose and amino acid levels. Glucagon is through to potentiate insulin release from β-cells (34), though it is unclear whether this paracrine effect plays a significant role *in situ* (35).

1.2.1.3. δ-cells

The δ-cells are found in the islet mantle and secrete somatostatin, a peptide known to suppress the secretion of several hormones, including insulin and glucagon (36). These cells can be distinguished by their dendritic-like projections, which project to neighbouring cells and capillaries (37). Though most of the somatostatin found in the blood originates from the gut, the somatostatin released within the islet is thought to play a regulatory role in the release of other islet hormones (36).

1.2.1.4. PP-cells

The PP-cells of the islet, also known as F-cells, secrete pancreatic polypeptide (PP), a 36 amino-acid peptide whose function remains unclear. In addition to being found in the islet periphery, PP-cells can also be found associated to pancreatic ducts and in acinar tissue (38).

1.3. HISTORY OF ISLET TRANSPLANT

Transplantation of only the islet tissue has several advantages over whole pancreas transplantation. Since the islets only constitute approximately 3% of the total mass of the pancreas, it is advantageous to avoid transplanting the exocrine component of the organ which can lead to complications following transplantation (39). In addition, whole organ transplantation is major surgery with an associated risk of morbidity and mortality, and is thus usually restricted to patients with end-stage renal failure (40). Islet transplantation, on the other hand, is a much simpler surgical procedure which could be more widely applicable (41).

The first attempt at islet transplantation as a treatment for diabetes occurred in 1893, when Dr. Watson-Williams transplanted 3 small pieces of pancreas from a sheep into the subcutaneous tissue of a 15 year old boy dying from uncontrolled ketoacidosis, the common cause of death in diabetic patients prior to the discovery of insulin 28 years later (42). Though the boy only survived for 3 days following the operation, a temporary improvement in blood glucose levels was observed. The discovery of insulin in 1922 by Banting, Best, McLeod, and Collip drastically improved the survival of diabetic patients (43), but exogenous insulin therapy could not prevent the development of chronic secondary complications.

It was proposed in 1967, by Paul Lacy, that transplantation of pancreatic islets could be a superior treatment for diabetes than exogenous insulin injections (44). In the early 1970s, several groups demonstrated that islet transplantation was able to restore normal glucose levels in rats rendered

diabetic by treatment with streptozotocin, a compound that is specifically toxic to β-cells (45-47). However, it wasn't until the development of the automated islet isolation method in 1986 that a sufficiently large number of islets could be isolated from a human pancreas for transplantation (48). Using this method, islet transplantation into a diabetic patient resulted in insulin independence for 22 days, after which point the graft failed, mostly likely due to inadequate immunosupression (49). In 1990, a series of patients at the University of Pittsburgh achieved prolonged insulin-independence following islet allotransplantation using a steroid-free immunosuppressive regime (50;51). However, these patients were undergoing abdominal exenteration with multivisceral resection for malignancy, so the success of these transplants could be related to the absence of an autoimmune diabetes background. During the 1990s, islet allotransplants were plagued by early failure, with only 12 per cent of patients from 1990-1998 remaining insulin-independent for more than 7 days (52).

An islet transplantation protocol was introduced in Edmonton in 1999 that included a steroid-free immunosuppressive regime and transplantation of much higher numbers of islets than ever before, which required multiple donor pancreata (53). An average of more than 11,000 islet equivalents (IEQ = an islet with a diameter of 150 μm) were transplanted in order to achieve insulin independence, which equates to a total of approximately 800,000 IEQ (54). Using this protocol, seven consecutive patients were able to achieve and maintain insulin-independence (53). Up until November of 2004, sixty-five

patients received islet transplants in Edmonton, of which 44 became insulin independent (54). However, of the patients achieving insulin independence, only 10% maintained insulin independence after 5 years. Furthermore, it is estimated that only 20% of the transplanted islet mass become functional grafts despite the high number of islets transplanted (55). Though the Edmonton protocol represented a significant improvement in the ability to achieve insulin independence through transplantation, a high number of islets is required, the function of these grafts is not ideal, and the grafts eventually fail.

In Miami, a recent study achieved a one-year insulin independence rate of 79% (11 out of 14 patients) using islets from one or two donors (56). However, two years after transplantation, only 43% of patients remained insulin independent. In this study, an average of 13,500 IEQ/kg were transplanted, which equates to 930,000 IEQ per patient, significantly higher than in the Edmonton protocol.

Recently, there has been much interest in single donor transplants in order to increase the number of patients that can be treated with the available organs. In a landmark study, Bernhard Hering's group at the University of Minnesota was able to achieve insulin independence in 8 consecutive patients using single donor islet transplantation (57). Five of these patients remained insulin independent for longer than 1 year. Patients were transplanted with an average of 7200 IEQ/kg obtained from one donor pancreas, which represents the first significant reduction in islet dose since the advent of the Edmonton protocol. Despite demonstrating the feasibility of single donor islet transplants, this study

also highlights the inefficient and unpredictable nature of the islet isolation procedure since only 8 of 18 consecutive isolations produced enough islets for transplantation.

1.4. POSSIBLE REASONS FOR THE FAILURE OF ISLET GRAFTS

Loss of viable islet cell mass can be divided into five main categories during the transplantation procedure: 1) islet loss during brain death and pancreas procurement, 2) islet loss during isolation, 3) islet loss during culture, 4) islet loss during the immediate post-transplantation period, and 5) islet loss following engraftment (Figure 1.2). In the first category, increased levels of inflammatory cytokines have been observed in donors following brain death (58), and it has been suggested this leads to decreased islet viability following isolation (59;60). In addition, unfavourable conditions during pancreas procurement and preservation can also affect islet viability (see section 1.8). Secondly, though the isolation procedure has been improved, it still remains inefficient at reliably isolating a high percentage of the total islet cell mass from donor organs. In the third phase, the injury sustained by the islet during isolation triggers islet cell death during subsequent culturing (61). Once transplanted, a significant percentage of islets are lost during the immediate post-transplantation period, a phenomenon known as primary non-function. It has been suggested that islet grafts trigger platelet activation, resulting in rapid infiltration and destruction of transplanted islets by leukocytes, a reaction termed the instant blood-mediated inflammatory reaction (IBMIR) (16). Hypoxia may also play a role

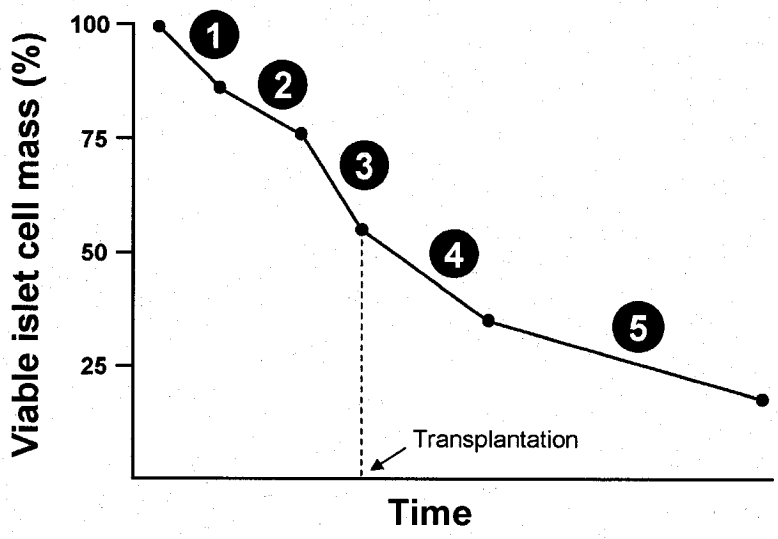


Figure 1.2. Loss of islet cell mass during islet cell transplantation. Loss of islet cell mass can be divided into four main categories during the transplantation procedure: 1) islet loss during brain death and pancreas procurement, 2) islet loss during isolation, 3) islet loss during culture, 4) islet loss during the immediate post-transplantation period, and 5) islet loss following engraftment.

in islet primary non-function since islets are avascular following isolation, and revascularization is not complete until 10-14 days following transplantation (62;63). However, the degree to which primary non-function of islet grafts is due to islet cell death triggered by the isolation procedure remains unclear. Thus it is plausible that some of the cell loss occurring in the 4th phase (immediately post-transplantation; Figure 1.2) is triggered by events occurring in phases 1-3. Finally, in the fifth phase, a slow loss of islet mass is evident during the subsequent years following transplantation; a poorly understood phenomena that could involve recurrent autoimmunity, chronic rejection, and/or diabetogenic effects of immunosuppressants (64).

The precise number of viable engrafted islets required to obtain insulin independence is not known. Patients who have undergone hemipancreatectomy generally do not display clinical diabetes (65-67), suggesting that transplantation of much less than 100 per cent of the total pancreatic islet cell mass could be sufficient to obtain normoglycemia. However, in Edmonton an average of more than 11,000 islet equivalents (IEQ = an islet with a diameter of 150 μm) were transplanted in order to achieve insulin independence, which equates to approximately 800,000 IEQ (54), far more than the estimated 500,000 IEQ present in a healthy human pancreas (16). Previous attempts using significantly less tissue were plagued by early graft failure, with only 12 per cent of patients from 1990-1998 remaining insulin-independent for more than 7 days (52). It is conceivable that the current number of transplanted islets required to achieve

normoglycemia includes a surplus of cells that are lost as a result of the isolation and transplantation process.

1.5. PROBLEM OF ISLET CELL DEATH FOLLOWING ISOLATION

Our laboratory made the original observation that isolated human islets undergo apoptosis following routine isolation (61;68). Similarly, high levels of apoptosis are observed in freshly isolated canine and rhesus monkeys islets (69;70). A significant increase in necrotic cell death with time in culture has also been observed (71). Isolated islet cell death results from a compound injury consisting of a barrage of several insults that, individually, are know to promote apoptosis, necrosis, or both. We will now consider the basic mechanisms of apoptosis and necrosis.

1.6. CELL DEATH MECHANISMS

1.6.1. Apoptosis

For a long time, eukaryotic cells have been thought to die by two distinct mechanisms: apoptosis or necrosis. Apoptosis is an energy-dependent process by which individual cells die by activating their own genetically programmed cell death mechanisms (72). There are many external cues that can trigger a cell to undergo apoptosis. These external signals lead to activation of various cellular signalling pathways that are responsible for mobilizing the apoptotic machinery, which includes the caspase family of cysteine proteases (73). During apoptosis, the effector caspases (i.e. caspase-3,-6,-7) cleave numerous cellular targets,

resulting in the systematic dismantling of the cell. Present in the cell as inactive zymogens, the effector caspases are activated by proteolytic cleavage by initiator caspases (i.e. caspase -8, -9, -10). The activation of the initiator caspases depends on interactions with adaptor molecules containing specific domains, such as death effector domains (DED) or caspase recruitment domains (CARD) (74;75). Different adaptor molecules present in the cell can respond to a variety of upstream signals, allowing for caspase activation in response to a range of cellular stressors. Many apoptotic signals converge on the mitochondria, leading to mitochondrial membrane permeabilization and subsequent release of cytochrome c from the intermembrane space (76). Once in the cytosol, cytochrome c complexes with apoptosis-protease activating factor-1 (APAF-1) and, in the presence of dATP, leads to activation of caspase-9.

The release of cytochrome c from the mitochondria is regulated by the Bcl-2 family of proteins (77;78). The proapoptotic Bcl-2 proteins can be subdivided into "multidomain" and "BH3-only" proteins. Multidomain proapoptotic proteins, such as Bax and Bak, contain the conserved Bcl-2 homology domains (BH) 1-3. Upstream of the multidomain proteins are the BH3-only proapoptotic proteins, such as Bid and Bad, which have only the amphipathic α-helical BH3 domain. Antiapoptotic Bcl-2 family members contain all four BH domains (BH1-4) and are generally thought to prevent apoptosis by sequestering the proapoptotic Bcl-2 proteins, although the exact mechanisms remain elusive (79). The anti-apoptotic members of the Bcl-2 family (i.e. Bcl-2, Bcl-X_L) are often upregulated by survival signals and can inhibit pro-apoptotic Bcl-2 proteins by heterodimerization

(80). It is often suggested that the Bcl-2 family acts as a switch regulating the decision to undergo apoptosis dependent on the ratio of survival and death signals (72;81). The proapoptotic multidomain proteins Bax and Bak play an essential role in mitochondrial dysfunction and cell death caused by a variety of stimuli (82). Bak is an integral membrane protein found mainly in the outer mitochondrial membrane, with a small fraction localized to the ER (83;84). In contrast, Bax normally resides in the cytosol of healthy cells, with a small percentage being localized to the ER, nuclear matrix, and the mitochondria (83;84). During apoptosis Bax changes conformation and translocates from the cytosol to the mitochondria and, along with Bak, mediates mitochondrial permeabilization and the release of cytotoxic proteins such as cytochrome c (78). Though the precise mechanism of mitochondrial permeabilization by proapoptotic Bcl-2 proteins is unclear, this step represents a critical checkpoint for survival beyond which the cell is committed to death.

1.6.2. Necrosis

Unlike apoptosis, necrosis does not require ATP and is much less regulated than apoptosis. Cells dying by necrosis typically swell and rupture, leading to a local inflammatory response. Necrosis is often associated with severe conditions such as ischemia, osmotic stress, or sheer stress. However, necrosis can also occur under normal physiological conditions, leading to the idea of programmed necrosis (85). Necrosis can be induced by overactivation of poly (ADP-ribose) polymerase-1 (PARP-1), leading to depletion of nicotinamide

adenine dinucleotide (NAD^+), impaired ATP production, and finally cell death (86). In addition, permeabilization of the mitochondria can lead to necrotic-like cell death, which could be attributed to the release of reactive oxygen species (ROS) following disruption of the mitochondrial membrane (85). Stimuli which cause an increase in nitric oxide have also been know to cause necrosis (87).

Many insults can lead to both necrosis and apoptosis. Moreover, in many circumstances, not all dying cells display the typical hallmarks of apoptosis or necrosis (88). This has led to the notion that perhaps apoptosis and necrosis represent two opposing ends of a complete range of intermediate forms of death (89). In many cases, the decision by a cell to undergo apoptosis or necrosis is based on the availability of sufficient energy to carry out apoptosis. Therefore, a stimulus know to induce apoptosis may induce necrosis when insufficient energy (glucose) is present.

1.7. SIGNALLING PATHWAYS REGULATING CELL DEATH

The ability of cells to react to environmental changes is dependent on the cooperation of intracellular signal transduction pathways to coordinate the cellular response. The integration of various external cues leads to regulation of physiological processes such as proliferation, differentiation, and death. A better understanding of these signals can provide insight into the mechanisms involved in islet failure following isolation and may provide potential therapeutic targets in order to increase islet survival and function.

1.7.1. The role of MAPKs in regulating islet cell death

The mitogen-activated protein kinases (MAPK) have been shown to play an important regulatory role in a variety or cellular processes (90). MAPKs are activated by a sequential cascade of protein phosphorylation in which MAPKs are phosphorylated by a MAPK kinase (MAPKK), which is itself activated by a MAPKK kinase (MAPKKK). This three-tiered kinase module is a common characteristic of all MAPK pathways (91). There are four main families of MAPK; the c-jun NH_2-terminal kinases (JNK), the p38 kinases, the extracellular-signal regulated kinase (ERK) family, and the ERK5 family (90). Little is known about the role of the ERK5 family in islets, therefore we will only consider the ERK, JNK and p38 families in the current discussion.

1.7.1.1. JNK

The c-jun NH_2-terminal kinases (JNK) are a group of MAPKs that play a role in apoptosis, proliferation, survival signalling, and embryonic morphogenesis (reviewed in (92)). JNK is activated by dual phosphorylation at Tyr185 and Thr183 by two dual specificity MAPKKs, MAPK kinase 4 (MKK4) and MAPK kinase 7 (MKK7). MKK4 and MKK7 are activated by a large group of MAPKKKs including the mitogen-activated protein/ERK kinase kinase (MEKK) family, the apoptosis signalling-regulating kinases (ASK), the mixed-lineage protein kinases (MLK), transforming growth factor β-activated kinase 1 (TAK1), and tumor progression locus 2 (TPL2) (93). Once activated, JNK can phosphorylate proteins in the nucleus and cytoplasm. Stress-induced JNK activation leads to

phosphorylation of transcription factors, including c-jun and activating transcription factor 2 (ATF2), which heterodimerize to bind divergent activator protein-1 (AP-1) sites in the c-jun promoter (94). However, despite the fact that c-jun expression is required in some cases for apoptosis, little is know about the genes that are involved. JNK-mediated induction of the Fas ligand (FasL) gene was proposed as a possible mechanism of JNK-induced apoptosis (95;96), however inhibition of Fas signalling does not prevent all forms of stress-induced apoptosis (97). A more direct link between JNK and the apoptotic machinery was observed in JNK-deficient cells which failed to release cytochrome c from the intermembrane space of the mitochondria in response to UV radiation (98). This effect is due to the ability of JNK to directly phosphorylate members of the BH3-only group of Bcl-2 family (Bim and Bmf), leading to Bax-dependent apoptosis (99;100). Therefore, JNK can regulate apoptosis by both transcription-dependent and independent mechanisms that are only now beginning to be elucidated. In addition, the duration of JNK activation appears to be important in determining the physiological outcome. Sustained JNK activation, in contrast with transient activation, appears to promote apoptosis in a variety of cell types (101-105). However, the consequences of JNK activation greatly depend on the type of stimulus and the cellular context (106).

JNK activation can be induced by growth factor withdrawal (107), detachment from the extracellular matrix (108), ischemia (109), osmotic stress (110), reactive oxygen species (111), and by cytokines (112), all of which are possible consequences of islet isolation. Our lab has demonstrated that freshly

isolated islets display high JNK phosphorylation that diminishes over the first 48 hours following isolation (113;114). This finding was recently confirmed elsewhere (115). This early activation of JNK coincides with the peak of islet apoptosis which occurs 24 hours following isolation (113;114). Addition of exogenous insulin to the culture media immediately following isolation reduced JNK activity and led to a decrease in DNA fragmentation after 24 hours in culture (113). Preservation of rat pancreata for 24 hours by the two-layer method (TLM), which uses oxygenated perfluorohydrocarbons to increase oxygen delivery to the organ, resulted in decreased apoptosis and lower JNK activity following isolation when compared to islets from pancreata stored in UW solution (116). In addition, the same study showed that islets from pancreata that were processed immediately following resection had significantly lower JNK activation than islet from pancreata stored for 24 hours by TLM or in UW solution. The most convincing evidence implicating JNK in transplanted islet survival comes from transplantation of rat islets overexpressing dominant negative (DN) JNK into streptozotocin-induced diabetic nude mice (117). Mice receiving DN-JNK islets displayed lower blood glucose levels, which could be attributed to maintenance of insulin gene transcription despite the presence of oxidative stress at the graft site as well as increased survival of islets due to impaired apoptotic signalling by JNK. Taken together, these findings point towards a role for JNK in mediating isolated islet apoptosis.

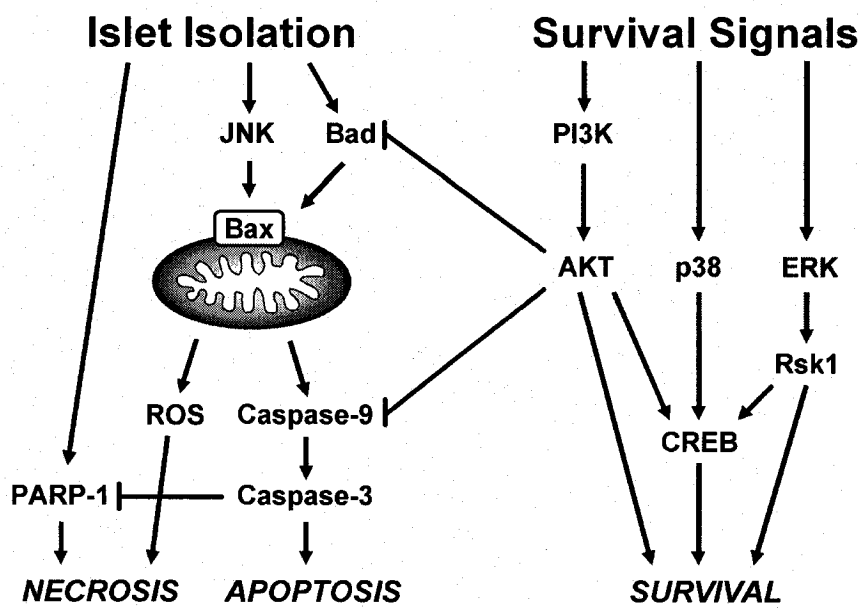


Figure 1.3. Selected intracellular signalling pathways regulating islet cell survival and death. See text for details.

1.7.1.2. p38

The p38 family of MAPKs play a role in regulation of inflammation, apoptosis, differentiation, and cell growth (93;118). There are five isoforms of p38 which have been identified to date (p38α,β,β2,γ,δ), which are activated to various degrees by MKK3, MKK4, and MKK6 in response to a range of stimuli including cytokines, growth factors, and environmental stress (119). Once active, p38 can target many substrates in both the nucleus and cytosol. As with JNK, the exact role of p38 in regulation of cell survival remains unclear as p38 appears to mediate both survival and apoptotic signals (93).

Isolated islets display increasing p38 activity over the first 7 days in culture following isolation (114). In porcine islets, high p38 activation following isolation correlated with decreased islet numbers over the first 36 hours following isolation, suggesting a role for p38 in mediating islet cell death (114). It has also been suggested that p38 can mediate β-cell survival, since inhibition of p38 potentiated IL-1β-induced cell death in a β-cell line (120). An increase in p38 phosphorylation was observed in isolated islets treated with insulin, which can act as a survival stimulus in many cell systems (113). This effect, however, is cell type-dependent since insulin has been shown to inhibit p38 activation in primary neuronal cultures (121). Nevertheless, IGF-I mediated survival has been shown to involve p38-dependent phosphorylation of cAMP-response element binding protein (CREB) and induction of Bcl-2 expression (122;123). Taken together, these data suggests that p38 could play a role in regulating islet survival, possibly by mediating growth factor signalling. It is also plausible that activation

of p38 in response to insulin represents an inhibitory feedback pathway to suppress insulin transcription in β-cells (124).

1.7.1.3. ERK

The ERK family of MAPKs regulate cellular processes such as mitosis, meiosis, survival, and cell growth (90;125). The two ERK isoforms (ERK1 and ERK2) are ubiquitously expressed and become activated in response to signals emanating from receptor tyrosine kinases, G-protein coupled receptors, and integrins. ERK becomes activated upon dual phosphorylation by MEK, which is itself activated by Raf-1. Raf-1 can be activated by Ras, a small G-protein proto-oncogene. Ras mutations occur in several human cancers, leading to persistent activation of ERK and increased cellular proliferation. ERK can suppress apoptosis in many cell types by transcriptionally-dependent and independent mechanisms (126). ERK directly regulates the activity of many transcription factors such as Elk-1, Ets1, c-myc, and c-jun (127). Many of the downstream effects of ERK on survival are mediated by the Rsk family of protein kinases (128). Activated Rsk is able to promote cell survival by phosphorylating and activating CREB, as well as by inhibitory phosphorylation of Bad (126;129).

In isolated porcine islets, high ERK phosphorylation immediately following isolation correlated with increased islet recovery after 36 hours in culture (114). Furthermore, inhibition of ERK activation using PD98059 induced apoptosis in purified rat β-cells (130). In contrast, ERK has been suggested to mediate IL-1β-induced NO production in β-cells and inhibition of ERK decreased cytokine-

induced cell death in purified rat β-cells (131;132). In addition, IGF-I mediated survival in INS-1 cells was not affected by PD98059 treatment despite the fact that inhibition of ERK activation decreased CREB phospohorylation (133). Taken together, these results indicate that the role of ERK in islet survival is still unclear.

1.7.2. AKT mediates cell survival

AKT (PKB) is a Ser/Thr kinase that regulates several cellular processes, including cell cycle progression, transcription, glucose uptake, and apoptosis. In particular, AKT plays a critical role in mediating cell survival in response to trophic factor stimuli and attachment to ECM (134). AKT is recruited to the plasma membrane following activation of phosphatidylinositol 3-kinase (PI3K) and is activated through phosphorylation by phosphoinositide-dependent kinase 1 and 2 (PDK1/2) (135;136). Activated AKT can directly phosphorylate several pro-apoptotic proteins, such as BAD (137;138), Caspase-9 (139), and FKHRL1 (140), leading to suppression of apoptotic signals (Figure 1.3). AKT can also phosphorylate the IκB kinase (IKK), leading to activation of NF-κB, which can promote the transcription of several pro-survival genes (141-143). AKT can also phosphorylate and activate CREB (144), which can promote the transcription of BCL-2. AKT has also been shown to suppress the JNK pathway by phosphorylating and negatively regulating the upstream kinases ASK1 (145), MLK3 (146), and MKK4 (SEK1) (147).

Several lines of evidence point towards a role for AKT in the survival of islets and β-cells. Transgenic mice expressing a constitutively active form of

AKT1 under the control of the insulin promoter display increased β-cell mass, improved glucose tolerance, and resistance to streptozotocin (STZ)-induced diabetes (148;149). Adenovirus-mediated overexpression of constitutively active AKT1 in isolated human islets increased *in vitro* β-cell proliferation, decreased apoptosis induced by serum/glucose withdrawal or high glucose, and decreased the number of transplanted islets required to reverse streptozotocin-induced diabetes in mice (150). In addition, PI3K/AKT signalling has been shown to mediate survival of isolated human islets (151), and insulinoma cell lines (133;152-154). Taken together, these findings suggest that the PI3K/AKT pathway plays a critical role in promoting the survival of β-cells.

1.8. ISLET ISOLATION PROCEDURE

Though many details have changed over the years, the basic steps of the present isolation procedure have changed very little since being introduced by Ricordi et al. (155). The main pancreatic duct is canulated and the pancreas is distended with cold Liberase enzyme blend via the pancreatic duct. The distended pancreas is then cut into pieces, and placed in the digestion chamber (known as the Ricordi chamber) along with marbles which assist in mechanical dissociation. The chamber is attached to a closed circuit via a 450 μm mesh, allowing digested tissue to be released from the chamber. The circuit is warmed to activate the Liberase and the chamber is shaken to aid the mechanical disruption of the tissue. Once the tissue is sufficiently digested, the circuit is cooled and the digestate, which contains both exocrine and endocrine tissue, is

collected and washed to remove the enzyme solution. Following a recovery period, islets are purified from exocrine cells using continuous density gradient centrifugation using a COBE 2991 centrifuge. The resulting islets are then placed in culture, awaiting transplantation.

Each step of the isolation incurs harm to the islets. By extension, islet isolation success can be greatly affected by the methods used during pancreas procurement and the quality of the gland. The resection of the pancreas begins a period of ischemia. It has long been known that reducing warm ischemia time improves the success of solid organ transplants. Minimizing the degree of warm and cold ischemia is also imperative for the success of the isolation. By resecting the pancreas before other organs during multi-organ harvest, warm ischemia time can be minimized, however this is not always possible. The Edmonton group has shown that addition and replenishment of iced saline slush around the anterior and posterior aspects of the pancreas during the procurement of the liver and kidneys significantly lowered the core pancreas temperature and greatly improved islet yield and function (156). In addition, care must be taken not to compromise the pancreatic capsule in order to prevent leakage of the digestion enzyme during distension. Loss of ductal integrity has been observed following cold storage of rat pancreas (157). In young human donors, islet yields were significantly higher when the pancreas was distended immediately following procurement rather than after 3 hours of cold ischemia (158). Conversely, the degree to which islets are affected by hypothermic injury sustained during organ procurement has not been examined.

Enzymatic digestion results in the loss of extracellular-matrix, cell-cell contact, the loss of peri-insular basement membrane, cleavage of cell surface components, and mechanical stress; all of which are likely to affect islet viability. There is evidence that intrinsic pancreatic proteases may affect the success of the isolation procedure (159;160). The Edmonton group has shown that inhibition of intrinsic serine protease activity within pancreases with cold ischemia times greater than 10 hours improves the isolation of viable islets (161). A serine protease inhibitor (Pefabloc) was added to the Liberase solution prior to injection into the pancreatic duct and it was shown to be effective at blocking serine protease activity during the isolation procedure without affecting the digestion (162).

Though the need for purification has been questioned (163;164), the current procedure for clinical islet allotransplant involves purification of islets from the rest of the pancreatic digestate. Purification by density gradient places the islets under severe osmotic stress, sheer stress, glucotoxicity, and potentially exposes them to endotoxin contamination.

1.9. TRIGGERS OF ISOLATED ISLET APOPTOSIS

The current method for isolation and culture of human islets prior to transplantation exposes islets to a unique set of insults that, individually, are known to induce apoptosis and/or necrosis in many cell types. We will now consider the mechanisms by which these insults can induce apoptosis.

1.9.1. Loss of ECM contact

The survival of many cell types is dependent on specific interactions with the extracellular matrix (ECM). These interactions are primarily mediated by the integrin family of cell surface receptors, which are heterodimers of α and β subunits that can activate cellular signalling pathways in response to cellular binding to the ECM (165;166). The signalling pathways mediated by integrins regulate many cellular functions, including cell cycle progression, survival, growth, and shape. Loss of attachment to the ECM causes apoptosis in many cell types, a process known as anoikis (167). Anoikis is important in development, tissue homeostasis, and the destruction of inappropriately located cells. Human islets are encapsulated by a peri-insular basement membrane that segregates endocrine tissue from exocrine (168). Cells on the periphery of the islet form cell-matrix attachments with the basement membrane, while cells within the core of the islet are though to be dependent on cell-cell interactions (168). As mentioned earlier, enzymatic digestion destroys the basement membrane surrounding the islets, disrupting cell-matrix interactions, leading to apoptosis (69;169). Furthermore, islet dispersion into single cells disrupts cell-cell interactions within the islet, inducing higher levels of cell death compared with intact islets (170). Therefore, maintenance of cell-cell and cell-matrix interactions within the islet are important in prevent cell death.

1.9.2. Loss of trophic support

Another consequence of islet isolation is the loss trophic support by the surrounding acinar and ductal tissue. Growth factors act to modulate a broad range of cellular responses including growth, proliferation, metabolism, and survival (171). Withdrawal of growth factors induces apoptosis in a variety of cell types (172). As with anoikis, growth factor dependence is thought to regulate tissue homeostasis and prevent inappropriate cell placement since growth factor independence is a hallmark of a transformed cancerous cell (173). In many cases, withdrawal of particular growth factors leads to activation of signalling pathways causing apoptosis. It has been hypothesized that growth factors, like ECM, suppress a constitutive death signal which becomes uncovered upon their removal (174). There are also significant metabolic changes which occur in cells following growth factor withdrawal that can trigger apoptosis (175). Growth factor-starved cells can display reduced glucose transport (176), lower glucose metabolism (177), disruption of the electron transport chain (178), and decreased ATP production (179). Isolated islets are separated from the acinar and ductal tissue which may provide growth factor support. Indeed, cells of the duct epithelium secrete IGF-II, which functions in a paracrine manner to provide trophic support to the β-cell (71). On the other hand, there is also evidence of significant nitric oxide production by the ductal epithelium in response to cytokines (180), and the ducts themselves can produce cytokines (181), providing an argument for duct-free islet preparations. The extent to which acinar tissue provides trophic support for islets remains to be determined. It is important

to note that many growth factors are secreted by islets themselves, and that this paracrine/autocrine support is likely to be maintained following isolation.

1.9.3. Hypoxia

Oxygen deprivation is known to induce cell death. In situations of low oxygen (0-0.5 %), cells will undergo apoptosis as long there is sufficient glucose to allow ATP production by glycolysis, otherwise necrosis will prevail (182). This process is dependent on Bax/Bak-mediated release of cytochrome c from the mitochondria and the subsequent activation of Caspase-9 (182;183). Hypoxia-induced cell death appears to be solely dependent on the mitochondrial pathway since overexpression of Bcl-2 or Bcl-X_L prevents apoptosis induced by oxygen deprivation (184). There is also evidence that hypoxia-induced apoptosis is transcriptionally regulated. Hypoxia-inducible factor-1 (HIF-1) is a basic helix-loop-helix transcription factor involved in transcription of genes during oxygen deprivation. HIF-1 is a heterodimer consisting of the constitutively expressed HIF-1β and the hypoxia-induced HIF-1α subunits (185). HIF-1 can regulate the transcription of genes involved in glycolysis, angiogenesis, and erythropoiesis (186). In addition, HIF-1 can induce the transcription of apoptotic genes, such as BNIP3, a pro-apoptotic member of the Bcl-2 family (187). Indeed, islets are highly vascularized structures whose function depends greatly on proper blood supply (188). Following isolation islets are avascular, and revascularization is not complete until 10-14 days following transplantation (62). During that time oxygen delivery to the islets is limited to diffusion. Islets are exposed to varying degrees

of ischemia throughout the isolation procedure, and culture techniques that are suitable for dispersed cell cultures may not be adequate for proper oxygen delivery throughout the entire islet. As mentioned previously, minimizing warm and cold ischemia times has a beneficial effect on islet yield and function, but it is difficult to attribute this effect solely to the prevention of apoptosis due to oxygen deficiency. Preservation of rat pancreata in University of Wisconsin solution for 24 hours prior to isolation resulted in increased islet apoptosis and necrosis immediately following isolation (116). Oxygen deprivation caused increased HIF-1α expression and apoptosis in cultured islets and in β-cell lines, and HIF-1α expression was thus proposed as a potential marker for islet ischemia (189).

1.9.4. Inflammatory cytokines

Inflammatory cytokines such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), or FasL, can bind to specific cell-surface receptors, leading to the activation of various signalling pathways that can activate the apoptotic machinery (190;191). Inflammatory cytokines are believed to mediate β-cell destruction in type I diabetes (192). In particular, IL-1β, TNF-α, and interferon-γ (INF-γ) are often elevated in type I diabetic patients, as well as in several animal models of diabetes, and can induce β-cell death in vitro (192;193). Additionally, intra-islet cytokine production has been suggested to contribute to apoptosis in cultured and transplanted islets (194-196). Increased levels of inflammatory cytokines have been observed in donors following brain death (58), and it has been suggested this leads to decreased islet viability following isolation (59;60).

Furthuremore, intra-islet cytokine production may result from endotoxin contamination of the enzyme preparation used during pancreatic digestion (194;195).

1.9.5. Endotoxin contamination

Endotoxin contamination has also been proposed as a cause of early islet failure (194;197). Significant levels of endotoxin have been detected in collagenase preparations and in Ficoll (polysucrose) (198), and despite the introduction of low-endotoxin Liberase (199), Ficoll is still commonly used gradient for human islet isolation. Endotoxin (lipid A) is the hydrophobic anchor of lipopolysaccharide (LPS), a glucosamine-based phospholipid found in the outer membranes of most Gram-negative bacteria (200). After binding to CD14 on the surface of animal cells, endotoxin activates Toll-like receptor 4 (TLR4), leading to activation of intracellular signalling pathways (201). Endotoxin triggers cytokine production from macrophages as well as tissue factor (TF) production from endothelial cells, the later of which increases the risk of intraportal thrombosis following islet transplantation (202). In addition, CD14 and TRL4 expression has been demonstrated within isolated human islets and treatment of isolated rat islets with LPS resulted in increased production of TNF-α, IL-1β, and IL-6 (203). Human islet apoptosis is induced by proinflammatory cytokines (TNF-α, IL-1β, INF-γ) *in vitro* (204), and it is therefore thought that endotoxin-induced cytokine production could contribute to apoptosis following isolation (194;195). The use of

endotoxin-free density gradients will hopefully eliminate the problem of endotoxin-mediated islet failure.

1.9.6. Oxidative stress

Many reactive oxygen species (ROS) are generated in healthy cells by cellular processes such as the mitochondrial respiratory chain (205). Cells are equipped with endogenous antioxidant proteins that can neutralize these ROS. Oxidative stress refers to the situation where the concentration of ROS overwhelms the endogenous antioxidant system, causing damage to proteins, lipids, carbohydrates, and DNA, which can result in cell death. Oxidative stress can also activate particular intracellular signalling pathways which regulate proliferation and apoptosis (206).

The β-cells of the islet are particularly sensitive to oxidative stress due to low expression levels of antioxidant proteins (26;27). In addition, oxidative stress has been proposed as a mechanism of β-cell failure in the development of diabetes (207). Oxidative stress has also been suggested to play a role in isolated islet failure (208). Indeed, ROS can be generated in response to many of the insults to which islets are exposed during isolation and culture, such as hypoxia (209-211), cytokines (212), inflammatory cells (213;214), and glucotoxicity (207;215). Therefore, strategies which target oxidative stress may lead to improved isolated islet survival.

1.10. CAUSES OF ISOLATED ISLET NECROSIS

Necrosis is often observed during situations of extreme environmental stress. Osmotic stress, mechanical stress, and sheer stress can all potentially lead to necrosis. As mentioned earlier, the decision by a cell to undergo apoptosis or necrosis is based on the availability of sufficient energy to carry out apoptosis. Thus, many of the aforementioned stimuli of apoptosis may induce necrosis if insufficient energy is present. Changes in ion concentrations, chronic decreased oxygen tension, and decreased ATP are all factors that could limit the availability of sufficient energy to ensure the execution of the apoptotic program (189;216;217).

1.11. RATIONALE

It has been more than 5 years since the introduction of the Edmonton protocol, yet islet cell transplantation is still not a reliable treatment for diabetes. The availability of donor tissue presents a major obstacle to the widespread introduction of this approach. Even with the available donors, the numbers of viable islets obtained are further diminished by the nature of the islet isolation procedure. Despite recent improvements to the isolation procedure, such as the use of the 2-layer method and a less toxic iodixanol gradient, many experienced groups isolate enough islets for transplantation from only 50% of isolations. Furthermore, a significant number of cells are lost due to cell death during the immediate post-isolation period (61). Indeed, islets are exposed to a unique set of circumstances during isolation that lead to the onset of apoptotic cell death, a

process that appears to continue even after transplantation. We suggest that this is a major reason underlying the rather substantial islet cell mass requirement to achieve successful transplantation. **A better understanding of the mechanisms controlling isolated islet cell death may lead to the development of strategies to improve islet survival following isolation.**

Although the original Edmonton protocol involved transplanting islets immediately following isolation, the recovery of islets in culture prior to transplantation has once again become common practice (56;57). One reason for culturing islets prior to transplantation is that a significant amount of islet cell death due to the isolation occurs during the 48 hours following isolation, and thus it is advantageous to allow these cells to die off in culture so as not to provoke additional inflammation local to the graft site. In addition, there is a much higher relative expression of inflammatory genes during the immediate post-isolation period when compared to islets cultured for one week (218). Culturing islets also provides an opportunity for therapies aimed at improving islet survival, inducing islet expansion, and reducing islet immunogenicity. **Therefore, the islet culture period could be utilized to prevent early islet cell loss following isolation.**

1.12. HYPOTHESIS

The conditions to which islets are exposed during isolation and post-isolation period in culture activate specific signalling pathways leading to the induction of cell death. Interventions that are designed to specifically respond to these signalling events can improve islet survival.

1.13. OBJECTIVES

1. Elucidate signal transduction events and related intracellular activities that are implicated in islet cell survival/death following islet isolation.
2. Develop therapeutic interventions to promote β-cell survival by targeting the key signalling events.

CHAPTER 2

Phosphatidylinositol 3-kinase Signalling to AKT Mediates Survival in Isolated Canine Islets of Langerhans

Reid Aikin, Lawrence Rosenberg, and Dusica Maysinger

2.1. ABSTRACT

The isolation of islet cells from the pancreas by enzymatic digestion causes many of these cells to undergo apoptosis. The aim of this work was to investigate the role of phosphatidylinositol 3-kinase (PI3K)/AKT signalling in mediating the survival of isolated islets. Insulin-like growth factor-1 (IGF-I) was examined as a potential culture media supplement that could rescue isolated islets from their apoptotic fate. Western blot analysis demonstrated that AKT phosphorylation peaks 20 hours after routine islet isolation. PI3K inhibition with wortmannin abolished both basal and IGF-I mediated AKT phosphorylation. IGF-I did not increase survival of isolated islets under normal conditions but it did have a protective effect against cytokine (TNF-α, IL-1β, INF-γ) mediated cell death. The protective effect of IGF-I against cytokine-stimulated apoptosis was blocked by wortmannin. In addition, inhibition of basal levels of PI3K activity caused a 31% decrease in islet survival, as shown by MTT assay. These results demonstrate that the PI3K/AKT pathway mediates survival of isolated islets of Langerhans.

2.2. INTRODUCTION

The islets of Langerhans of the pancreas are defined collections of endocrine cells consisting primarily of insulin producing β-cells. Recently, the transplantation of isolated islets as tissue into patients with type-1 diabetes mellitus has been shown to be effective in restoring euglycemia (53). The availability of donor tissue, however, presents a major obstacle to the widespread introduction of this approach (219). Even with the available donors, the numbers of viable islets obtained are further diminished by the nature of the islet isolation procedure. The isolation of islet cells from the pancreas by enzymatic digestion causes many of these cells to undergo apoptosis (61;68;169). The loss of trophic support, combined with the severe conditions to which islets are exposed during isolation are likely factors leading to islet cell destruction (69-71). Identification of factors that improve islet survival following isolation, together with improvements in the isolation procedure itself, would be major steps towards increasing the availability and quality of donor islets.

AKT is a Ser/Thr kinase that has been shown to block apoptosis and promote cell survival in response a to growth factor stimulus (134;220). Growth factor binding to cell surface receptors leads to the activation of several kinases, including phosphatidylinositol 3-kinase (PI3K). PI3K stimulation results in the recruitment of AKT to the plasma membrane where the constitutively active phosphoinositide-dependent kinase-1 (PDK1) phosphorylates AKT on its Thr-308 residue (221;222). AKT becomes fully activated upon autophosphorylation at its Ser-473 position (223). Once activated, AKT can then protect against apoptosis

by targeting proteins such as Bad(137;138), Caspase-9(139), and FKHRL1(140). Isolated rat islets have previously been demonstrated to express both AKT (224) and PI3K(225).

Insulin-like growth factor-1 (IGF-I) is a trophic factor that has been shown to promote cell survival via PI3K/AKT dependent signalling (137;220;226), and Type I IGF receptors (IGF-IR) have been characterized on rat pancreatic islets (227). IGF-I has previously been demonstrated to block cytokine induced apoptosis in isolated islets from pre-diabetic NOD mice (228), neonatal rat islets (229), and adult rat islets (230). The purpose of the present study was to examine whether stimulation or inhibition of the PI3K/AKT pathway could effect survival of isolated canine islets of Langerhans. We demonstrate that IGF-I did not increase survival of isolated islets under normal culture conditions but that it did have a PI3K dependent protective effect against cytokine mediated cell death. In addition, inhibition of basal levels of PI 3-kinase activity by wortmannin caused a 31% decrease in islet survival. These results demonstrate that the PI 3-kinase pathway, presumably acting through AKT, mediates survival of isolated islets of Langerhans.

2.3. MATERIALS AND METHODS

Canine Islet Isolation. Canine pancreata were used as a model of large-scale islet isolation because of the reliability and reproducibility of the canine islet isolation process. All procedures were in compliance with CCAC standards and were approved by the institutional Animal Care Committees. Pancreata were harvested from 2-4 year old mongrel dogs under general anesthesia by sterile surgical technique using previously defined protocols (231). Pancreata were placed in ice-cold saline prior to dissection of fatty and connective tissue. Islets were isolated using the method of Ricordi et al. (48). Briefly, a 30°C solution of 2 mg/ml Liberase CI enzyme blend (Roche Molecular, Indianapolis, IN, USA) in Hanks' balanced salt solution (HBSS) (Mediatech, Herndon, VA, USA) supplemented with 0.2 mg/ml DNase I (Boehringer-Mannheim, Montreal, Que., Canada) was infused into the pancreatic ducts of the head and tail portions of the gland using a syringe. The distended pancreas was placed in a sterilized aluminum digestion chamber (Bio-Rep, Miami, FL, USA) through which HBSS supplemented with penicillin (100,000 U/l) and fungizone (2500 g/l) (Gibco, Burlington, Ont., Canada) was recirculated at 37°C. Extent of tissue digestion was assessed by staining aliquots of digestate with dithizone (Sigma, St. Louis, MO, USA), and visualizing the islets under an inverted light microscope (Nikon, Montreal, Que., Canada). The digestion process was terminated by cooling the circuit to between 5 and 10°C once the majority of islets were free of surrounding acinar tissue. The digestate was collected, centrifuged (400×*g*) and washed three times in HBSS-FCS solution and islets were purified on a discontinuous

EuroFicoll density gradient (Eurocollins solution and Polysucrose 400, Mediatech, Herndon, VA, USA; Ficoll, Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) using a COBE 2991 Cell Processor (COBE BCT, Denver, CO, USA).

Islet Culture. Isolated canine islets were cultured in serum-free CMRL 1066 (Gibco, Burlington, Ont., Canada) with 1000 IEQ/ml of medium, at 37°C in a humidified atmosphere of 5% CO_2. When required, medium was changed every other day. IGF-I (Upstate Biotechnology, Lake Placid, NY, USA) stimulation experiments were carried out on day 0 (immediately following isolation) because of the low basal levels of AKT phosphorylation (see Figure 1). IGF-I was used at 100ng/ml unless otherwise indicated. In some experiments, the PI3K inhibitor wortmannin (100 nM) (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) was added to the cells 30 minutes prior to IGF-I stimulation. In some cases, culture media was supplemented with 100 ng/ml TNF-α, 100 U/ml IL-1β, and 100ng/ml INF-γ (R&D Systems Inc., Minneapolis, MN, USA).

Islet Cell Lysis. The islet samples (2500 IEQ) were spun down for 2 minutes at 900 rpm and 4°C. The pellet was washed twice with ice cold PBS and then dissolved in lysis buffer (50 mM Tris-HCl, pH8.0, 1.37 mM NaCl, 1% (v/v) nonident P-40, 10% (v/v) glycerol, 0.1 mM sodium orthovanadate, Complete protease inhibitor cocktail tablet (Boehringer-Mannheim)). The samples were sonicated and spun down for 20 minutes at 14000 rpm, 4°C, and the supernatant was kept for analysis. The protein content was determined using BIO-RAD

Protein Assay Dye Reagent (BIO-RAD, Hercules, CA, USA). Lysates were diluted 6:1 with 6X Laemmli sample buffer (0.375M Tris-HCl pH 6.8, 12% w/v SDS, 3% v/v glycerol, 0.2% w/v bromophenol blue, 12% β-mercaptoethanol in double distilled water) and boiled for 3 minutes.

Western Blot Analysis. An equal amount of protein (75 μg) was loaded for each sample into a 12% polyacrylamide gel run at 100 V. Transfer onto nitrocellulose was conducted at 250 mA for 90 minutes. Membranes were blocked with 2% bovine albumin in washing buffer (25mM Tris, 150 mM NaCl, 0.05% Tween 20 in double distilled water). Blocked membranes were then probed with primary antibodies. Anti-AKT and Anti-phospho-AKT (Ser-473) (N.E. Biolabs, Beverly, MA, USA) were used at 1:1000 dilutions. Anti-ERK1 (Santa Cruz Biotechnology, CA, USA) was used at 1:5000 while anti-phospho-ERK (Promega Corp., Madison, WI, USA) was used at 1:5000. Following primary antibody incubation, blots were washed for 1 hour in washing buffer then incubated for 1 hour in anti-rabbit horseradish peroxidase-linked antibody (1:4000) (Amersham LifeSciences Inc., Buckinghamshire, England). Following another 1 hour washing, the blots were developed using the ECL chemiluminescence system (Amersham) and Kodak X-OMAT film (Kodak, Rochester, NY, U.S.A.). Membranes were stripped by incubating at 65°C for half an hour in stripping buffer (100 mM β-mercaptoethanol, 2% w/v SDS and 62.5 mM Tris-HCl pH 6.7) and reprobed with primary antibody. Developed blots were scanned on a UMAX Astra 2000P scanner using Presto! PageManager version 4.20.02 (NewSoft, Inc.).

MTT Assay. Aliquots containing 500 IEQ were placed in sterile eppendorf tubes and 50 μl of stock MTT (5mg/ml) (Sigma) was added to each sample. The samples were incubated at 37°C for 2h, washed twice with cold PBS, and lysed with 200 μl of DMSO (Sigma). Two 100μ aliquots from each sample were loaded onto a 96-well plate and the absorbance was measured at 595 nm using a Benchmark Microplate Reader (BIO-RAD). Data from 48 hour time points were expressed as IEQ by calibrating the assay using the absorbance of 500 IEQ immediately following isolation. Each experiment was performed in triplicate.

PI/FDA Fluorescent Microscopy. Performed as described previously (232), with slight modifications. Briefly, aliquots containing 100 IEQ were resuspended in PBS containing 4 μM propidium iodide (PI) (Molecular Probes Inc., Eugene, OR, USA) and 0.67 μM fluorescein diacetate (FDA) (Molecular Probes). The stained islets were placed on glass slides with cover-slips and incubated for 60 minutes at room temperature in a foil-covered container with a moist paper-towel. Slides were visualized under a fluorescent Olympus BX60 microscope connected via a video camera to a PC. Images were analyzed using Image Pro Plus 4.0 software.

Data Analysis. The scanned Western blots were semi-quantified using Scion Image version beta-3b (Scion Corp.). All experiments were performed at least three times from separate isolations. All results are expressed as mean ±

standard error of the mean (SEM). Statistical significance was determined using a one-way *ANOVA* with a post-hoc Bonferroni's test as well as the Student's t-test using SYSTAT (SPSS Inc., Chicago, IL, USA). Differences were considered significant where $P < 0.05$.

2.4. RESULTS

AKT phosphorylation levels were examined over the 6 days following isolation (Figure 2.1). Western blot analysis of phospho-AKT (Thr-308) and AKT expression levels were performed for isolated canine islets cultured in serum-free CMRL 1066. Immediately following isolation, AKT phosphorylation levels were at their lowest. A rise in AKT phosphorylation over the next 12-16 hours resulted in a peak in AKT phosphorylation that lasted into the fourth or fifth day post-isolation. AKT expression levels remained constant over the 6-day culture period. The observed increase in AKT signalling could be an adaptive response to the change in islet environment.

Based on the observed time-course of AKT phosphorylation, AKT activation appeared lowest immediately following isolation. Therefore, all IGF-I stimulation experiments were performed on the day of the isolation (day 0). The dose-response and time-course of AKT activation by IGF-I were performed on freshly isolated islets (Figure 2.2). The dose-response was maximal between 10 and 100 ng/ml (Figure 2.2A), and the dose of 100 ng/ml was then used for the remaining experiments. AKT activation was already maximal after 10 minutes of IGF-I stimulation and the signal lasted for at least 2 hours (Figure 2.2B). It should be noted that the dose-response and time-course experiments were also performed 24 hours after isolation, however because the basal levels of AKT-phosphorylation were already so high, no effect was seen (data not shown).

In order to show that IGF-I stimulation of AKT was dependent on PI3K activity, islet cells were pretreated with wortmannin, a noncompetitive,

irreversible inhibitor of PI3K (233;234). Pretreatment of islet cells with 100 nM wortmannin for 20 minutes was sufficient to completely block all AKT activation caused by 30 minute exposure to 100ng/ml IGF-I (Figure 2.3A). Wortmannin had no significant effect on the activation of the extracellular-signal-regulated kinase (ERK), a member of the mitogen-activated protein kinase (MAPK) family that had been implicated in survival signalling by various growth factors [reviewed in (90)] (Figure 2.3B).

Islet cell survival/viability was assessed using the MTT assay, which has previously been demonstrated to reflect the state of islet cell metabolism (235). The assay is based on the ability of viable cells to reduce a tetrazolium salt into water-insoluble coloured formazan crystals (236). Islet cultured with 100 ng/ml IGF-I for 48 hours showed no increase in survival compared to the control (Figure 2.4). However, the addition of 100 nM wortmannin to the culture medium, with or without IGF-I, caused a significant decrease in islet survival, demonstrating a role for PI3K in islet cell survival. The addition of wortmannin alone caused a 31% decrease in islet survival after 48 hours in culture. In the presence of IGF-I (Figure 2.4, bars 2 and 4), wortmannin caused a 35% decrease in islet survival. The MTT assay has been shown previously to be unaffected directly by wortmannin in β-TC3 cells (225). Qualitative assessment of islet survival by PI/FDA staining confirmed that IGF-I had no effect on islet survival while wortmannin notably decreased islet survival, as seen by the increase in PI staining (Figure 2.5).

IGF-I has previously been demonstrated to block cytokine induced apoptosis in isolated islets from pre-diabetic NOD mice (228), neonatal rat islets (229), and adult rat islets (230). We thus examined whether PI3K signalling, presumably acting through AKT, is responsible for this protective effect of IGF-I against cytokine induced cell death. The addition of 100 ng/ml TNF-α, 100 U/ml IL-1β, and 100 ng/ml INF-γ caused a significant decrease in islet survival (Figure 2.6). The negative effect of cytokine stimulation on islet survival was blocked by 100 ng/ml IGF-I. This protective effect of IGF-I was abolished by wortmannin (100 nM), demonstrating that PI3K propagates IGF-I mediated survival signals under cytokine-stimulated conditions.

Figure 2.1. Time course of AKT phosphorylation in canine islets of Langerhans following routine isolation and culture in serum-free CMRL 1066 medium. (A) Western blot analysis of AKT phosphorylation and expression. **(B)** Densitometric analysis of AKT phosphorylation corrected for expression. Blots are representative of four independent experiments.

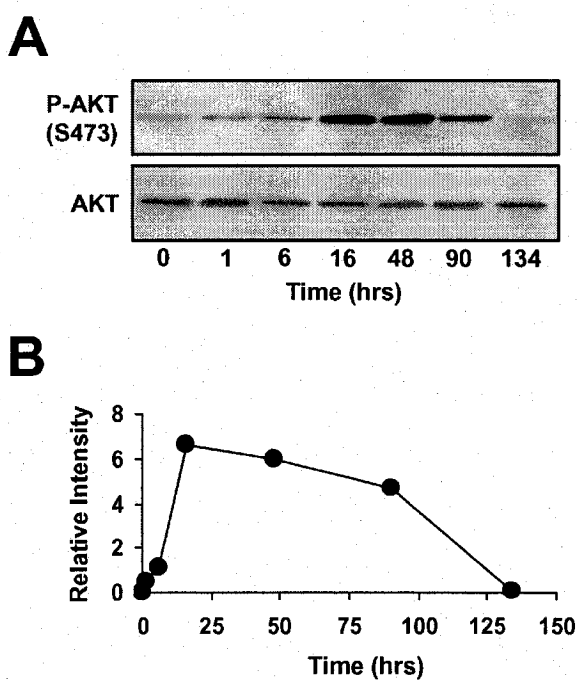

A
P-AKT
(S473)
AKT
0 1 6 16 48 90 134
Time (hrs)
B
Relative Intensity
0 2 4 6 8
0 25 50 75 100 125 150
Time (hrs)

Figure 2.2. Dose- and time-dependent AKT phosphorylation mediated by IGF-I. (A) Freshly isolated canine islets were cultured in serum-free CMRL 1066 with increasing doses of IGF-I (1-1000 ng/ml) for 30 minutes. **(B)** Islets were treated with 100 ng/ml IGF-I for 10-120 minutes. Lysates were prepared as described in Materials and Methods and AKT phosphorylation and expression levels were determined by Western blot analysis. Blots shown are representative of three independent experiments.

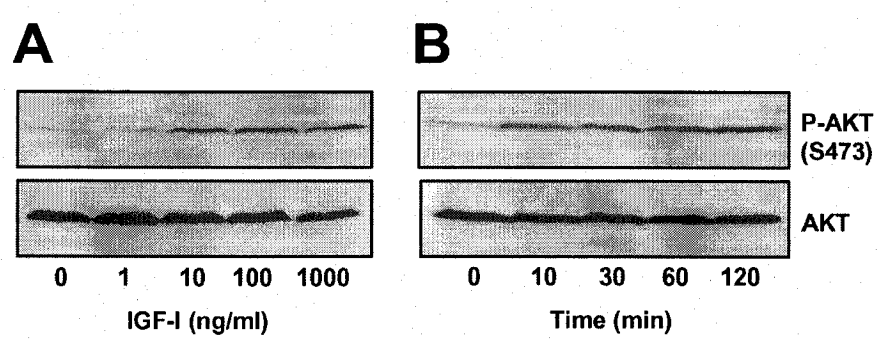

A
B
P-AKT
(S473)
AKT
0 1 10 100 1000
IGF-I (ng/ml)
0 10 30 60 120
Time (min)

Figure 2.3. Effect of PI3K inhibition on IGF-I mediated signalling to AKT and ERK. Isolated canine islets were pretreated for 20 minutes with 100 nM wortmannin followed by 30-minute incubation with 100 ng/ml IGF-I. Lysates were prepared as described in Materials and Methods and subjected to Western blot analysis of phosphorylation and expression levels of **(A)** AKT and **(B)** ERK. Blots shown are representative of three independent experiments.

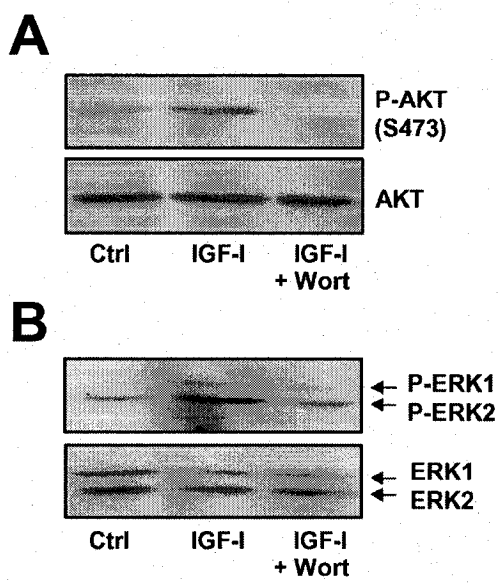

A
P-AKT
(S473)
AKT
Ctrl
IGF-I
IGF-I
+ Wort
B
P-ERK1
P-ERK2
ERK1
ERK2
Ctrl
IGF-I
IGF-I
+ Wort

Figure 2.4. Effects of IGF-I and wortmannin on isolated canine islet cell survival measured by MTT assay. Islets were cultured for 48 hours in serum-free CMRL 1066 media supplemented with combinations of IGF-I (100 ng/ml) and wortmannin (100 nM). Bars represent means ± S.E.M. for triplicate experiments.

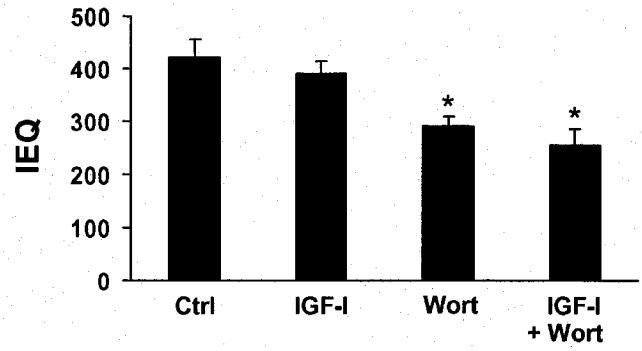

500
400
300
200
100
0
IEQ
*
*
Ctrl
IGF-I
Wort
IGF-I
+ Wort

Figure 2.5. Effects of IGF-I and PI3K inhibition on survival of isolated canine islets of Langerhans. (A) Islets were cultured for 24 hours in serum-free CMRL 1066 media supplemented with **(B)** 100 ng/ml IGF-I or **(C)** IGF-I and 100 nM wortmannin. Islet cell survival was visualized by fluorescent microscopy using propidium iodide and fluorescein diacetate dyes. The bar represents 25 μm.

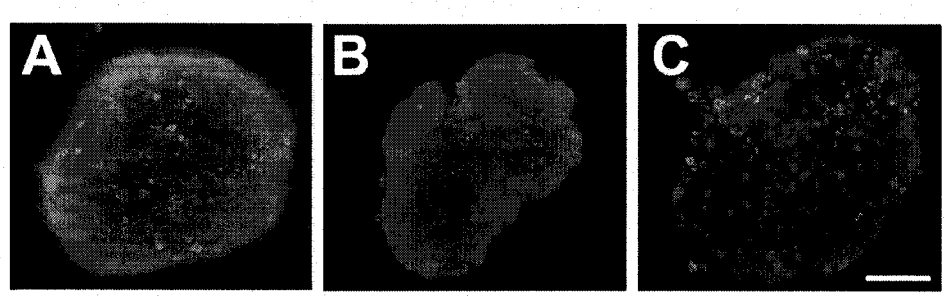
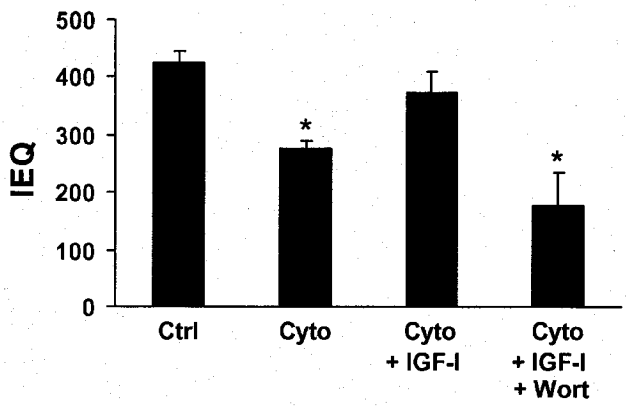

A
B
C

Figure 2.6. Effects of IGF-I and wortmannin on cytokine-stimulated isolated canine islet survival measured by MTT assay. Islets were cultured for 48 hours in serum-free CMRL 1066 media supplemented with combinations of IGF-I (100 ng/ml), wortmannin (100 nM), and cytokines (100 ng/ml TNF-α, 100 U/ml IL-1β, 100 ng/ml INF-γ). Bars represent means ± S.E.M. for triplicate experiments.


500
400
300
200
100
0
IEQ
*
*
Ctrl
Cyto
Cyto
+ IGF-I
Cyto
+ IGF-I
+ Wort

2.5. DISCUSSION

The present study demonstrates that PI3K plays a role in survival signalling in isolated canine islets of Langerhans. Under normal culture conditions, PI3K inhibition substantially decreases islet survival (31% decrease). However, PI3K/AKT stimulation using IGF-I did not increase islet survival under normal conditions. In islets receiving an acute cytokine insult, IGF-I did have a protective effect that was PI3K dependent.

Ligation of type I IGF receptors (IGF-IR) with IGF-I induces autophosphorylation of the receptor resulting in activation of the intrinsic tyrosine kinase activity of the IGF-IR. The activated receptor can then interact with insulin receptor substrate-1 (IRS-1) leading to activation of the PI3K pathway (237). PI3K activity results in the generation of 3-phosphorylated inositide lipids, namely phosphatidylinositol 3,4,5-trisphosphate (PIP_3) and phosphatidylinositol 3,4-bisphosphate [$PI(3,4)P_2$] (238;239). Various proteins are then recruited to the membrane-localized phospholipids via a pleckstrin homology (PH) domain. Recruitment of AKT and PDK1 to the plasma membrane allows the constitutively active PDK1 to phosphorylate AKT on its Thr-308 residue (221;222). AKT becomes fully activated by autophosphorylation at its Ser-473 position (223). Once activated, AKT can then protect against apoptosis by phosphorylating proteins such as Bad (137;138), Caspase-9 (139), glycogen synthase kinase-3 (GSK-3) (240), IκB-kinase (IKK) (141;241), and FKHRL1 (140). Pugazhenthi et al. have outlined an AKT dependent IGF-I signalling pathway that leads to CREB induction of the Bcl-2 promoter (242).

PI3K can also modulate other pathways besides AKT, including the Ras-ERK pathway, PKC, PLC-γ, c-Jun N-terminal kinase (JNK), and $p70^{s6k}$ [reviewed in (243)]. Of these, ERK is tightly linked to cell survival (107). ERK has recently been shown to cause Bad phosphorylation via Rsk (126) and it has been implicated in AKT-independent protection from apoptosis (244;245). We have previously demonstrated that ERK signalling in isolated islet cells is associated with increased survival following isolation (114). In the present study, inhibition of PI3K did not cause a significant decrease in ERK activation (Figure 2.3B), thus ERK signalling appears not to be regulated by PI3K under these conditions. ERK activation independent of PI3K may still be occurring, but the fact that wortmannin completely blocked the protective effect of IGF-I demonstrates that the PI3K/AKT pathway is probably the main survival pathway utilized by IGF-I in islet cells.

In some instances, the protective effect afforded by IGF-I is only observed when it is administered prior to the stressing agent (230;246). This could explain why IGF-I had little effect on islet cell death caused by the isolation procedure. In addition, the canine islets used displayed low p38 activation immediately following isolation (data not shown), as we have observed previously (169). In our experience in porcine islets, low levels of p38 activation immediately following isolation correlated with improved survival after 36h in culture (114). Therefore, in the present study, the islets may not have been damaged to a large enough degree to benefit from the protective effect of IGF-I. Perhaps IGF-I would

have a greater impact on islets that have high initial p38 levels and are thus less likely to survive (114).

Cytokine mediated signalling in pancreatic islet cells has been extensively studied due to the involvement of cytokines in the autoimmune destruction of islet cells in diabetes [reviewed in (247)]. Nitric oxide (NO) has been demonstrated to be a key player in cytokine mediated islet cell death (30;131;248-251). Inhibition of nitric oxide synthesis has been shown to protect neonatal rat islet cells from a cytokine insult (230). In the same study, IGF-I was able to block the expression and activation of iNOS in islets exposed to IL-1β (230). IGF-I also blocked NOS-2 expression in Ins-1 cells, a rat β-cell line, exposed to LPS and INF-γ (252). The inhibitory effect of IGF-I on NOS-2 expression was shown to be wortmannin sensitive. The same study showed that IGF-I inhibited cytokine stimulated Bax expression and it caused an increase in Bcl-2 expression. Elevated Bcl-2 expression alone is sufficient to protect islet cells from cytokine mediated cell death (253-256). Members of the Bcl-2 family of proteins are thought to effect mitochondrial integrity (81), an effect also mediated by NO (257). Taken together with the present work, these results suggest that IGF-I signalling through AKT could block cytokine mediated cell death upstream of NO production and at the mitochondrial level through the induction of the Bcl-2 promoter and phosphorylation of Bad. It remains to be determined if the protective effect of IGF-I in response to cytokine stimulation is necessarily dependent on transcription.

Following isolation, the activity of AKT increases to a maximal level by 20h post-isolation (Figure 2.1). This time-course of activation is the exact opposite of JNK following isolation, which we demonstrated previously (114). It has been reported that PI3K signalling suppresses activation of JNK in cultured neurons (258). IGF-1 has also been shown to have a negative effect on JNK activation in human embryonic kidney 293 cells (246). The decrease in JNK activation observed in islet cells following isolation could be a result of the cells attempt to increase survival via the AKT pathway. It remains unclear, however, what stimulated the AKT pathway in these islet cells. Some environmental change has lead to increased phosphorylation of AKT after 20 hours, an effect that may be dependent on changes in expression of modulators of the AKT pathway. Chronic AKT activation induced by environmental changes such as those experienced by isolated islet cells has not been reported in other studies. The present work, combined with our previous results (114), have demonstrated that large-scale isolation and subsequent culturing of islet cells results in a specific cellular response, the understanding of which could be invaluable in attempting to increase islet survival.

CONNECTING TEXT

The previous chapter demonstrated a role for the PI3K/AKT signalling pathway in isolated canine islet survival and that AKT became highly phosphorylated after 16 hours in culture. In contrast, we previously demonstrated strong JNK phosphorylation immediately after islet isolation that decreases over the next 24 hours (114). The temporal correlation between these events suggested that AKT could be acting to suppress the JNK pathway in islets following isolation. Therefore, the aim of Chapter 3 was to explore the possibility of AKT-JNK cross-talk in islets following isolation and the relevance of JNK suppression on islet survival.

CHAPTER 3

Cross-talk between PI3K/AKT and JNK mediates survival of isolated human islets

Reid Aikin, Dusica Maysinger, and Lawrence Rosenberg

3.1. ABSTRACT

Therapeutic strategies aimed at the inhibition of specific cell death mechanisms may increase islet yield and improve cell viability and function following routine isolation. The aim of the current study was to explore the possibility of AKT-JNK cross-talk in islets following isolation and the relevance of JNK suppression on islet survival. Following routine isolation, increased AKT activity correlated with suppression of JNK activation, suggesting that they may be related events. Indeed, the increase in AKT activation following isolation correlated with suppression of ASK1, a kinase acting upstream of JNK, by phosphorylation at Ser83. We therefore examined whether modulators of PI3K/AKT signalling affected JNK activation. PI3K inhibition led to increased JNK phosphorylation and islet cell death which could be reversed by the specific JNK inhibitor SP600125. In addition, IGF-I suppressed cytokine-mediated JNK activation in a PI3K-dependent manner. We also demonstrate that inhibition of PI3K rendered islets more susceptible to cytokine-mediated cell death. SP600125 transiently protected islets from cytokine-mediated cell death, suggesting that JNK may not be necessary for cytokine-induced cell death. When administered immediately following isolation, SP600125 improved islet survival and function, even 48 hours after removal of SP600125, suggesting that JNK inhibition by SP600125 may be a viable strategy for improving isolated islet survival. Taken together, these results demonstrate that PI3K/AKT suppresses the JNK pathway in islets and this cross-talk represents an important anti-apoptotic consequence of PI3K/AKT activation.

3.2. INTRODUCTION

The ability of cells to react to environmental changes is dependent on the cooperation of intracellular signal transduction pathways to coordinate the cellular response. The integration of various external cues leads to regulation of physiological processes such as proliferation, differentiation, and cell death. The mitogen-activated protein kinases (MAPK) have been shown to play an important regulatory role in a variety or cellular processes (90). MAPKs are activated by a sequential cascade of protein phosphorylation in which MAPKs are phosphorylated by a MAPK kinase (MAPKK), which is itself activated by a MAPKK kinase (MAPKKK). This three-tiered kinase module is a common characteristic of all MAPK pathways (91).

The c-jun NH_2-terminal kinases (JNK) are a group of MAPKs that play a role in apoptosis, proliferation, survival signalling, and embryonic morphogenesis (reviewed in (92)). JNK is activated by dual phosphorylation at Tyr185 and Thr183 by two dual specificity MAPKKs, MAPK kinase 4 (MKK4) and MAPK kinase 7 (MKK7). MKK4 and MKK7 are activated by a large group of MAPKKKs including the MEKK family, ASK1, MLKs, TAK1, and TPL2 (93). Once activated, JNK can phosphorylate proteins in the nucleus and cytoplasm. Stress-induced JNK activation leads to phosphorylation of transcription factors, including c-jun and ATF2, which heterodimerize to bind divergent AP-1 sites in the c-jun promoter (94). However, despite the fact that c-jun expression is required in some cases for apoptosis, little is know about the genes that are involved. JNK-mediated induction of the Fas ligand (FasL) gene was proposed as a possible

mechanism of JNK-induced apoptosis (95;96), however blockage of Fas signalling does not prevent all forms of stress-induced apoptosis (97). A more direct link between JNK and the apoptotic machinery was observed in JNK-deficient cells which failed to release cytochrome c from the intermembrane space of the mitochondria in response to UV radiation (98). This effect is due to the ability of JNK to directly phosphorylate members of the BH3-only group of Bcl-2 family (Bim and Bmf), leading to Bax-dependent apoptosis (99;100). Therefore, JNK can regulate apoptosis by both transcription-dependent and independent mechanisms that are only now beginning to be elucidated. In addition, the duration of JNK activation appears to be important in determining the physiological outcome. Sustained JNK activation, in contrast with transient activation, appears to promote apoptosis in a variety of cell types (101-105). However, the consequences of JNK activation greatly depend on the type of stimulus and the cellular context (106).

ASK1 is a MAPKKK which activates the JNK and p38 MAPK pathways in response to pro-inflammatory cytokines, oxidative stress and ER stress (259). ASK1 can be activated by interaction with the TNF receptor-associated factor (TRAF) domain of TRAF-2 and TRAF-6, and is required for TNF-α-induced JNK activation (260). ASK1-deficient cells are resistant to apoptosis induced by TNF-α or H_2O_2 and do not exhibit sustained JNK activation in response to these insults (105). Overexpression of ASK1 induces apoptosis through induction of the mitochondrial pathway (261-264). The NH_2-terminal of ASK1 contains an inhibitory domain which can interact with other proteins such as thrioredoxin and

14-3-3, which prevent ASK1 activation (265-267). ASK1 is also inhibited by interaction with the HIV-1 protein Nef, providing infected T-cells with protection from TNF-α and Fas, demonstrating the importance of ASK1 in cytokine-mediated cell death (268).

AKT is a Ser/Thr kinase that has been shown to be a critical mediator of cell survival in response to growth factor stimuli (134;269). AKT is recruited to the plasma membrane following activation of PI3K and is activated through phosphorylation by PDK1/2 (135;136). AKT can directly phosphorylate several pro-apoptotic proteins leading to suppression of apoptotic signals. We and others have observed strong activation of AKT within 12-24 hours following islet isolation (151;270). In contrast, strong JNK activity is observed immediately after islet isolation and decreases over the next 24 hours (114). The temporal correlation between these events suggested that AKT could be acting to suppress the JNK pathway in islets following isolation. Indeed, it has been demonstrated that AKT can regulate several proteins in order to suppress the JNK pathway, such as MLK3 (146), JNK-interacting protein 1 (JIP1) (271), and MKK4 (147). In particular, AKT was demonstrated to directly phosphorylate ASK1, leading to suppression of JNK signalling (145). We therefore explored the possibility of AKT-JNK cross-talk in islets following isolation and the impact of JNK inhibition on islet survival. In the present study, we demonstrate that PI3K/AKT signalling suppresses JNK activation and inhibits JNK-mediated islet cell death.

3.3. MATERIALS AND METHODS

Human Islet Isolation. Pancreata were retrieved from heart-beating cadaveric donors at the time of multi-organ harvest for transplantation. Consent for donation of tissues for research was obtained by the local organ procurement organization. Warm ischemia time was approximately 5 minutes and cold ischemia time, using University of Wisconsin (UW) solution perfusion, was between 30 minutes and 6 hours. The main pancreatic duct was canulated and a cold (6-8°C) solution of 2 mg/ml Liberase enzyme blend (Roche, Montreal, QC, Canada) in perfusion solution (Mediatech, Herndon, VA, USA) supplemented with 0.2 mg/mL DNAse I (Roche) was infused through the canula into the pancreas using a syringe. The distended pancreas was placed in a sterilized aluminium digestion chamber (Bio-Rep, Miami, FL, USA) through which the perfusion solution was recirculated at 37°C. The extent of tissue digestion was assessed by staining aliquots of digestate with dithizone (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) and visualizing the islets under an inverted light microscope (Nikon, Montreal, QC, Canada). The digestion process was terminated by cooling the circuit to between 5 and 10°C when the majority of the islets were seen to be free of surrounding acinar tissue. The digestate was collected, centrifuged, and washed three times with wash solution (Mediatech) and islets were purified on a continuous density gradient (Biocoll separating solution; Biochrom AG, Berlin, Germany) using a COBE 2991 Cell Processor (COBE BCT, Denver, CO, USA). The number of islet equivalents (IEQ) and islet purity were assessed using dithizone (Sigma), a zinc chelater which stains the

zinc rich β-cells. Glucose-stimulated insulin secretion was routinely assessed to ensure islet functionality.

Islet cell culture. Isolated islets (>90% purity) were cultured in CMRL 1066 media (Gibco, Burlington, ON, Canada) containing 10% fetal bovine serum (FBS) (Wisent, St. Bruno, QC, Canada), at 37°C in a humidified atmosphere of 5% CO_2. Medium was changed every other day. For acute cytokine stimulation experiments, islets were treated with 100 ng/mL TNF-α, 2 ng/mL IL-1β, and 100 ng/mL INF-γ (R&D Systems Inc., Minneapolis, MN, USA). The biological activities of the cytokines are 10 U/ng (TNF-α), 50 U/ng (IL-1β), 10 U/ng (INF-γ). Insulin-like growth factor-1 (IGF-I) (Upstate Biotechnology, Lake Placid, NY, USA) was used at a concentration of 100 ng/ml. The PI3K inhibitor wortmannin (Sigma) was used at 100 nM. The inhibitors of JNK (SP600125) and of p38 (SB203580) (both from Calbiochem, San Diego, CA, USA) were used at a final concentration of 20 μM. The general caspase inhibitor Z-VAD-fmk (Calbiochem) was added following isolation to the culture media at a final concentration of 50 μM. For all compounds prepared in DMSO, the final concentration of DMSO in the culture media was kept below 0.1%. Vehicle controls were prepared for all treatments.

Caspase-3 Assay. The islet samples (2000 IEQ) were spun down for 2 minutes at 900 rpm and 4°C. The pellet was washed twice with ice cold PBS and then dissolved in lysis buffer (50 mM Tris-HCl, pH 8.0, 1.37 mM NaCl, 1% (v/v) nonident P-40, 10% (v/v) glycerol). The samples were sonicated and spun down

for 20 minutes at 14000 rpm, 4°C, and the supernatant was kept for analysis. The protein content was determined using BIO-RAD Protein Assay Dye Reagent (BIO-RAD, Mississauga, Ontario, Canada). In a 96-well plate, lysate containing 100 μg of protein was added to caspase buffer (50 mM HEPES, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, 10 mM dithiothreitol, 100 mM NaCl, pH 7.2) containing 200 μM Ac-DEVD-*p*NA. Samples were incubated at 37°C for 90 minutes and the release of *p*NA was analyzed by measuring the absorbance at 405 nm using a Benchmark Microplate Reader (BIO-RAD).

MTT Assay. Aliquots containing 500 IEQ in 500 μl of media were placed in sterile eppendorf tubes and 50 μL of stock 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/mL) (Sigma) was added to each sample. The samples were incubated at 37°C for 2 hours, washed twice with cold PBS, and lysed with 200 μL of DMSO (Sigma). Two 100 μL aliquots from each sample were loaded onto a 96-well plate and the absorbance was measured at 595 nm using a Benchmark Microplate Reader (BIO-RAD). Four independent samples were analyzed per experiment and each experiment was performed at least three times.

Mitochondrial Depolarization. Islets were dispersed by first washing with dispersion solution (Gibco) followed by a 10 minute incubation at 37°C with trypsin/EDTA (Gibco). Trypsinized islets were washed with cold CMRL 1066 with 10% FBS, pipetted gently, and resuspended in PBS. JC1 (Molecular Probes Inc.,

Eugene, OR, USA) was used according to manufacturers directions to identify cells with depolarized mitochondria. The stained islets were placed on glass slides with cover-slips and incubated for 15 minutes at room temperature in a foil-covered container with a moist paper-towel. Slides were visualized under a fluorescent Olympus BX60 microscope connected via a digital video camera to a PC. Five hundred cells were counted per slide, with three slides per group for at least three independent experiments. Images were analyzed using Image Pro Plus 4.0 software (Media Cybernetics Inc., Silver Spring, MD, USA).

Glucose-Stimulated Insulin Release. Cultured islets (100 IEQ per group in duplicate) were washed with CMRL 1066 and incubated in Hank's buffered saline containing 2.2 mM glucose for two consecutive periods of 60 minutes at 37°C. Next, islets were incubated for 30 minutes with 22 mM glucose and then another 30 minutes with 22 mM glucose with 50 μM 3-isobutil-1-methylxanthine (IBMX), an inhibitor of cyclic-AMP (cAMP) phosphodiesterase. Finally, islets were washed with Hank's buffered saline and incubated for 1h in 2.2 mM glucose. The supernatants were kept following each incubation and analyzed for insulin content using a commercially available insulin ELISA kit (ALPCO diagnostics, Windham, NH, USA). Insulin release was normalized to the protein content of the pellet determined using BIO-RAD Protein Assay Dye Reagent (BIO-RAD).

Western Blotting. The islet samples (2500 IEQ) were spun down for 2 minutes at 900 rpm and 4°C. The pellet was washed twice with ice cold PBS and then

dissolved in lysis buffer (50 mM Tris-HCl, pH 8.0, 1.37 mM NaCl, 1% (v/v) nonident P-40, 10% (v/v) glycerol, 0.1 mM sodium orthovanadate, complete protease inhibitor cocktail tablet (Roche)). The samples were sonicated and spun down for 20 minutes at 14000 rpm, 4°C, and the supernatant was kept for analysis. The protein content was determined using BIO-RAD Protein Assay Dye Reagent (BIO-RAD). Lysates were diluted 6:1 with 6X Laemmli sample buffer (0.375 M Tris-HCl pH 6.8, 12% (w/v) SDS, 3% (v/v) glycerol, 0.2% (w/v) bromophenol blue, 12% (v/v) β-mercaptoethanol in double distilled water) and boiled for 5 minutes. An equal amount of protein (75 μg) was loaded for each sample into a 12% polyacrylamide gel run at 100 V for 90 minutes. Transfer onto nitrocellulose was conducted at 250 mA for 90 minutes. Membranes were blocked with 2% bovine serum albumin in washing buffer (25 mM Tris, 150 mM NaCl, 0.05% Tween 20 in double distilled water). Blocked membranes were then probed with primary antibodies. Anti-phospho-JNK (Promega Corp., Madison, WI, USA) was used at a 1:5000 dilution. Anti-JNK1 and anti-JIP1 (Santa Cruz Biotechnology, CA, USA) were used at a dilution of 1:1000. Anti-AKT, anti-phospho-AKT (Ser-473), anti-c-jun, anti-phospho-c-jun (Ser-73), anti-ASK1, and anti-phospho-ASK1 (Ser-83) (all from Cell Signalling Tech., Beverly, MA, USA) were used at a dilution of 1:1000. Following primary antibody incubation, blots were washed for 1 hour in washing buffer then incubated for 1 hour in anti-rabbit horseradish peroxidase-linked antibody (1:4000) (Amersham LifeSciences Inc., Buckinghamshire, England). Following another 1 hour washing, the blots were developed using the ECL chemiluminescence system (Amersham) and Kodak X-

OMAT film (Kodak, Rochester, NY, U.S.A.). Membranes were stripped by incubating at 65°C for half an hour in stripping buffer (100 mM β-mercaptoethanol, 2% (w/v) SDS and 62.5 mM Tris-HCl pH 6.7) and reprobed with primary antibody.

Statistical Analysis. All results are expressed as mean ± standard error of the mean (SEM). Statistical significance was determined using a one-way *ANOVA* with a post-hoc Bonferroni's test as well as the Student's t-test using SYSTAT 9 (SPSS Inc., Chicago, IL, USA). Differences were considered significant where $P < 0.05$.

3.4. RESULTS

Following routine isolation decreased JNK phosphorylation correlates with increased AKT phosphorylation. Using Western blot analysis, the levels of JNK and AKT phosphorylation were examined during the first 72 hours following isolation (Figure 3.1A). We observed that JNK phosphorylation levels decreased concomitantly with a rise in AKT phosphorylation (Figure 3.1B). By 16 hours following isolation AKT is highly activated whereas JNK activation begins to decrease. The tight temporal correlation between these two events supported the notion that they might be related.

We examined the possibility that an upstream kinase of JNK was inhibited, causing the observed decrease in JNK phosphorylation. ASK1 is a key regulator of the JNK pathway amenable to inhibition by AKT-mediated phosphorylation at Ser83 (145). We therefore examined the levels of ASK1 phosphorylation at Ser83 over the first 72 hours following isolation (Figure 3.1A). ASK1 phosphorylation was evident by 16 hours and remained elevated, consistent with the pattern of AKT activation. Expression of JIP1, a scaffold protein which can regulate JNK activity in islets (272), remained unchanged (Figure 3.1A).

PI3K inhibition leads to increased JNK-mediated apoptosis. To test whether increased AKT-mediated suppression of ASK1 is responsible for the observed decrease in JNK activity, we employed the PI3K inhibitor wortmannin. Islets were cultured for 48 hours, at which point AKT is highly active and JNK activity is low, and then treated with wortmannin. An increase in JNK phosphorylation was detectable following 12 hours of wortmannin treatment and

increased over the ensuing 24 hours (Figure 3.2A). Wortmannin treatment completely blocked AKT phosphorylation and consequently ASK1 phosphorylation at Ser83. These results suggest that PI3K/AKT activation in isolated islets is responsible for the suppression of JNK via inactivation of ASK1.

We have previously demonstrated that wortmannin treatment induced cell death in isolated canine islets (270). We thus examined the possibility that PI3K inhibition leads to JNK-mediated cell death. Human islets were pretreated for 30 minutes with a selective JNK inhibitor (SP600125) prior to wortmannin treatment and JNK phosphorylation was assessed after 24 hours in culture. SP600125 was able to completely block wortmannin induced JNK phosphorylation (Figure 3.2B). In addition, SP600125 was able to prevent decreased MTT reduction following 24 and 72 hours of wortmannin treatment (Figure 3.2C).

IGF-I suppresses cytokine-mediated JNK activation in a PI3K-dependent manner. To study whether activators of AKT can lead to suppression of JNK signalling, we examined the effects of IGF-I on cytokine-mediated JNK activation. Isolated islets were cultured for 48 hours prior to treatment, at which time JNK activity is reduced (Figure 3.1A). Cytokine treatment led to increased JNK phosphorylation and a 30% decrease in MTT reduction following 24 hours in culture (Figure 3.3). Pretreatment with IGF-I abolished cytokine-mediated JNK activation and protected islets against the cytokine-induced decrease in MTT reduction. Wortmannin pretreatment abolished the protective effect of IGF-I and was able to restore cytokine-mediated activation of JNK, suggesting that the protective effect of IGF-I is mediated by PI3K-dependent suppression of JNK.

Indeed, JNK inhibition by SP600125 blocked JNK phosphorylation and partially protected islets against cytokine-induced cell death (Figure 3.3). These findings suggest that the protective effect of IGF-I against cytokine-induced cell death is partially mediated by PI3K-mediated suppression of JNK signalling.

Inhibition of PI3K/AKT renders islets more susceptible to cytokine-mediated cell death. To examine the possibility that the rise in AKT activation in untreated isolated islets reduces their susceptibility to cytokine-mediated cell death, we treated islets with cytokines in the presence of wortmannin. Treatment with either wortmannin alone or cytokines alone led to increased JNK phosphorylation, increased caspase-3 activity, and reduced viability (Figure 3.4). However, co-treatment of islets with both wortmannin and cytokines led to increased levels of JNK phosphorylation when compared with islets treated with only cytokines (Figure 3.4A). Inhibition of PI3K also increased cytokine-induced caspase-3 activation and caused a significant decrease in MTT reduction when compared with cytokine-treated islets (Figure 3.4, B and C). These findings indicate that the elevated levels of AKT activity occurring in cultured islets could render these cells less susceptible to cytokine-mediated cell death.

The JNK inhibitor SP600125 transiently protects islets from cytokine-mediated cell death. JNK has previously been implicated in cytokine-mediated β-cell death (120;273;274). Therefore, we examined whether JNK inhibition by pretreatment with SP600125 is sufficient to block cytokine-mediated cell death. As expected, SP600125 was able to completely block cytokine-induced JNK and c-jun phosphorylation following 24 hour treatment (Figure 3.5A). SP600125 also

suppressed the cytokine-mediated decrease in islet viability following 24 hours in culture, as measured by MTT assay (Figure 3.5B). However, by 72 hours the protective effect of SP600125 against cytokines was diminished. These data indicate that SP600125 can delay, but not prevent, cytokine-induced cell death.

SP600125 improves isolated islet survival and function following routine isolation. We have previously shown that freshly isolated islets display high JNK activity immediately following isolation which diminishes over the first 48 hours following isolation (113;114). This early activation of JNK coincides with the peak of islet apoptosis which occurs 24 hours following isolation (113;114;275). We therefore examined the effects of SP600125 treatment immediately following isolation on islet survival and function after 48 hours in culture. SP600125 treatment led to a 37% increase in islet viability after 48 hours in culture (Figure 3.6A). In addition, SP600125 reduced caspase-3 activity by 47% (Figure 3.6B).

We previously demonstrated increased mitochondrial depolarization in islets following 72 hours in culture (Chapter 5)(275), which could account for increased necrotic-like cell death in islets. Since JNK has been shown to be required for cytochrome c release (98), we examined whether SP600125 could diminish mitochondrial depolarization in isolated islets. Indeed, SP600125 treatment caused a 62% decrease in the number of cells with depolarized mitochondria (11.4 ± 1.2 *vs.* 4.3 ± 0.7) as assessed by JC1 staining (Figure 3.6C).

To examine the effect of SP600125 on islet function, static glucose-stimulated insulin release was assessed in islets following 48 hours in culture with or without SP600125. Insulin secretion by SP600125 treated islets was not significantly different than that of untreated islets at both low and high glucose levels (Figure 3.6D). However, the addition of IBMX resulted in increased insulin secretion by the SP600125 treated islets, indicating that either these islets released more insulin per β-cell or contained a higher percentage of β-cells. Both SP600125 treated and control islets were able to shut-off insulin secretion in response to low glucose (Figure 3.6D).

To ascertain whether islets treated with SP600125 maintained their increased viability when SP600125 was withdrawn, we cultured islets for 96 hours during which SP600125 was administered either during the first 48 hours and then withdrawn, during the last 48 hours only, or during the whole 96 hour incubation. Islets that received SP600125 during the first 48 hours only (approximate culture period prior to transplantation) showed a 32% increase in MTT reduction despite the removal of SP600125 after 48 hours (Figure 3.6E). Islets that received SP600125 throughout the 96 hour culture period were equally viable, displaying a 28% increase in MTT reduction. Surprisingly, islets treated with SP600125 during only the last 48 hours displayed a 21% increase in MTT reduction compared to the untreated control, despite the fact that JNK activation has decreased significantly at this point.

Sustained JNK activation caused by wortmannin or cytokines does not require caspase activation. Caspase activity is required in some cases for

sustained JNK activation (276). We therefore examined the effects of general caspase inhibition on JNK activation by wortmannin. Pretreatment of islets with 50 μM Z-VAD-fmk had no effect on JNK phosphorylation induced by 24 hour treatment with wortmannin (Figure 3.7A). Similarly, Z-VAD-fmk had no effect on JNK phosphorylation following 24 hours of cytokine treatment (Figure 3.7B).

Inhibition of p38 with SB203580 has a negative effect on isolated islet survival. Many stimuli that cause JNK activation also activate the p38 pathway, since these two kinases share many common upstream regulators. In particular ASK1, which in addition to acting upstream of JNK also leads to p38 activation (261). Furthermore, just as AKT can have a suppressive effect on JNK, AKT has also been demonstrated to suppress the p38 pathway (277). We therefore explored the possibility that p38 was also contributing to cytokine and wortmannin induced cell death using the p38 inhibitor SB203580. Pretreatment of islets with SB203580 had no effect on cytokine-induced cell death (Figure 3.8A). Similarly, SB203580 had no protective effect against the wortmannin-induced decrease in islet viability (Figure 3.8A). When administered immediately following isolation, SB203580 led to decreased viability, suggesting that p38 may act as a survival pathway in isolated islets (Figure 3.8B).

Figure 3.1. Increased AKT phosphorylation correlates with decreased JNK phosphorylation. (**A**) Whole cell lysates were taken at the indicated times following isolation and analyzed by Western blotting. The blots are representative of six independent experiments. (**B**) Semi-quantification of representative blots using densitometric analysis. Open circles represent AKT phosphorylation and closed circles denote JNK phosphorylation.

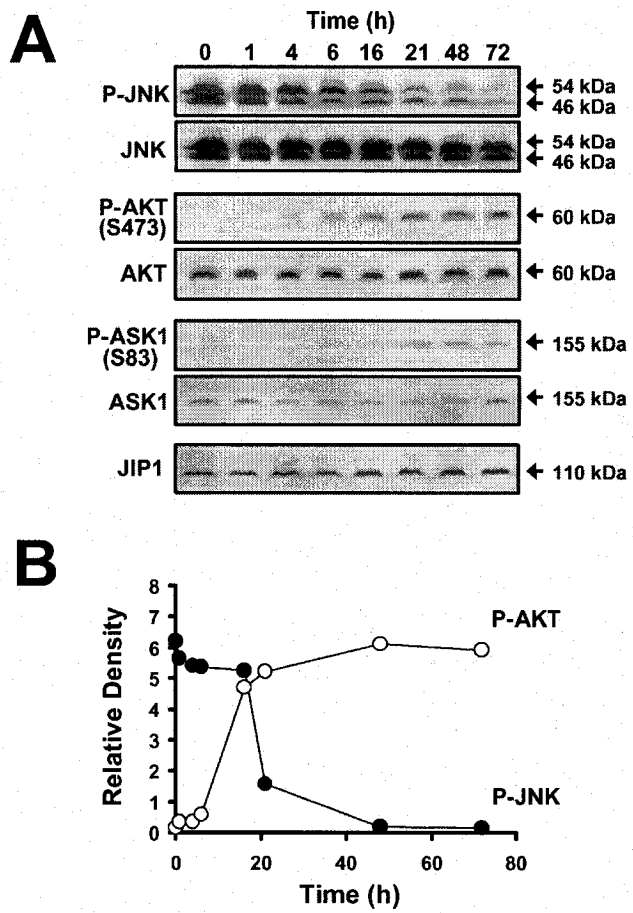

A
Time (h)
0 1 4 6 16 21 48 72
P-JNK
54 kDa
46 kDa
JNK
54 kDa
46 kDa
P-AKT (S473)
60 kDa
AKT
60 kDa
P-ASK1 (S83)
155 kDa
ASK1
155 kDa
JIP1
110 kDa
B
Relative Density
P-AKT
P-JNK
Time (h)

Figure 3.2. PI3K inhibition leads to increased JNK-mediated cell death. (**A**) Freshly isolated islets were cultured for 48 hours prior to the addition of 100 nM wortmannin. Whole cell lysates were taken following 12, 24, and 36 hours of treatment and analyzed by Western blotting. The blot is representative of three independent experiments. (**B**) Isolated islets were cultured for 48 hours and pretreated with SP600125 (20 μM) for 30 minutes prior to the addition of wortmannin (100 nM). Whole cell lysates were taken following 24 hours of treatment and analyzed by Western blotting. The blot is representative of three independent experiments. (**C**) Isolated islets were cultured for 48 hours and pretreated with SP600125 (20 μM) for 30 minutes prior to the addition of wortmannin (100 nM). Islet viability was assessed by MTT assay after 24 and 72 hours of treatment. Bars represent the mean MTT reduction expressed as a percentage of the control ± SEM for quadruplicate samples from five independent experiments. *, $P<0.05$ compared with untreated controls (Ctrl).

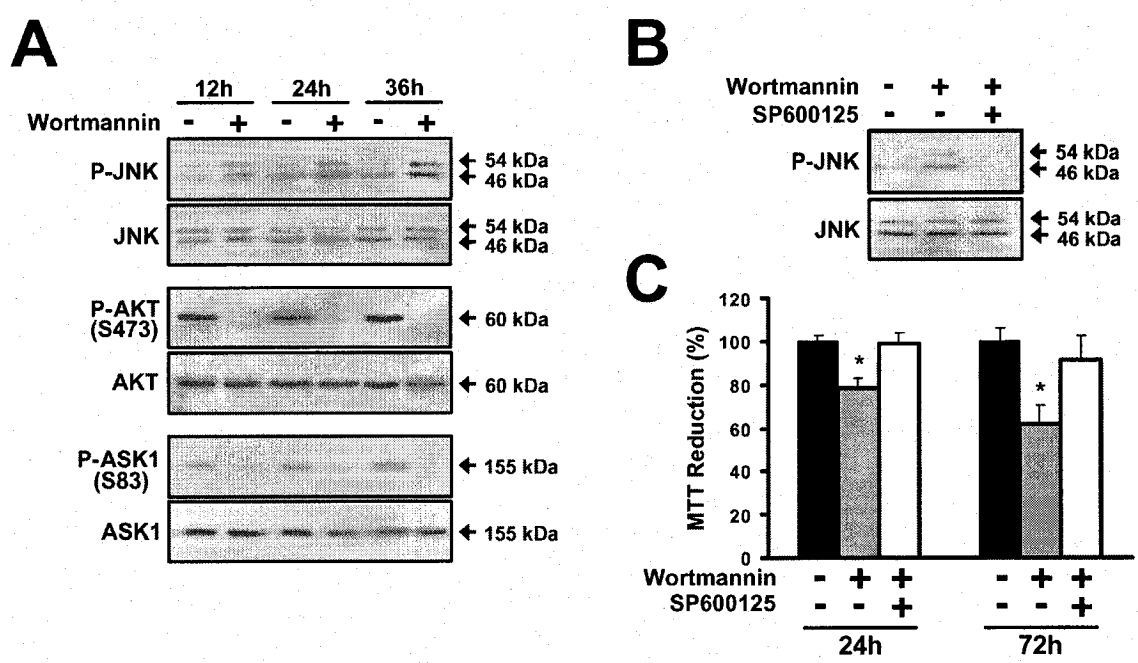

A
12h
24h
36h
Wortmannin - + - + - +
P-JNK
54 kDa
46 kDa
JNK
54 kDa
46 kDa
P-AKT (S473)
60 kDa
AKT
60 kDa
P-ASK1 (S83)
155 kDa
ASK1
155 kDa
B
Wortmannin - + +
SP600125 - - +
P-JNK
54 kDa
46 kDa
JNK
54 kDa
46 kDa
C
MTT Reduction (%)
120
100
80
60
40
20
0
*
*
Wortmannin - + + - + +
SP600125 - - + - - +
24h
72h

Figure 3.3. IGF-I suppresses cytokine-mediated JNK activation in a PI3K-dependent manner. Freshly isolated islets were cultured for 48 hours and then treated with or without cytokines (Cyto; 100 ng/ml TNF-α, 2 ng/mL IL-1β, 100 ng/ml INF-γ), IGF-I (100 ng/ml), wortmannin (Wort; 100 nM), and SP600125 (SP; 20 μM) for 24 hours. (**A**) Whole cell lysates were taken and JNK phosphorylation was analyzed by Western blotting. The blot is representative of three independent experiments. (**B**) Islet viability was assessed by MTT assay. Bars represent the mean MTT reduction expressed as a percentage of the control ± SEM for quadruplicate samples from three independent experiments. *, $P<0.05$; **, $P<0.01$ compared with untreated controls.

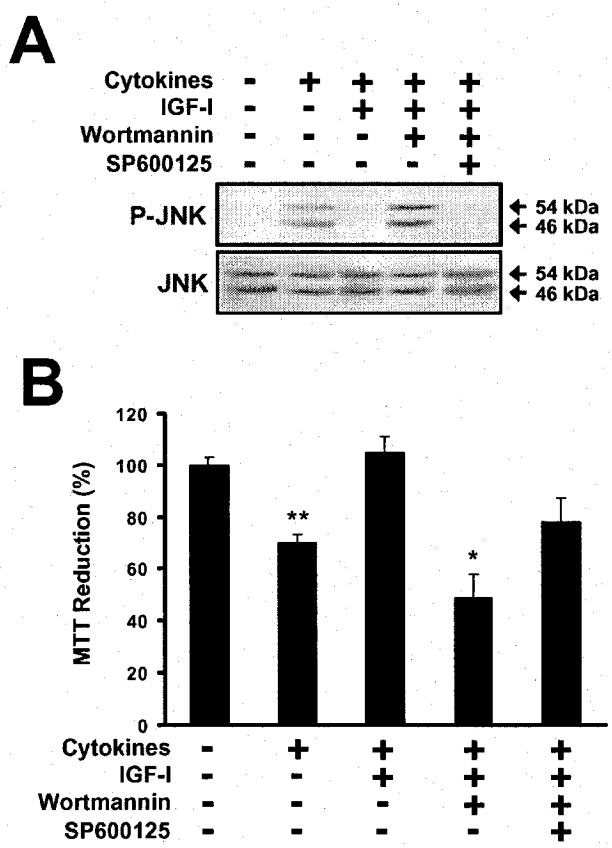

A
Cytokines - + + + +
IGF-I - - + + +
Wortmannin - - - + +
SP600125 - - - - +
P-JNK
54 kDa
46 kDa
JNK
54 kDa
46 kDa
B
MTT Reduction (%)
120
100
80
60
40
20
0
**
*
Cytokines - + + + +
IGF-I - - + + +
Wortmannin - - - + +
SP600125 - - - - +

Figure 3.4. Inhibition of PI3K/AKT renders islets more susceptible to cytokine-mediated cell death. Islets were culture for 48 hours prior to the addition of wortmannin (Wort; 100 nM) and/or cytokines (Cyto; 100 ng/ml TNF-α, 2 ng/mL IL-1β, 100 ng/ml INF-γ). The islets were then cultured for an additional 24 hours. (**A**) Lysates were taken and JNK phosphorylation was analyzed by Western blotting. The blot shown is representative of three independent experiments. (**B**) Caspase-3 activity of islet cell lysates was assessed by measuring the cleavage of DEVD-*p*NA. The amount of *p*NA released was measured by analyzing the absorption at 405 nm. For each graph, bars represent the mean values ± SEM for three independent experiments. (**C**) Islet viability was assessed by MTT assay. Bars represent the mean MTT reduction expressed as a percentage of the control ± SEM for quadruplicate samples from four independent experiments. *, $P<0.05$; **, $P<0.01$ compared with untreated controls (Ctrl).

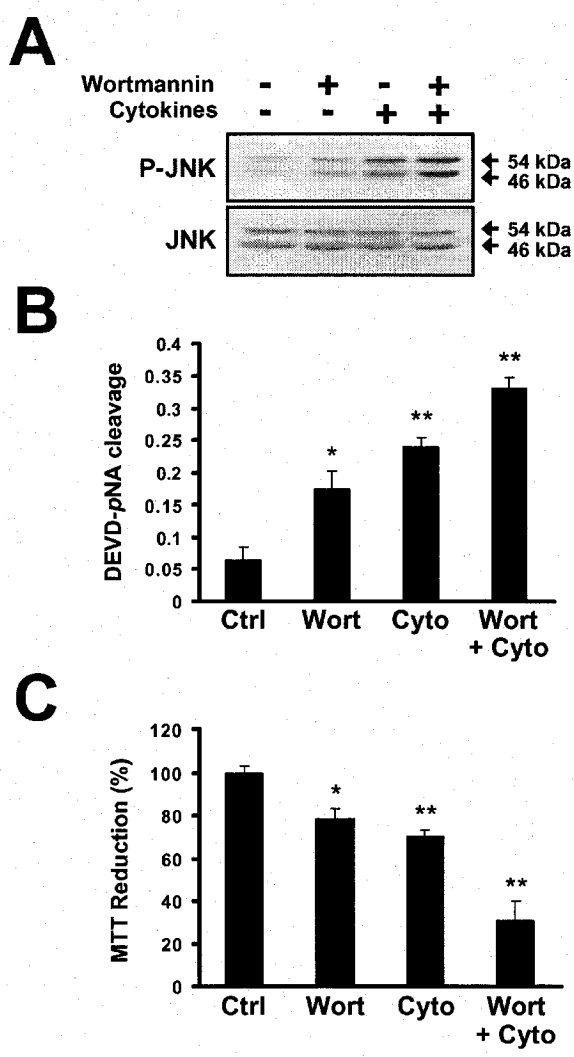

A
Wortmannin - + - +
Cytokines - - + +
P-JNK
54 kDa
46 kDa
JNK
54 kDa
46 kDa
B
DEVD-pNA cleavage
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
*
**
**
Ctrl
Wort
Cyto
Wort + Cyto
C
MTT Reduction (%)
120
100
80
60
40
20
0
*
**
**
Ctrl
Wort
Cyto
Wort + Cyto

Figure 3.5. The JNK inhibitor SP600125 transiently protects islets from cytokine-mediated cell death. Freshly isolated islets were cultured for 48 hours and then treated with cytokines (100 ng/ml TNF-α, 2 ng/mL IL-1β, 100ng/ml INF-γ) with or without SP600125 (20 μM). (**A**) Lysates were taken after 24 hours and JNK and c-jun phosphorylation were analyzed by Western blotting. The blots shown are representative of three independent experiments. (**B**) Islet viability was assessed after 24 and 72 hours by MTT assay. Bars represent the mean MTT reduction expressed as a percentage of the control ± SEM for quadruplicate samples from four independent experiments. *, $P<0.05$; **, $P<0.01$ compared with untreated controls.

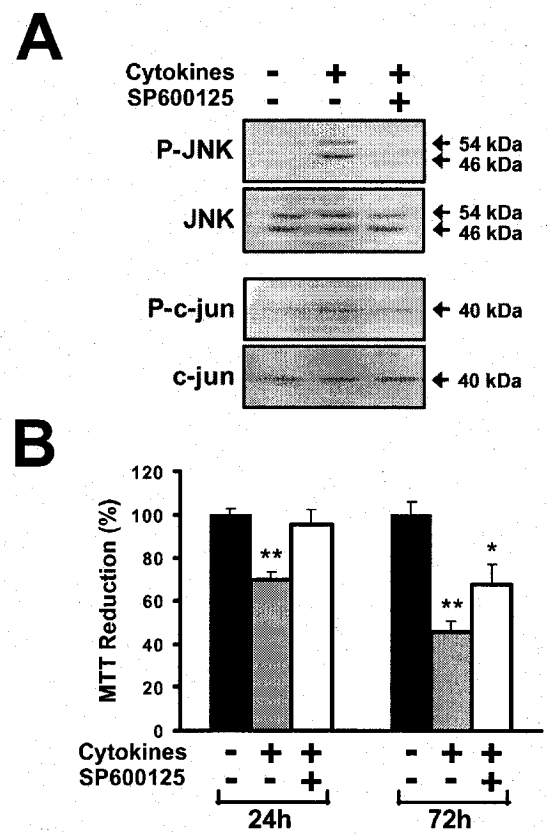

A
Cytokines - + +
SP600125 - - +
P-JNK
54 kDa
46 kDa
JNK
54 kDa
46 kDa
P-c-jun
40 kDa
c-jun
40 kDa
B
MTT Reduction (%)
120
100
80
60
40
20
0
**
**
*
Cytokines - + + - + +
SP600125 - - + - - +
24h
72h

Figure 3.6. SP600125 improves isolated islet survival and function following routine isolation. Immediately following isolation, islets were cultured with or without the addition of SP600125 (20 μM) to the culture media. (**A**) Following 48 hours in culture, islet viability was assessed by MTT assay. Bars represent the mean MTT reduction expressed as a percentage of the control ± SEM for quadruplicate samples from five independent experiments. (**B**) Caspase-3 activity was assessed by measuring the cleavage of DEVD-*p*NA by islet cell lysates taken after 48 hours in culture. The amount of *p*NA released was measured by analyzing the absorption at 405 nm. Bars represent the mean values ± SEM for three independent experiments. (**C**) Mitochondrial depolarization was assessed following 48 hours in culture by JC1 staining. The number of cells with depolarized mitochondria was expressed as a percentage of the total cells counted. Bars represent the mean values ± SEM for three independent experiments. (**D**) Glucose-stimulated insulin release was examined following 48 hours in culture. Bars represent mean values ± SEM for three independent experiments. (**E**) Islets were cultured with or without the addition of SP600125 (20 μM) to the culture media for the indicated periods. Following 96 hours in culture, islet viability was assessed by MTT assay. Bars represent the mean MTT reduction expressed as a percentage of the control ± SEM for quadruplicate samples from five independent experiments. *, $P<0.05$; **, $P<0.01$ compared with untreated controls (Ctrl).

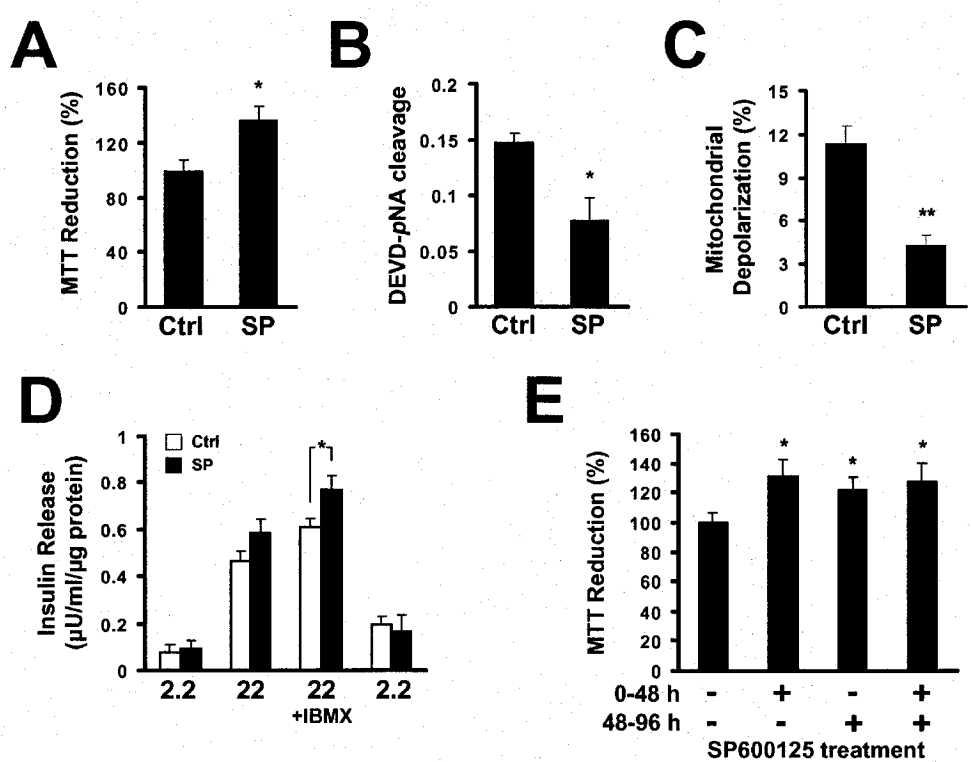

A
MTT Reduction (%)
160
120
80
40
0
*
Ctrl
SP
B
DEVD-pNA cleavage
0.2
0.15
0.1
0.05
0
*
Ctrl
SP
C
Mitochondrial Depolarization (%)
15
12
9
6
3
0
**
Ctrl
SP
D
Insulin Release (µU/ml/µg protein)
1
0.8
0.6
0.4
0.2
0
Ctrl
SP
*
2.2
22
22
2.2
+IBMX
E
MTT Reduction (%)
160
140
120
100
80
60
40
20
0
*
*
*
0-48 h - + - +
48-96 h - - + +
SP600125 treatment

Figure 3.7. Sustained JNK activation caused by wortmannin or cytokines does not require caspase activation. (**A**) Freshly isolated islets were cultured for 48 hours prior to the addition of 100 nM wortmannin with or without 50 μM Z-VAD-fmk or 20 μM SP600125. Whole cell lysates were taken following 24 hours of treatment and JNK phosphorylation was analyzed by Western blotting. The blot is representative of three independent experiments. (**B**) Freshly isolated islets were cultured for 48 hours and then treated with cytokines (100 ng/ml TNF-α, 2 ng/mL IL-1β, 100 ng/ml INF-γ) with or without 50 μM Z-VAD-fmk or 20 μM SP600125. Whole cell lysates were taken following 24 hours of treatment and JNK phosphorylation was analyzed by Western blotting. The blot is representative of three independent experiments.

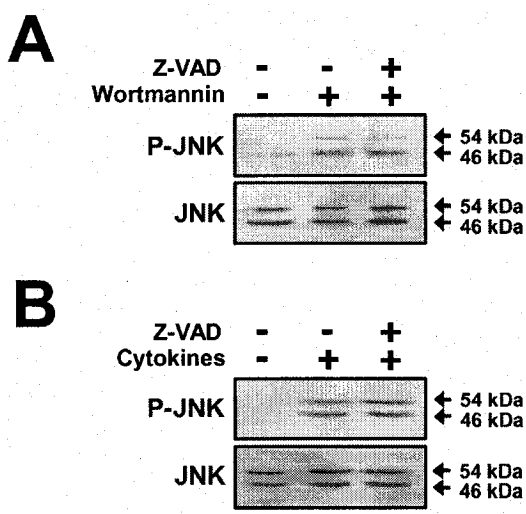
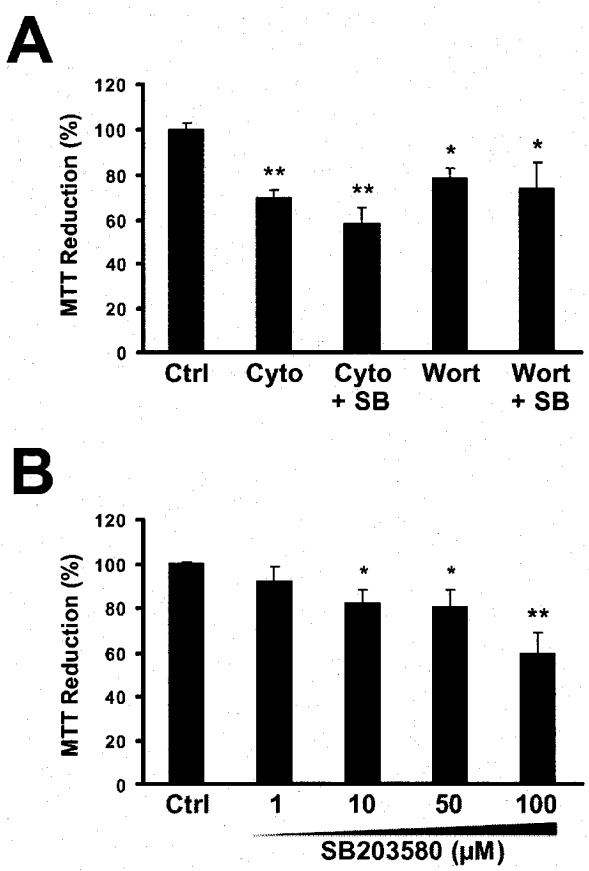

A
Z-VAD - - +
Wortmannin - + +
P-JNK
54 kDa
46 kDa
JNK
54 kDa
46 kDa
B
Z-VAD - - +
Cytokines - + +
P-JNK
54 kDa
46 kDa
JNK
54 kDa
46 kDa

Figure 3.8. Inhibition of p38 with SB203580 has a negative effect on isolated islet survival. (**A**) Freshly isolated islets were cultured for 48 hours and then treated with or without cytokines (Cyto; 100 ng/ml TNF-α, 2 ng/mL IL-1β, 100 ng/ml INF-γ), wortmannin (Wort; 100 nM), and SB203580 (SB; 20 μM) for 24 hours. Islet viability was assessed by MTT assay. Bars represent the mean MTT reduction expressed as a percentage of the control ± SEM for quadruplicate samples from three independent experiments. (**B**) Immediately following isolation, islets were treated with increasing concentrations of SB203580 (1-100 μM). Following 48 hours in culture, islet viability was assessed by MTT assay. Bars represent relative MTT reduction values expressed as a percentage of the control ± SEM for quadruplicate samples from four independent experiments. *, $P<0.05$; **, $P<0.01$ compared with untreated controls (Ctrl).


A
MTT Reduction (%)
120
100
80
60
40
20
0
**
**
*
*
Ctrl
Cyto
Cyto + SB
Wort
Wort + SB
B
MTT Reduction (%)
120
100
80
60
40
20
0
*
*
**
Ctrl
1
10
50
100
SB203580 (μM)

3.5. DISCUSSION

Our results suggest that there is a cross-talk between PI3K/AKT and JNK in human islets via negative regulation of ASK1. The ability of AKT to suppress the JNK pathway has been observed in a variety of cell systems (246;278;279). AKT has been shown to suppress the JNK pathway by phosphorylating and negatively regulating ASK1 (145), MLK3 (146), and MKK4 (SEK1) (147). In addition, AKT has also been suggested to prevent JNK activation by directly interacting with JIP1 and preventing the recruitment of upstream kinases to JNK (271). It has also been suggested that AKT may suppress JNK through upregulation of JIP1 (278), but we observed no changes in JIP1 expression following isolation (Figure 3.1A). Another mechanism of suppressing JNK signalling is through the upregulation of MKP-1, an inducible phosphatase which is thought to preferentially dephosphorylate p38 and JNK (280), yet in some cases it can also inactivate ERK (281). We have shown that following islet isolation, MKP-1 expression increases over the first 72 hours following isolation (114). This increase in MKP-1 expression coincides with the decrease in JNK phosphorylation and could represent another mechanism of JNK suppression in isolated islets. Therefore, there are several mechanisms by which AKT can suppress JNK activation, indicating that JNK suppression is an important consequence of AKT-mediated survival. However, it is plausible that the particular mechanisms of JNK suppression will depend on the nature of the stimuli and the cellular context. It is interesting that cytokine treatment caused JNK activation in islets despite high AKT activity, suggesting that AKT may

suppress JNK activation only by particular stimuli. Indeed, there is an assortment of kinases acting upstream of JNK, not all of which may be suppressed by AKT. In addition, the subcellular distribution of particular kinases may play an important role in permitting particular interactions between signalling molecules.

Our results demonstrate that inhibition of PI3K/AKT signalling in human islets results in a time-dependent increase in JNK activation (Figure 3.2). A similar result was obtained in cerebellar granule neurons in which inhibition of PI3K led to an increase in JNK activation (258). In addition, expression of dominant negative AKT in human vascular smooth muscle cells led to increased JNK activation and FasL induction (282). These findings suggest that either the inhibition of PI3K/AKT itself stimulates the JNK pathway or that in the absence of PI3K/AKT signalling an external activator of the JNK pathway becomes apparent. The stimuli responsible for the activation of JNK in the absence of PI3K/AKT signalling could be any number of insults to which islets are subjected to during the isolation procedure. Indeed, the same stimuli that are responsible for JNK activation immediately following isolation could still be present, and are uncovered through derepression of the JNK pathways by inhibition of PI3K/AKT.

JNK activation can be induced by growth factor withdrawal (107), detachment from the extracellular matrix (108), ischemia (109), osmotic stress (110), reactive oxygen species (111), and by cytokines (112), all of which are possible consequences of islet isolation. We have demonstrated that freshly isolated islets display high JNK activity which diminishes over the first 48 hours following isolation (113;114). This early activation of JNK coincides with the peak

of islet apoptosis which occurs 24 hours following isolation (113;114;275). Addition of exogenous insulin to the culture media immediately following isolation reduced JNK activity and led to a decrease in DNA fragmentation after 24 hours in culture (113). Preservation of rat pancreata for 24 hours by the two-layer method (TLM), which uses oxygenated perfluorohydrocarbons to increase oxygen delivery to the organ, resulted in decreased apoptosis and lower JNK activity following isolation when compared to islets from pancreata stored in UW solution (116). In addition, the same study showed that islets from pancreata which were processed immediately following resection had significantly lower JNK activation than islet from pancreata stored for 24 hours by TLM or in UW solution. The most convincing evidence implicating JNK in transplanted islet survival comes from transplantation of rat islets overexpressing dominant negative (DN) JNK into streptozotocin-induced diabetic nude mice (117). Mice receiving DN-JNK islets displayed lower blood glucose levels, which could be attributed to maintenance of insulin gene transcription despite the presence of oxidative stress at the graft site as well as increased survival of islets due to impaired apoptotic signalling by JNK. Taken together, these findings point towards a role for JNK in mediating isolated islet apoptosis. The ability of SP600125 to improve isolated islet survival and function supports the notion that JNK is an important regulator of islet death.

When administered immediately following isolation, SP600125 improved islet survival and function, even 48 hours after removal of SP600125 (Figure 3.6E). This finding indicates that islets rescued by JNK inhibition remain viable

despite the removal of SP600125. Therefore, SP600125 could be a beneficial constituent of islet culture media, and perhaps other solutions during the isolation. JNK inhibition was also able to improve isolated islet survival when treatment was commenced 48 hours after isolation, at a point when JNK activation is significantly reduced (Figure 3.6E). The beneficial effects of SP600125 even when JNK activity is reduced could be due to the fact that JNK is required for mitochondria-mediated cell death (98-100), which appears to be occurring at later time points in islets (Chapter 5)(275). We previously suggested that the inability of caspase inhibition to prevent islet cell death may be due to the fact that the caspases lie downstream of any mitochondrial dysfunction, and thus their inhibition has no effect on mitochondria-mediated cell death (Chapter 5)(275). In this case, caspase inhibition led to a more necrotic-like mode of islet cell death. In contrast, SP600125 significantly reduced mitochondrial depolarization, suggesting that JNK regulates events upstream of mitochondrial dysfunction in islets. Recently, SP600125 (20 μM) has been shown to result in rapid activation of cAMP-response element binding protein (CREB) in MIN6 cells under basal conditions (283), which could also explain the positive effect of SP600125 at later time-points following isolation since CREB is implicated in islet survival (284;285). However, we did not observe an increase in p38 or CREB phosphorylation following 30 minutes of SP600125 treatment in isolated human islets (data not shown). Similarly, SP600125 did not result in increased p38 phosphorylation in stimulated Jurkat T cells (286). Therefore, it is plausible that

SP600125-induced CREB activation is only observable under non-stimulated conditions, as suggested by Vaishnav et al. (283).

There is considerable evidence supporting a role for JNK in IL-1β-mediated β-cell apoptosis (120;273;274;287). However, JNK inhibition using SP600125 only afforded transient protection of human islets against a combination of IL-1β, TNF-α, and INF-γ (Figure 3.5). This may be due to the ability of these cytokines to activate several pathways in addition to JNK known to regulate islet apoptosis, including STATS (288;289), production of reactive oxygen species (290), increased cytosolic Ca^{2+} levels (291), and decreased BcL-2 (285). In addition, the ability of cytokines to induce apoptosis through FADD-mediated recruitment of caspase-8 (extrinsic pathway) is also possible, although perhaps not likely due to the high levels of expression of the endogenous caspase-8 inhibitor FLIP in human islets (292). Our results suggest that JNK is necessary for proper execution of cytokine-stimulated cell death in human islets, but the precise role of JNK in cytokine-mediated islet cell death remains unclear.

In addition to increased islet viability, we observed increased insulin secretion by human islets treated with SP600125 (Figure 3.6D). Previously, SP600125 has been shown to increase insulin release in MIN6 cells, and overexpression of dominant negative JNK led to increased insulin gene transcription (293). A role for JNK in stress-mediated suppression of insulin gene transcription has also been demonstrated in rat islets (117). These effects could be explained by the suppressive effect of c-jun on insulin gene transcription

(294). Therefore, in addition to promoting isolated islet survival, JNK inhibition can also improve insulin secretion.

Many stressors that activate JNK also activate p38. However, as with JNK, the exact role of p38 in regulation of cell survival remains unclear because p38 appears to mediate both survival and apoptotic signals (93). Our current results suggest that p38 activity may represent a survival signal in isolated human islets since inhibition of p38 increased isolated islet cell death (Figure 3.8). Similarly, p38 inhibition has previously been demonstrated to aggravate cytokine-mediated human islet cell death (295). In addition, inhibition of p38 potentiated IL-1β-induced cell death in a β-cell line (120). These findings are in contrast to those in rat islets, in which p38 inhibition reduced apoptosis induced by IL-1β and INF-γ (296). We have previously observed an increase in p38 phosphorylation in canine islets treated with insulin, which can act as a survival stimulus in many cell systems (113). This effect, however, is cell-type dependent since insulin has been shown to inhibit p38 activation in primary neuronal cultures (121). IGF-I mediated survival has been shown to involve p38-dependent phosphorylation of CREB and induction of Bcl-2 expression (122;123). Taken together, these data suggest that p38 could play a role in regulating islet survival, possibly by mediating growth factor signalling. It is also plausible that activation of p38 in response to insulin represents an inhibitory feedback pathway to suppress insulin transcription (124).

In summary, our results demonstrate that PI3K/AKT suppresses the JNK pathway in human islets and that this cross-talk represents an important anti-

apoptotic consequence of PI3K/AKT activation. In addition, due to its beneficial effects on islet survival and function, JNK inhibition by SP600125 may be a viable strategy for improving isolated islet survival.

3.6. ACKNOWLEDGMENTS

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CONNECTING TEXT

In Chapter 2 we demonstrated that AKT becomes highly phosphorylated in isolated canine islets after 16 hours in culture. In Chapter 3, we confirmed this finding in human islets and established that signalling through the PI3K/AKT pathway is important for islet survival. However, it remained unclear why AKT would become highly phosphorylated under such presumably stressful conditions. Therefore, the aim of Chapter 4 was to identify the cause for the observed increase in AKT phosphorylation in isolated human islets.

CHAPTER 4

Autocrine insulin action activates AKT and increases survival of isolated human islets

Reid Aikin, Dusica Maysinger, Stephen Hanley, Mark Lipsett, Mauro Castellarin, Steven Paraskevas, and Lawrence Rosenberg

4.1. ABSTRACT

The PI3K/AKT pathway plays a critical role in promoting the survival of pancreatic β-cells. AKT becomes activated in isolated human islets following overnight culture despite significant levels of cell death. The aim of the current study was to identify the cause for the observed increase in AKT phosphorylation in isolated islets. We hypothesized that a local factor, secreted by the isolated islets in culture, was acting in an autocrine manner to activate AKT. We demonstrate that islet conditioned medium induced AKT phosphorylation in freshly isolated human islets, whereas frequent media replacement decreased AKT phosphorylation. Following overnight culture, islet conditioned medium contained significantly elevated levels of insulin, indicating that insulin may be responsible for the observed increase in AKT phosphorylation. Indeed, treatment with an anti-insulin antibody suppressed AKT phosphorylation, leading to decreased islet survival. In addition, dispersion of islets into single cells also suppressed AKT phosphorylation and induced islet cell death, indicating that islet integrity is also required for maximal AKT phosphorylation. Our findings demonstrate that insulin acts in an autocrine manner to activate AKT and mediate the survival of isolated human islets. These findings provide new information on how culturing islets prior to transplantation may be beneficial to their survival by allowing for autocrine activation of the pro-survival AKT pathway.

4.2. INTRODUCTION

Although successful single donor islet transplantations have been reported recently, the proportion of islet isolations producing sufficient viable islets for transplantation remains low (57). Even with recent improvements to the isolation procedure, such as the use of the 2-layer method and a less toxic iodixanol gradient, many experienced groups isolate enough islets for transplantation from only 50% of isolations. Furthermore, a significant number of cells are lost due to apoptosis and necrosis during the immediate post-isolation period (61;275). A better understanding of the mechanisms controlling isolated islet cell death may lead to the development of strategies to improve islet survival following isolation.

AKT is a Ser/Thr kinase that regulates several cellular processes, including cell cycle progression, transcription, glucose uptake, and apoptosis. In particular, AKT plays a critical role in mediating cell survival in response to growth factor stimuli (134). AKT is recruited to the plasma membrane following activation of PI3K and is activated through phosphorylation by PDK1/2 (135;136). Activated AKT can directly phosphorylate several pro-apoptotic proteins leading to suppression of apoptotic signals. Transgenic mice expressing a constitutively active form of AKT1 under the control of the insulin promoter display increased β-cell mass, improved glucose tolerance, and resistance to streptozotocin-induced diabetes (148;149). Adenovirus-mediated overexpression of constitutively active AKT1 in isolated human islets increased *in vitro* β-cell proliferation, decreased apoptosis induced by serum/glucose withdrawal or high glucose, and decreased the number of transplanted islets required to reverse streptozotocin-induced

diabetes in mice (150). In addition, PI3K/AKT signalling has been shown to mediate survival of isolated human islets (151;297), canine islets (270), and insulinoma cell lines (133;152-154). Taken together, these findings indicate that the PI3K/AKT pathway plays a critical role in promoting the survival of β-cells.

Although the original Edmonton protocol involved transplanting islets immediately after isolation, the recovery of islets in culture prior to transplantation has once again become common practice (56;57). One reason for culturing islets prior to transplantation is that a significant amount of islet cell death due to the isolation occurs during the 48 hours following isolation, and thus it is advantageous to allow these cells to die off in culture so as not to provoke additional inflammation local to the graft site. In addition, there is a much higher relative expression of inflammatory genes during the immediate post-isolation period when compared to islets cultured for one week (218). Culturing islets also provides an opportunity for therapies aimed at improving islet survival, inducing islet expansion, and reducing islet immunogenicity. We and others have demonstrated that immediately following isolation, human islets display low levels of AKT phosphorylation (151;297). However, following overnight culture in either serum containing or serum-free medium, AKT becomes highly phosphorylated in a PI3K-dependent manner (151;270;297). This increased AKT phosphorylation affords islets a certain degree of protection against insults such as inflammatory cytokines (297). The aim of the current study was to identify the cause for the observed increase in AKT phosphorylation in isolated human islets. We hypothesized that a local factor, secreted by the isolated islets in culture, was

acting in an autocrine manner to activate AKT. Our findings indicate that autocrine insulin action is responsible for stimulating the AKT pathway in cultured human islets.

4.3. MATERIALS AND METHODS

Human Islet Isolation. Pancreata were retrieved from heart-beating cadaveric organ donors (17-66 years of age) at the time of multi-organ harvest for transplantation. Consent for donation of tissues for research was obtained by the local organ procurement organization (Quebec-Transplant). Warm ischemia time was approximately 5 minutes and cold ischemia time, using refrigerated University of Wisconsin (UW) solution perfusion, was between 3-8 hours. Islets were isolated using the method of Ricordi et al. (48). Briefly, the main pancreatic duct was canulated and a cold (6-8°C) solution of 1.4 mg/ml Liberase enzyme blend (Roche, Montreal, QC, Canada) dissolved in Perfusion Solution (Mediatech, Herndon, VA, USA) was infused through the canula into the pancreas over 10 minutes using a 60 ml syringe. The distended pancreas was placed in a sterilized digestion chamber (Bio-Rep, Miami, FL, USA) through which perfusion solution supplemented with 0.2 mg/ml DNAse I (Roche) was recirculated at 37°C. The extent of tissue digestion was assessed by staining aliquots of digestate with dithizone (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada), a zinc chelater which stains the zinc rich β-cells, and visualizing the islets under an inverted light microscope (Carl Zeiss Canada Ltd., Toronto, Ontario, Canada). When the majority of the islets were seen to be free of surrounding acinar tissue, the digestion process was terminated by cooling the circuit to between 5 and 10°C and flushing the system with Dilution Solution (Mediatch) supplemented with 10% fetal bovine serum (Montreal Biotech Inc., Montreal, Quebec, Canada). The digestate was collected, centrifuged, and

washed three times with Wash Solution (Mediatech) and islets were purified on a continuous density gradient (Biocoll separating solution; Biochrom AG, Berlin, Germany) using a COBE 2991 Cell Processor (COBE BCT, Denver, CO, USA). The number of islet equivalents (IEQ) and islet purity were assessed using dithizone (Sigma). Glucose-stimulated insulin secretion was routinely assessed to ensure islet functionality.

Islet cell culture. Isolated islets (>90% purity) were cultured in CMRL 1066 medium (Gibco, Burlington, ON, Canada) containing 10% fetal bovine serum (Montreal Biotech Inc.), at 37°C in a humidified atmosphere of 5% CO_2. Medium was changed every other day. In some experiments, medium was supplemented with insulin (Eli Lilly, Indianapolis, IN, USA) at the indicated concentration. Monoclonal azide-free anti-insulin (Acris Antibodies, Hiddenhausen, Germany) and anti-IGF-1 (Upstate, Lake Placid, NY, USA) were added to culture medium to a final concentration of 1:50. Hydroxy-2-naphthalenylmethylphosphonic acid trisacetoxymethyl ester (HNMPA-$(AM)_3$; Calbiochem, San Diego, CA, USA) was used at 50 μM unless otherwise indicated. 3-isobutyl-1-methylxanthine (IBMX; Sigma) was used at 50 μM. For all compounds prepared in DMSO, the final concentration of DMSO in the culture medium was kept below 0.1%. Vehicle controls were prepared for all treatments. In some cases, islets were dispersed by first washing with Dispersion Solution (Gibco) followed by a 10 minute incubation at 37°C with trypsin/EDTA (Gibco). Trypsinized islets were washed with cold CMRL 1066 containing 10% FBS, pipetted gently, and resuspended in

CMRL 1066 containing 10% FBS. The insulin content of islet medium was assessed using a commercially available insulin ELISA kit (ALPCO diagnostics, Windham, NH, USA).

MTT Assay. Mitochondrial metabolic activity was used as an indicator of islet viability (235). Aliquots containing 500 IEQ in 500 µl of medium were placed in sterile eppendorf tubes and 50 µL of stock 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/ml) (Sigma) was added to each sample. The samples were incubated at 37°C for 2 hours, washed twice with cold PBS, and lysed with 200 µL of DMSO (Sigma). Two 100 µL aliquots from each sample were loaded onto a 96-well plate and the absorbance at 595 nm was measured using a Benchmark Microplate Reader (BIO-RAD). Four independent samples were analyzed per experiment and each experiment was performed at least three times.

Fluorescent Microscopy. Whole islets were dispersed (see above) and resuspended in PBS containing 2 µM propidium iodide (PI) (Molecular Probes Inc., Eugene, OR, USA) and 0.67 µM fluorescein diacetate (FDA) (Molecular Probes). The stained islets were placed on glass slides with cover-slips and incubated for 15 minutes at room temperature in a humid foil-covered container. Slides were visualized under a fluorescent Olympus BX60 microscope connected via a digital video camera to a PC. Five hundred cells were counted per slide, with three slides per group for at least three independent experiments. The

percentage of PI-positive cells was determined by dividing the number of PI positive cells by the total number of cells counted. Images were analyzed using Image Pro Plus 4.0 software (Media Cybernetics Inc., Silver Spring, MD, USA). Confocal microscopy was performed using a Zeiss LSM 510 Confocal Laser Scanning Microscope (Carl Zeiss Canada Ltd.).

Western Blotting. The islet samples (2500 IEQ) were centrifuged for 2 minutes at 900 rpm and 4°C. The tissue pellet was washed twice with ice cold PBS and then dissolved in lysis buffer (50 mM Tris-HCl, pH 8.0, 1.37 mM NaCl, 1% (v/v) nonident P-40, 10% (v/v) glycerol, 0.1 mM sodium orthovanadate, complete protease inhibitor cocktail tablet (Roche)). The samples were sonicated and centrifuged for 20 minutes at 14000 rpm, 4°C, and the supernatant was kept for analysis. The protein content was determined using BIO-RAD Protein Assay Dye Reagent (BIO-RAD). Lysates were diluted 6:1 with 6X Laemmli sample buffer (0.375 M Tris-HCl pH 6.8, 12% (w/v) SDS, 3% (v/v) glycerol, 0.2% (w/v) bromophenol blue, 12% (v/v) β-mercaptoethanol in double distilled water) and boiled for 5 minutes. An equal amount of protein (50 μg) was loaded for each sample into a 12% polyacrylamide gel run at 100 V for 90 minutes. Transfer onto nitrocellulose was conducted at 250 mA for 90 minutes. Membranes were blocked with 2% bovine serum albumin in washing buffer (25 mM Tris, 150 mM NaCl, 0.05% Tween 20 in double distilled water). Blocked membranes were then probed with primary antibodies. Anti-AKT and anti-phospho-AKT (Ser-473) (both from Cell Signalling Tech., Beverly, MA, USA) were used at a dilution of 1:1000.

Anti-phospho-JNK (Promega Corp., Madison, WI, USA) was used at a 1:5000 dilution. Anti-JNK1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at a dilution of 1:1000. Following primary antibody incubation, the membranes were washed for 30 minutes in washing buffer then incubated for 1 hour in anti-rabbit horseradish peroxidase-linked antibody (1:4000) (Amersham LifeSciences Inc., Buckinghamshire, England). Following another 30 minute wash, the blots were developed using the ECL chemiluminescence system (Amersham) and Kodak X-OMAT film (Kodak, Rochester, NY, U.S.A.). Membranes were stripped by incubating at 65°C for half an hour in stripping buffer (100 mM β-mercaptoethanol, 2% (w/v) SDS and 62.5 mM Tris-HCl pH 6.7) and reprobed with primary antibody.

Statistical Analysis. All results are expressed as mean ± standard error of the mean (SEM). Statistical significance was determined using a one-way *ANOVA* with a post-hoc Bonferroni's test as well as the Student's t-test using SYSTAT 9 (SPSS Inc., Chicago, IL, USA). Differences were considered significant where $P < 0.05$.

4.4. RESULTS

AKT phosphorylation is detectable 3 hours following isolation and is maximally activated after 12 hours. We have previously demonstrated that AKT becomes highly phosphorylated in isolated human and canine islets after 12-16 hours in culture (270;297). In order to determine the kinetics of this signal, we examined the levels of AKT phosphorylation in human islets over the course of the first 24 hours following isolation. Using Western blot analysis, an increase in AKT phosphorylation was detectible as early as 3 hours following isolation (Figure 4.1A). The level of AKT phosphorylation reached a maximum by 12 hours, consistent with our previous findings (270;297).

Islet conditioned medium induces AKT phosphorylation in freshly isolated islets. If isolated islets were secreting a factor that was acting in an autocrine manner to induce AKT phosphorylation, then addition of islet conditioned medium to freshly isolated islets would be expected to induce AKT phosphorylation. To address this possibility, conditioned medium was used from islets which had been cultured for 24 hours, a point at which AKT is highly phosphorylated. Islets from the ensuing isolation were then treated with conditioned medium within an hour after isolation and AKT phosphorylation was examined after 30 minutes. Islets treated with conditioned medium showed increased AKT phosphorylation compared to islets which received fresh, untreated medium (Figure 4.1B). Conditioned medium that was heated to 65°C for 30 minutes lost the ability to induce AKT phosphorylation, suggesting that a heat-labile factor was responsible for augmenting AKT phosphorylation.

Regular media replacement decreases AKT phosphorylation. Since AKT becomes highly phosphorylated after 12-16 hours in culture, it is possible that the autocrine factor must accumulate in the medium to have its maximal effect. We therefore examined whether frequent media replacement would prevent the accumulation of the secreted factor in the medium, resulting in lower AKT phosphorylation. Replacing the islet culture medium every 6 hours over a 24 hour period resulted in decreased AKT phosphorylation compared with islets which were replated with the same medium (Figure 4.1C). This finding supports the notion that a local secreted factor is indeed responsible for the increase in AKT phosphorylation in isolated islets.

Treatment with an anti-insulin antibody decreases AKT phosphorylation and human islet survival. Since insulin is secreted by β-cells, is a strong activator of AKT (298), and is heat sensitive (299), we examined whether insulin was the factor responsible for the observed rise in AKT phosphorylation. Using an insulin ELISA, we found that following 24 hours, islet conditioned medium contained significantly elevated levels of insulin (conditioned medium = 59.2 mU/ml ± 9.6; fresh medium = 1.08 μU/ml ± 0.062) (Figure 4.2A). Freshly isolated human islets were then cultured with or without an anti-insulin antibody to sequester insulin secreted into the islet culture medium. Addition of anti-insulin antibody significantly decreased AKT phosphorylation following 24 hours in culture (Figure 4.2B). However, treatment with an anti-IGF antibody, which binds both IGF-I and IGF-II, had no effect on AKT phosphorylation (Figure 4.2B). Treatment with anti-insulin antibody also resulted in a 27% reduction in

islet viability, as measured by the MTT assay (Figure 4.2C). On the other hand, an anti-IGF antibody had no significant effect on islet viability. Addition of the anti-insulin antibody to the islet culture medium also caused an almost 2-fold increase in the number of propidium iodide (PI)-positive cells (Figure 4.2D). Thus, isolated islets release high levels of insulin into the surrounding medium which activates AKT and improves islet survival.

Insulin, but not glucose, leads to increased AKT phosphorylation in isolated human islets. Immediately following isolation, AKT phosphorylation levels are low while JNK is highly phosphorylated. Over the ensuing 24 hours, JNK phosphorylation levels decrease concomitantly with a rise in AKT phosphorylation (114;297). Since JNK is known to suppress insulin signalling (300), it is possible that insulin signalling pathway is suppressed in islets immediately following isolation and AKT only becomes activated following suppression of the JNK pathway. We therefore examined whether exogenous insulin could induce AKT phosphorylation in islets immediately following isolation, at a time when JNK is highly activated. Within 1 hour following isolation, human islets were treated with increasing concentrations of insulin for 15 minutes and AKT phosphorylation was assessed. Insulin was able to cause an increase in AKT phosphorylation at a concentration of 100 nM, (Figure 4.3A), which is within the concentration range found in islet conditioned medium (59.2 mU/ml ˜ 360 nM). Therefore, signalling to AKT via the IR is functional in isolated human islets, supporting our previous finding that, in fact, the PI3K/AKT pathway suppresses

JNK activation (297). This point is further demonstrated by the finding that the treatment of isolated islets with insulin decreases JNK phosphorylation (113).

Previous studies have suggested that glucose-induced activation of intracellular signalling pathways is mediated by the autocrine action of secreted insulin. Treatment of βTC3 cells for 2 minutes with 15 mM glucose resulted in increased IR tyrosine phosphorylation and increased association of IRS-1 with the 85 kDa subunit of PI3K (301), and similar findings were observed in isolated rat islets (302). Furthermore, 16.7 mM glucose was able to activate AKT after only 5 minutes in the HIT-T15 β-cell line (303). Based on our finding that basal insulin secretion activates AKT following isolation, we examined whether inducers of insulin secretion can accelerate AKT phosphorylation following isolation. Treatment of freshly isolated human islets with 22.2 mM glucose for 15 minutes had no effect on AKT phosphorylation (Figure 4.3B). Similarly, a combination of 22.2 mM glucose and IBMX, an inhibitor of cyclic-AMP phosphodiesterase that causes elevated intracellular cAMP levels that lead to increased exocytosis of insulin (304), had no significant effect on AKT phosphorylation. Therefore, in our hands, glucose was unable to induce AKT phosphorylation in human islets immediately following isolation. Similarly to these findings, treatment of isolated rat islets with high glucose had no effect on AKT phosphorylation, though 100 nM insulin did (305;306).

In order to determine the role of the IR in the activation of AKT in isolated islets we employed a cell-permeable IR tyrosine kinase inhibitor, HNMPA-$(AM)_3$ (307). HNMPA-$(AM)_3$ has previously been shown to decrease glucose-stimulated

AKT activation in HIT-T15 cells (303). Surprisingly, pretreatment with 50 μM HNMPA-$(AM)_3$ for 30 minutes led to increased AKT phosphorylation (Figure 4.3B). In addition, HNMPA-$(AM)_3$ also induced high JNK phosphorylation. Since these effects were observed in the presence of high glucose, we also examined the effects of HNMPA-$(AM)_3$ alone on isolated human islets under normal culture conditions.

The IR tyrosine kinase inhibitor HNMPA-$(AM)_3$ induces islet cell death and AKT phosphorylation. To furthure explore the effects of HNMPA-$(AM)_3$, freshly isolated islets were cultured with or without HNMPA-$(AM)_3$ for 24 hours. Islets displayed a dose-dependent decrease in viability in response to HNMPA-$(AM)_3$ treatment (Figure 4.4A). Addition of 50 μM HNMPA-$(AM)_3$ for 24 hours resulted in a 4-fold increase in PI-positive cells (Figure 4.4B). Consistent with the above findings, 24 hour treatment with HNMPA-$(AM)_3$ resulted in a dose-dependent increase in AKT phosphorylation (Figure 4.4C). Thus, non-specific effects of HNMPA-$(AM)_3$ could be responsible for the observed rise in AKT and JNK phosphorylation, since HNMPA-$(AM)_3$ can also inhibit other kinases (307). Therefore, further studies are warranted to examine the specificity of HNMPA-$(AM)_3$.

Dispersion of islets into single cells suppresses AKT phosphorylation and induced islet cell death. Though diminished, AKT phosphorylation was still observed in the presence of anti-insulin antibody, suggesting that the local concentration of insulin within the islet might be higher than in the surrounding medium and that anti-insulin antibody is not able to

completely sequester intra-islet insulin. We therefore examined whether dispersion of isolated human islets into single cells affects AKT phosphorylation. Isolated human islets were trypsinized and dispersed into single cells immediately following isolation. An identical number of islet equivalents (IEQ) was cultured in both the dispersed and control cultures (1000 IEQ/ml of medium). Therefore, the dispersed islet culture (1X) contained the same number of cells/ml as the undispersed control islets (Ctrl). Dispersion of islets into single cells significantly decreased AKT phosphorylation and increased cell death (Figure 4.5). If the decrease in AKT phosphorylation caused by dispersion was due to reduced local concentration of insulin, then increasing the cell density would increase the insulin concentration and AKT phosphorylation. However, increasing the cell density up to eight fold had no significant effect on AKT phosphorylation (Figure 4.5A). These findings demonstrate that islet integrity is required for maximal AKT activation and cell survival.

Figure 4.1. Islet conditioned medium contains a heat-labile factor that induces AKT phosphorylation in freshly isolated human islets. (**A**) Whole cell lysates were taken at the indicated times following islet isolation and analyzed by Western blotting. The blots are representative of four independent experiments. (**B**) Following isolation, human islets were cultured for 1 hour after which the medium was replaced with either fresh medium (Ctrl), islet conditioned medium (CM), or heat-inactivated conditioned medium (HCM). After 30 minutes of treatment, whole cell lysates were prepared and analyzed by Western blotting. The blots are representative of three independent experiments. (**C**) Isolated islets were placed in culture and the culture medium was replaced every 6 hours (MR) or cells were replated with the same medium (Ctrl). Whole cell lysates were prepared after 24 hours and analyzed by Western blotting. The blots shown are representative of three independent experiments.

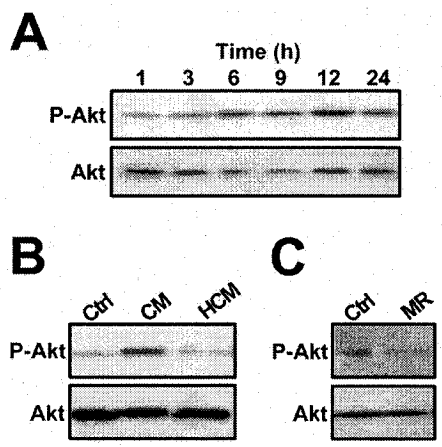
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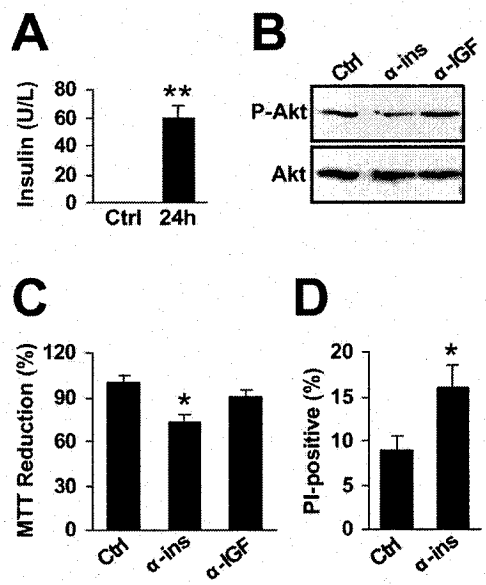
Figure 4.2. Anti-insulin antibody decreases AKT phosphorylation and induces cell death of isolated human islets. (**A**) Insulin content of fresh medium (Ctrl) and medium from islets cultured for 24 hours was assessed by ELISA. Bars represent the mean insulin content ± SEM for duplicate samples from three independent experiments. (**B**) Immediately following isolation, islets were cultured with or without the addition of anti-insulin antibody (1:50) or anti-IGF (1:50) to the culture medium. Whole cell lysates were prepared after 24 hours and assessed by Western blot analysis. The blots shown are representative of three independent experiments. (**C**) Islet viability was assessed by MTT assay after 24 hours. Bars represent the mean MTT reduction expressed as a percentage of the control ± SEM for quadruplicate samples from three independent experiments. (**D**) Membrane permeability was assessed following 24 hours in culture using PI/FDA staining. The number of PI-positive cells was expressed as a percentage of the total cells counted. Bars represent the mean values ± SEM for three independent experiments. *, $P<0.05$, ***, $P<0.001$ compared with untreated controls (Ctrl).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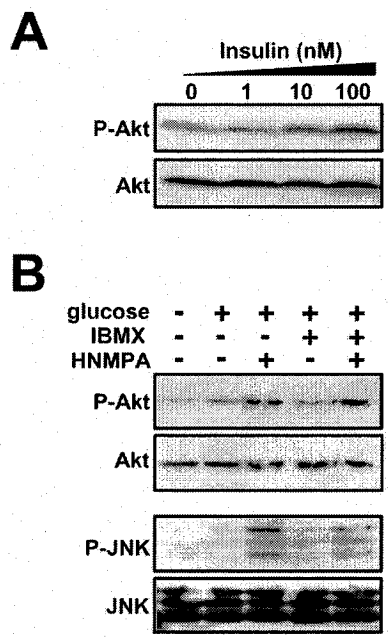
Ctrl
α-ins

Figure 4.3. Insulin, but not glucose, leads to increased AKT phosphorylation in isolated human islets. (**A**) Isolated islets were cultured for 1 hour following isolation and then treated with increasing concentrations of insulin. After 15 minutes of treatment, whole cell lysates were prepared and analyzed by Western blotting. The blots are representative of three independent experiments. (**B**) Freshly isolated islets were cultured for 1 hour after which time they were pretreated with or without HNMPA-$(AM)_3$ (50 μM) for 30 minutes. Islets were then treated for 15 minutes with glucose (22.2 mM) or glucose in combination with IBMX (50 μM). Whole cell lysates were prepared and examined by Western blot analysis. The blots shown are representative of three independent experiments.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Insulin (nM)
0 1 10 100
P-Akt
Akt
B
glucose - + + + +
IBMX - - - + +
HNMPA - - + - +
P-Akt
Akt
P-JNK
JNK

Figure 4.4. Inhibition of IR tyrosine kinase activity with HNMPA-$(AM)_3$ induced islet cell apoptosis. (**A**) Immediately following isolation, islets were cultured with increasing concentrations of HNMPA-$(AM)_3$. Following 24 hours in culture, islet viability was assessed by MTT assay. Bars represent the mean MTT reduction expressed as a percentage of the control ± SEM for quadruplicate samples from three independent experiments. (**B**) Immediately following isolation, islets were treated with 50 μM HNMPA-$(AM)_3$ and membrane permeability was assessed following 24 hours in culture using PI/FDA staining. The number of PI-positive cells was expressed as a percentage of the total cells counted. Bars represent the mean values ± SEM for three independent experiments. (**C**) Immediately following isolation, islets were cultured with increasing concentrations of HNMPA-$(AM)_3$. Following 24 hours in culture, whole cell lysates were prepared and AKT phosphorylation was examined by Western blot analysis. The blot is representative of three independent experiments. *, $P<0.05$ compared with untreated controls (Ctrl).

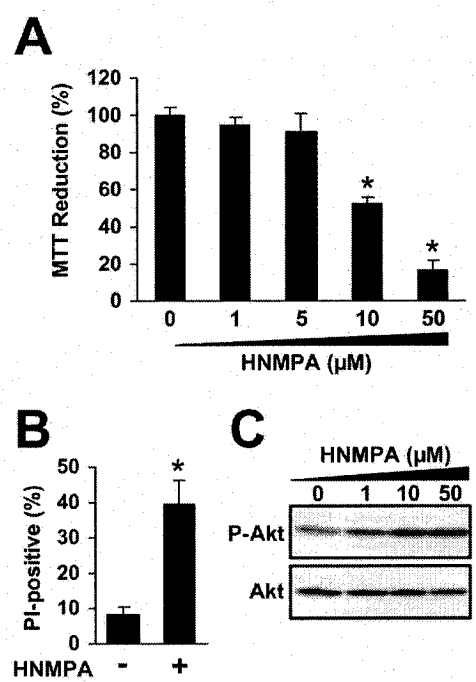
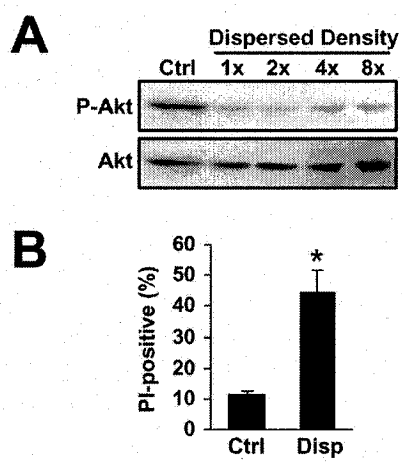

A
MTT Reduction (%)
120
100
80
60
40
20
0
*
*
0
1
5
10
50
HNMPA (μM)
B
PI-positive (%)
50
40
30
20
10
0
*
HNMPA
-
+
C
HNMPA (μM)
0
1
10
50
P-Akt
Akt

Figure 4.5. Dispersion of islets into single cells suppresses AKT phosphorylation and induced islet cell death. Immediately following isolation, islets were dispersed into single cells (see materials and methods). Dispersed cells were cultured at the indicated density, where the lowest density (1X) corresponds to the same number of cells/ml as the undispersed control islets (Ctrl). (**A**) Whole cell lysates were prepared after 12 hours in culture and examined by Western blot analysis. The blots are representative of three independent experiments. (**B**) Membrane permeability was assessed following 24 hours in culture using PI/FDA staining. The number of PI-positive cells was expressed as a percentage of the total cells counted. Bars represent the mean values ± SEM for three independent experiments. *, $P<0.05$ compared with untreated controls (Ctrl).


A
Dispersed Density
Ctrl
1x
2x
4x
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P-Akt
Akt
B
PI-positive (%)
60
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40
30
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10
0
*
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Disp

4.5. DISCUSSION

The current findings provide new information on how culturing islets prior to transplantation may improve graft survival. Indeed, treatments which activate AKT during islet culture can improve graft survival (151), indicating that elevated AKT activity could render islets less susceptible to injury during the immediate post-transplantation period. Therefore, it is plausible that culturing islets prior to transplantation allows for secreted insulin to activate AKT in an autocrine fashion, yielding islets which are more likely to survive the insults encountered immediately following transplantation.

We have previously demonstrated that the rise in AKT phosphorylation in cultured islets is dependent on PI3K activity and that inhibition of PI3K induced islet cell death (270;297). Here we demonstrate that treatment with an anti-insulin antibody decreased AKT phosphorylation and induced cell death, indicating that insulin secreted by the islets is acting in an autocrine manner to mediate islet survival. A similar finding was observed in dispersed rat islets, where treatment with anti-insulin antibody increased the amount of TUNEL-positive cells (308). Furthermore, supplementing culture medium with insulin leads to improved survival of isolated canine islets (113), and dispersed rat islets (308). Taken together, these findings indicate that insulin improves the survival of isolated islets and support the supplementation of islet culture medium with insulin immediately following isolation. Moreover, is could be reasoned that the negative effects of calcineurin inhibitor-based immunosuppressants on islet survival is due

to the fact that these compounds suppress insulin secretion (309;310), thus decreasing autocrine insulin-mediated islet survival.

It is unclear why cell death is still occurring despite high levels of AKT activation following overnight culture of isolated islets (61;151;297). This could be due to the energy requirements of AKT-mediated survival (175). Indeed, AKT-mediated survival in the absence of growth factors is dependent on the presence of glucose (311). Furthermore, AKT cannot prevent apoptosis under hypoxic conditions (182). Islets are highly vascularized structures whose function depends greatly on proper blood supply (188). Following isolation islets are avascular, and revascularization is not complete until 10-14 days following transplantation (62). During that time oxygen delivery to the islets is limited to diffusion. Islets are exposed to varying degrees of ischemia throughout the isolation procedure, and culture techniques that are suitable for dispersed cell cultures may not be adequate for proper oxygen delivery throughout the entire islet. Therefore, efforts to improve oxygen delivery during culture should further improve islet survival.

We did not observe any increase in AKT phosphorylation following treatment with high glucose. Similarly to our findings, treatment of isolated rat islets with high glucose had no effect on AKT phosphorylation, but 100 nM insulin did (305;306). In addition, no effect of glucose on AKT was observed in INS-1 cells treated for under 1 hour (152;306;312;313). However, high glucose has been shown to activate AKT after 1 hour in INS-1 cells (312;314), and after 2 hours in MIN6 cells and mouse islets (154), which does not coincide with the

kinetics of glucose-stimulated insulin release. Taken together, these data suggest that the ability of glucose to activate AKT in β-cells after at least 1 hour may be independent of glucose-stimulated insulin secretion (305). It is also possible that during glucose-stimulated insulin release, the insulin signalling pathway is suppressed in β-cells for a short period of time to avoid over stimulation of insulin-mediated signalling.

The current results demonstrate that the IR kinase inhibitor HNMPA-$(AM)_3$ increases the phosphorylation of AKT, despite inducing high levels of cell death. It is unclear why HNMPA-$(AM)_3$ induced AKT phosphorylation in our model. Other studies have shown that HNMPA-$(AM)_3$ decreased AKT phosphorylation after 8 hours, but not 3 hours, in detached human umbilical vein endothelial cells (315). This correlated with accelerated apoptosis in detached cells, though the effect was much less apparent in adherent cultures. In addition, HNMPA-$(AM)_3$ partially blocked PI3K activation by 17β-estradiol in retinal neuronal cells (316). HNMPA-$(AM)_3$ has also been shown to block insulin-stimulated glucokinase and insulin promoter activity in the HIT-T15 β-cell line (303). In the current study, we also demonstrate that HNMPA-$(AM)_3$ leads to rapid JNK phosphorylation, a kinase known to mediate islet cell death (273;297). Similar findings have been observed in an embryonic mosquito cell line, where JNK phosphorylation was greatly increased by HNMPA-$(AM)_3$ treatment for 1 hour (317). Non-specific effects of HNMPA-$(AM)_3$ could also be responsible for the observed rise in AKT and JNK phosphorylation, since HNMPA-$(AM)_3$ can also inhibit other kinases

(307). Therefore, caution should be used when interpreting results using HNMPA-$(AM)_3$.

Our findings demonstrate that dispersion of human islets into single cells suppresses AKT phosphorylation and induced islet cell death. Previous studies have also demonstrated high levels of cell death in dispersed islet cultures (170;308). One explanation for the increase in cell death upon dispersion of islets into single cells is the loss of contact with the ECM, which is mediated by the integrin receptor family. Integrin ligation is known to mediate survival through activation of AKT (318), and the loss of ECM contact upon islet dispersion could explain the observed decrease in AKT (Figure 6). Concordantly, culturing dispersed islets in the presence of ECM proteins decreases apoptosis (130). It is also possible that dispersed islets secrete less insulin, since aggregates of β-cells have been shown to secrete more insulin than free β-cells (319). In addition, the local concentration of insulin within the islet could be higher than in the surrounding medium. However, if the decrease in AKT phosphorylation caused by dispersion was due to reduced local concentration of insulin, then increasing the insulin concentration by increasing the cell density would restore AKT phosphorylation, which it did not (Figure 6). It is also possible that synergism between growth factor and integrin signalling could be affected in dispersed islets (320). Overall, the specific reasons for the poor survival of dispersed islets remain unclear, but are likely due to the disruption of the islet microenvironment.

It remains to be determined what receptor is responsible for the observed autocrine effect of insulin, since at high concentrations insulin can also activate

IGFR-1 (321). In addition, it is possible that insulin signalling is partially suppressed immediately following isolation, since 100 nM insulin only induced a mild increase in AKT phosphorylation. A combination of JNK inhibition and insulin treatment immediately following islet isolation may further increase AKT activation and survival. In summary, the present work demonstrates that insulin acts in an autocrine manner to activate AKT and mediate the survival of isolated human islets.

4.6. ACKNOWLEDGMENTS

This work was supported by the Juvenile Diabetes Research Foundation, the Canadian Institutes for Health Research (CIHR), and the Canadian Diabetes Association (CDA). Organs were obtained in collaboration with Quebec-Transplant. L. Rosenberg is a National Scientist supported by the Fonds de la Recherches en Santé du Quebec. S. Hanley is supported by a fellowship from the CDA and the CIHR. M. Lipsett is supported by a fellowship from the Diabetic Children's Foundation and the CIHR. The authors would also like to thank J. Ding for technical assistance and J. Tam for critically reviewing the manuscript.

CONNECTING TEXT

Our previous results demonstrated that apoptosis was occurring in isolated islets, however, the relative contributions of apoptosis and necrosis to total cell death remained unknown. Furthermore, the rise in phosphorylation of the antiapoptotic AKT protein, combined with a decrease in DNA fragmentation over the first 7 days following isolation (113), suggested that perhaps death mechanisms other than apoptosis were at play. The aim of Chapter 5 was to examine the effects of caspase and PARP-1 inhibition on islet survival.

CHAPTER 5

Inhibition of caspase-mediated PARP-1 cleavage results in increased necrosis in isolated islets of Langerhans

Reid Aikin, Lawrence Rosenberg, Steven Paraskevas, and Dusica Maysinger

5.1. ABSTRACT

The current procedure for isolation of islet cells from the pancreas for transplantation by enzymatic digestion is accompanied by significant islet cell loss. Therapeutic strategies aimed at the inhibition of islet cell damage could be expected to increase islet yield and improve cell viability, thereby making more efficient use of available donor tissue. The aim of the present work was to examine the effects of caspase and PARP-1 inhibition on islet survival. We demonstrate that following isolation, islets become increasingly necrotic and display a PARP-1 cleavage pattern typical of necrotic cells, characterized by the appearance of a 50 kDa cleavage product. Caspase inhibition using Z-VAD-fmk resulted in increased necrosis in both human and canine islets by a nicotinamide-sensitive mechanism. Necrosis was also induced by DEVD-fmk, but not by YVAD-cmk, indicating that only inhibitors of caspase-3 were able to cause necrosis. Moreover, increased mitochondrial depolarization was observed in islets following 72 hours in culture, which correlated with increased expression of Bax. Mitochondrial depolarization was also visible in islets treated with both Z-VAD-fmk and nicotinamide, indicating that mitochondrial dysfunction may account for the necrotic-like death observed in the absence of PARP-1 and caspase activity. Our results demonstrate that inhibition of PARP-1 cleavage results in increased levels of PARP-1-mediated necrotic cell death, highlighting the importance of PARP-1 cleavage in assuring the execution of the apoptotic program. Taken together, these findings reveal the interdependence of necrosis and apoptosis in isolated islets, suggesting therapeutic strategies which target early events in cell death signalling in order to prevent multiple forms of islet cell death.

5.2. INTRODUCTION

Islet cell transplantation has been shown to be an effective therapy for the treatment of type 1 diabetes (53). Nonetheless, many islet transplant recipients demonstrate abnormal glucose homeostatic profiles, and several have been placed back on exogenous insulin periodically as required. In addition, the current protocol for islet isolation is still extremely inefficient, with average yields representing less than 50% of the original islet cell mass (322;323). Hence, multiple organ donors are often necessary to meet the requirements of a single patient (53;55). Additionally, the harshness of the isolation procedure exposes islets to significant stress, which could contribute to decreased viability following isolation (69;71;322). We have previously demonstrated that the commonly employed method of islet isolation leads to the induction of islet cell apoptosis (61;169). However, the degree to which primary non-function of islet grafts is due to islet cell death triggered by the isolation procedure remains unclear. It is plausible that the current number of islets required to achieve normoglycemia includes a surplus of cells which die as a result of the stress of being isolated. Thus, therapeutic strategies aimed at the inhibition of islet cell death could be expected to increase islet yield and improve cell viability, thereby making more efficient use of the available donor tissue.

In general, eukaryotic cells are often thought to die in two distinct ways: apoptosis or necrosis. Apoptosis is a process by which individual cells die after activating their own genetically programmed cell death mechanisms. There are many external cues that can trigger a cell to undergo apoptosis. These external signals lead to activation of various cellular signalling pathways that are

responsible for mobilizing the apoptotic machinery, which includes the caspase family of cysteine proteases (reviewed in (73)). Necrosis, on the other hand, is much less controlled than apoptosis. Cells dying by necrosis typically swell and rupture leading to a local inflammatory response. Necrosis often involves overactivation of PARP-1, leading to depletion of NAD^+, impaired ATP production, and finally cell death (86). Many insults, however, can lead to both necrosis and apoptosis. Moreover, in many circumstances, not all dying cells display the typical hallmarks of apoptosis or necrosis (88). This has lead to the notion that perhaps apoptosis and necrosis represent two opposing ends of a complete range of intermediate forms of death (89).

The aim of the current work was to examine the effects of caspase inhibition and PARP-1 inhibition on islet survival. However, in doing so, we uncovered the interdependence of apoptosis and necrosis in isolated islets. Our results demonstrate that inhibition of caspase-mediated PARP-1 cleavage results in increased necrosis in isolated islets of Langerhans.

5.3. MATERIALS AND METHODS

Human Islet Isolation. Pancreata (n=4) were retrieved from heart-beating cadaveric donors at the time of multi-organ harvest for transplantation. Consent for donation of tissues for research was obtained by the local organ procurement organization. Donors were between the ages of 45 and 60 years old with no history of diabetes or metabolic disorders. Warm ischemia time was approximately 5 minutes and cold ischemia time, with University of Wisconsin solution perfusion, was between 30 minutes and 6 hours. Islets were isolated using the method of Ricordi, et al. (155). Briefly, the main pancreatic duct was canulated and a cold (6-8°C) solution of 2 mg/ml Liberase enzyme blend (Roche, Montreal, QC, Canada) in perfusion solution (Mediatech, Herndon, VA, USA) supplemented with 0.2 mg/ml DNAse I (Roche) was infused, through the canula, into the pancreas using a syringe. The distended pancreas was placed in a sterilized aluminum digestion chamber (Bio-Rep, Miami, FL, USA) through which the perfusion solution was recirculated at 37°C. Extent of tissue digestion was assessed by staining aliquots of the digestate with dithizone (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) and visualizing the islets under an inverted light microscope (Nikon, Montreal, QC, Canada). The digestion process was terminated by cooling the circuit to between 5°C and 10°C when the majority of the islets were seen to be free of surrounding acinar tissue. The digestate was collected, centrifuged, and washed three times with wash solution (Mediatech) and islets were purified on a continuous density gradient (Biocoll separating solution; Biochrom AG, Berlin, Germany) using a COBE 2991 Cell Processor

(COBE BCT, Denver, CO, USA). Islet purity was assessed by dithizone staining (Sigma), and glucose-stimulated insulin secretion (69) was routinely assessed to ensure islet functionality.

Canine Islet Isolation. All procedures were in compliance with national animal care standards and were approved by institutional animal care committees. Pancreata (n=7) were canulated and harvested from 2-4 year old mongrel dogs under general anesthesia and sterile conditions. Pancreata were placed in ice-cold saline prior to dissection of fatty and connective tissue. Islet isolation and purification were performed in an identical manner as for human islets, but Liberase CI enzyme blend (Roche) was used instead of Liberase enzyme blend.

Islet cell culture. Isolated islets (>90% purity) were cultured in CMRL 1066 (Gibco, Burlington, ON, Canada) with 1000 IEQ/mL of medium, at 37°C in a humidified atmosphere of 5% CO_2. When required, medium was changed every other day. The general caspase inhibitor Z-VAD-fmk, the caspase-3 like inhibitor Ac-DEVD-fmk, and the caspase-1 inhibitor Ac-YVAD-cmk (all from Calbiochem) were added following isolation to the culture media at a final concentration of 50 μM. Nicotinamide (Sigma) was used at a final concentration of 10 mM. For acute cytokine stimulation experiments, islets were recovered overnight in medium supplemented with 10% fetal bovine serum (FBS) (Wisent, St. Bruno, QC, Canada) and treated with 100 ng/mL TNF-α, 100 U/mL IL-1β, and 100 ng/mL INF-γ (R&D Systems Inc., Minneapolis, MN, USA). For all compounds prepared

in DMSO, the final concentration of DMSO in the culture media was kept below 0.1%. Vehicle controls were prepared for all treatments.

Cell Death ELISA. The assay was performed according to the manufacturer's instructions using a commercially available kit (Roche). The ELISA, based on a sandwich-enzyme immunoassay principle, detects histone-associated DNA fragments in the cell cytoplasm that are characteristic of apoptotic cells. Lysates were prepared in lysis buffer (50 mM Tris-HCl, pH 8.0, 1.37 mM NaCl, 1% (v/v) nonident P-40, 10% (v/v) glycerol, 0.1 mM sodium orthovanadate, complete protease inhibitor cocktail tablet (Roche)). Variations in sample size were corrected by measuring total sample DNA content and expressing the results as (absorbance at 405 nm)/mg DNA.

Fluorescent Microscopy. Islets were dispersed by first washing with dispersion solution (Gibco) followed by a 10 minute incubation at 37°C with trypsin/EDTA (Gibco). Trypsinized islets were washed with cold CMRL 1066 with 10% FBS, pipetted gently, and resuspended in PBS. Dispersed islets were resuspended in PBS containing 2 μM propidium iodide (PI) (Molecular Probes Inc., Eugene, OR, USA) and 0.67 μM fluorescein diacetate (FDA) (Molecular Probes). The percentage of necrotic cells was determined by dividing the number of PI positive cells which did not display nuclear condensation or fragmentation by the total number of cells counted. Dispersed islets were also stained with 2 μM PI and 25 μM Newport green (NG) (Molecular Probes), a zinc chelator which can identify β-

cells (324). Annexin-V-Flous (Roche) was used according to the manufacturer's directions, in combination with PI, in order to discern apoptotic (green) from necrotic (green with red nuclei) cells. Annexin-V positive cells which displayed PI positive fragmented nuclei were also considered to be apoptotic. JC1 (Molecular Probes) was used according to manufacturers directions to identify cells with depolarized mitochondrial membranes. For all dyes, the stained islets were placed on glass slides with cover-slips and incubated for 15 minutes at room temperature in a foil-covered container with a moist paper-towel. Slides were visualized under a fluorescent Olympus BX60 microscope connected via a digital video camera to a PC. Five hundred cells were counted per slide, with three slides per group for at least three independent experiments. Images were analyzed using Image Pro Plus 4.0 software (Media Cybernetics Inc., Silver Spring, MD, USA).

Caspase-3 Assay. The islet samples (2,000 IEQ) were spun down for 2 minutes at 900 rpm and 4°C. The pellet was washed twice with ice cold PBS and then dissolved in lysis buffer (50 mM Tris-HCl, pH 8.0, 1.37 mM NaCl, 1% (v/v) nonident P-40, 10% (v/v) glycerol). The samples were sonicated and spun down for 20 minutes at 14,000 rpm, 4°C, and the supernatant was kept for analysis. The protein content was determined using BIO-RAD Protein Assay Dye Reagent (BIO-RAD). In a 96-well plate, lysate containing 100 μg of protein was added to caspase buffer (50 mM HEPES, 1 mM EDTA, 0.1% CHAPS, 10% Sucrose, 10 mM dithiothreitol, 100 mM NaCl, pH 7.2) containing 200 μM Ac-DEVD-*p*NA.

Samples were incubated at 37°C for 90 minutes and the release of *p*NA was analyzed by measuring the absorbance at 405 nm using a Benchmark Microplate Reader (BIO-RAD).

MTT Assay. Aliquots containing 500 IEQ in 500 µl of media were placed in sterile eppendorf tubes and 50 µL of stock MTT (5mg/mL) (Sigma) was added to each sample. The samples were incubated at 37°C for 2 hours, washed twice with cold PBS, and lysed with 200 µL of DMSO (Sigma). Two 100 µL aliquots from each sample were loaded onto a 96-well plate and the absorbance was measured at 595 nm using a Benchmark Microplate Reader (BIO-RAD). Four independent samples were analyzed per experiment and each experiment was performed at least three times.

Western Blotting. The islet samples (2,500 IEQ) were spun down for 2 minutes at 900 rpm and 4°C. The pellet was washed twice with ice cold PBS and then dissolved in lysis buffer (50 mM Tris-HCl, pH8.0, 1.37 mM NaCl, 1% (v/v) nonident P-40, 10% (v/v) glycerol, 0.1 mM sodium orthovanadate, Complete protease inhibitor cocktail tablet (Roche)). The samples were sonicated and spun down for 20 minutes at 14,000 rpm, 4°C, and the supernatant was kept for analysis. The protein content was determined using BIO-RAD Protein Assay Dye Reagent (BIO-RAD). Lysates were diluted 6:1 with 6X Laemmli sample buffer (0.375 M Tris-HCl pH 6.8, 12% w/v SDS, 3% v/v glycerol, 0.2% w/v bromophenol blue, 12% β-mercaptoethanol in double distilled water) and boiled for 5 minutes.

An equal amount of protein (75 μg) was loaded for each sample into a 12% polyacrylamide gel run at 100 V for 90 minutes. Transfer onto nitrocellulose was conducted at 250 mA for 90 minutes. Membranes were blocked with 2% bovine albumin in washing buffer (25 mM Tris, 150 mM NaCl, 0.05% Tween 20 in double distilled water). Blocked membranes were then probed with primary antibodies. Anti-PARP-1 (Santa Cruz Biotechnology, CA, USA) was used at 1:1000, anti-active caspase-3 (Cell Signalling Tech., Beverly, MA, USA) at 1:1000, and anti-Bax (Santa Cruz) was used at 1:800. Following primary antibody incubation, blots were washed for 1 hour in washing buffer then incubated for 1 hour in anti-rabbit horseradish peroxidase-linked antibody (1:4000) (Amersham LifeSciences Inc., Buckinghamshire, England). Following another 1 hour washing, the blots were developed using the ECL chemiluminescence system (Amersham) and Kodak X-OMAT film (Kodak, Rochester, NY, U.S.A.). Membranes were stripped by incubating at 65°C for half an hour in stripping buffer (100 mM β-mercaptoethanol, 2% w/v SDS and 62.5 mM Tris-HCl pH 6.7) and reprobed with primary antibody.

Statistical Analysis. All results are expressed as mean ± SEM. All statistics were performed using SYSTAT 9 (SPSS Inc., Chicago, IL, USA). Statistical significance was determined using the Student's t-test and, where applicable, a one-way *ANOVA* with a post-hoc Tukey's test. Differences were considered significant where $P < 0.05$.

5.4. RESULTS

The mode of cell death in isolated canine islets changes from predominantly apoptosis to necrosis. Using Annexin-V and propidium iodide (PI) staining, both apoptotic (green) and necrotic (green with red nuclei) cells could be identified (Figure 5.1A, B). At 12 hours following isolation, cell death was predominantly apoptotic (65.2 ± 7.8% of dead cells), while after 72 hours in culture, islet cell death became increasingly necrotic (64.4 ± 3.8% of total dead cells). Dual staining with Newport green (NG) and PI was used to confirm that cell death was occurring in β-cells (Figure 5.1A, bottom two panels). The amount of apoptosis occurring was also quantified by cell death ELISA (Figure 5.1C). The peak of DNA fragmentation occurred on the day after isolation and declined significantly over the ensuing 6 days.

Isolated canine islets display a PARP-1 cleavage pattern characteristic of necrotic cells. It has previously been demonstrated that cells undergoing necrotic cell death can exhibit a pattern of PARP-1 cleavage which differs from the typical apoptotic fragments of 89 kDa and 24 kDa (325;326). These necrotic cells can display a major fragment at 50 kDa. We therefore examined the PARP-1 cleavage pattern in isolated canine islets in order to see which cleavage products were present. After 24 hours of culture, these islets display a major PARP-1 fragment at 50 kDa as well as minor fragments at 89 kDa and 65 kDa (Figure 5.2). PARP-1 degradation to the 50 kDa fragment increased with time in culture, consistent with the increase in necrosis shown in Figure 5.1. In addition, the 89 kDa PARP-1 fragment is only seen 24 hours after

isolation (Figure 5.2), at a time when nucleosomal fragmentation is at its peak (Figure 5.1C). A combination of cytokines (TNF-α, INF-γ, and IL-1β), which has previously been shown to induce both apoptosis and necrosis in islets (256;327), led to increased levels of the 89 kDa, 65 kDa, and 50 kDa PARP-1 fragments (Figure 5.2).

Nicotinamide decreases necrosis but increases caspase-3 activation and apoptotic PARP-1 cleavage. Since nicotinamide has previously been shown to decrease necrotic cell death through its inhibitory effect on PARP-1 (86), we investigated its potential to increase islet survival. The addition of nicotinamide alone to the islet culture media caused a mild, but significant, decrease in necrosis (Figure 5.3A). This decrease in necrosis, however, did not translate into a statistically significant increase in islet viability as measured by the MTT assay (Figure 5.3B). Interestingly, nicotinamide treated islets displayed an increase in the 89 kDa PARP-1 fragment as well as an increase in active caspase-3 (Figure 5.4). Therefore, nicotinamide appears to inhibit necrosis while at the same time increase apoptosis.

General caspase inhibition leads to increased necrotic cell death by a nicotinamide-sensitive mechanism in human and canine islets. We have previously shown that apoptosis occurs in isolated human and canine islets and therefore hypothesized that caspase inhibition would be a viable approach to increasing islet cell survival following isolation. Surprisingly, use of the general caspase inhibitor Z-VAD-fmk was associated with an 18.4% decrease in canine islet cell viability and a 16.8% decrease in human islet cell viability after 72 hours

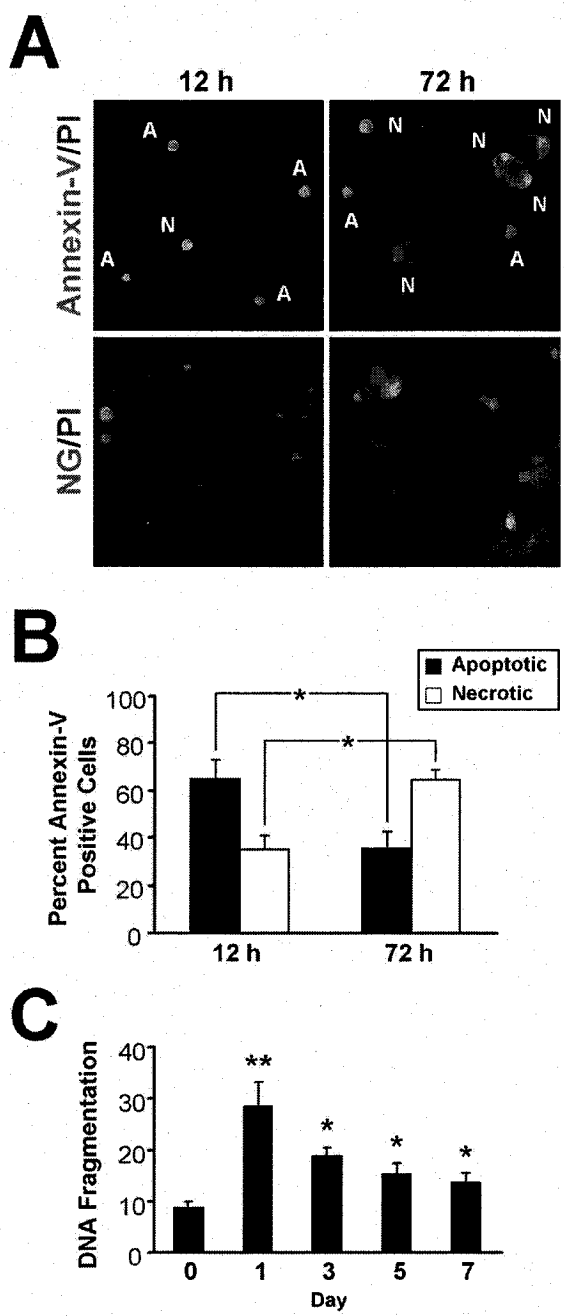
in culture (Figure 5.3B). Using PI/FDA fluorescent staining, we demonstrate a two-fold increase in necrotic cell death in canine islets treated with Z-VAD-fmk (Figure 5.3A). However, co-treatment with nicotinamide abolished the harmful effects of Z-VAD-fmk treatment in both canine and human islets, indicating that the increase in necrosis caused by caspase inhibition was PARP-1-mediated. Consistent with this increase in necrosis, treatment with Z-VAD-fmk led to an increase in necrotic PARP-1 cleavage, an effect which was also abolished by the addition of nicotinamide (Figure 5.4).

Only caspase inhibitors which block caspase-3 activity induce necrosis in isolated islets. In order to demonstrate that Z-VAD-fmk was promoting necrosis by inhibiting caspase-mediated PARP-1 cleavage, we employed the caspase-3-like inhibitor DEVD-fmk and the caspase-1 specific inhibitor YVAD-cmk. As expected, DEVD-fmk treatment also led to the induction of islet cell necrosis (Figure 5.5A). On the other hand, treatment with YVAD-cmk, which does not inhibit caspase-3 activity (Figure 5.5B), had no significant effect on islet necrosis (Figure 5.5A). The caspase substrate sequence used to assay caspase-3 activation corresponds to the amino acid sequence of the caspase-3 cleavage site in PARP-1. Therefore, inhibition of caspase activity towards the PARP-1 cleavage sequence resulted in increased necrosis.

Isolated islets express increasing levels of Bax following isolation, which correlates with increased mitochondrial membrane depolarization. The fact that necrotic cells were still evident in the presence of nicotinamide alone, or in combination with Z-VAD-fmk, indicated that other cell death

mechanisms were at play in islets following isolation. Overexpression of Bax has previously been shown to induce cell death, even in the presence of caspase inhibitors (328). In fact, even in the absence of caspase inhibition, disruption of the mitochondrial membrane by Bax caused cell death which can display both necrotic and apoptotic features. In addition, human islets have been shown to contain high levels of Bax mRNA and Bax protein (329). We therefore examined the expression of Bax in isolated islets following routine isolation. Indeed, Bax levels increased significantly over the first 72 hours in culture following isolation (Figure 5.6A), consistent with results from isolated human islets (329). This increase in Bax expression correlated with an increase in mitochondrial membrane depolarization (Figure 5.6B,C). Mitochondrial depolarization was also visible in islets treated with both Z-VAD-fmk and nicotinamide (Figure 5.6D, arrows), indicating that mitochondrial dysfunction may account for the cell death observed in the absence of PARP-1 and caspase activity.

Figure 5.1. The mode of cell death in isolated canine islets changes from predominantly apoptosis to necrosis. (**A**) Isolated canine islets were dispersed and stained with both Annexin-V and PI at the indicated time points following isolation (top two panels). Apoptotic cells are labeled "A" and necrotic cells are labeled "N". Newport green, a zinc chelating dye, and PI were used to confirm the presence of dying β-cells (bottom two panels). (**B**) Apoptotic and necrotic cells identified by Annexin-V/PI staining were counted and these values were expressed as a percentage of the total dead cells counted ± SEM for four independent isolations. (**C**) Cultured canine islets were lysed at the indicated times following isolation and apoptotic death was quantified using a cell death ELISA measuring oligonucleosomal fragmentation. The bars represent the mean values ± SEM for triplicate samples from four independent experiments in which the absorbance values (abs 405 nm) were standardized for DNA content. * $P<0.05$, ** $P<0.01$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12 h
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C
DNA Fragmentation
40
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20
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1
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Day

Figure 5.2. Isolated canine islets display a PARP-1 cleavage pattern characteristic of necrotic cells. Whole cell lysates were taken at the indicated times following isolation. A positive control group (Cyt) was treated for 24 hours with mixture of cytokines (TNF-α, 100 ng/mL; INF-γ 100 ng/mL; IL-1β, 100 U/mL). PARP-1 cleavage was analyzed by Western blotting. The blot is representative of four independent experiments.

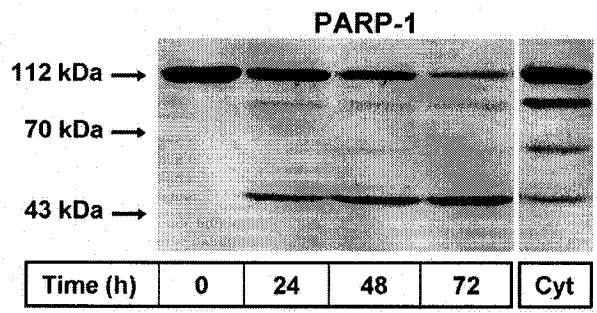

PARP-1
112 kDa
70 kDa
43 kDa
Time (h)
0
24
48
72
Cyt

Figure 5.3. Z-VAD-fmk causes increased cell death by a nicotinamide sensitive mechanism. Following isolation, human or canine islets were treated with either Z-VAD-fmk (50 μM) and/or nicotinamide (10 mM) for 72 hours. (**A**) Necrosis was assessed by propidium iodide (PI) and fluorescein diacetate (FDA) staining of dispersed canine islets. PI positive cells were expressed as a percentage of the total cells counted. Bars represent mean values ± SEM for three independent experiments. (**B**) Islet viability was assessed by MTT assay. Canine islets are represented by black bars and human islets by white bars. Bars represent mean absorbance values ± SEM for quadruplicate samples from four independent experiments. * $P<0.05$

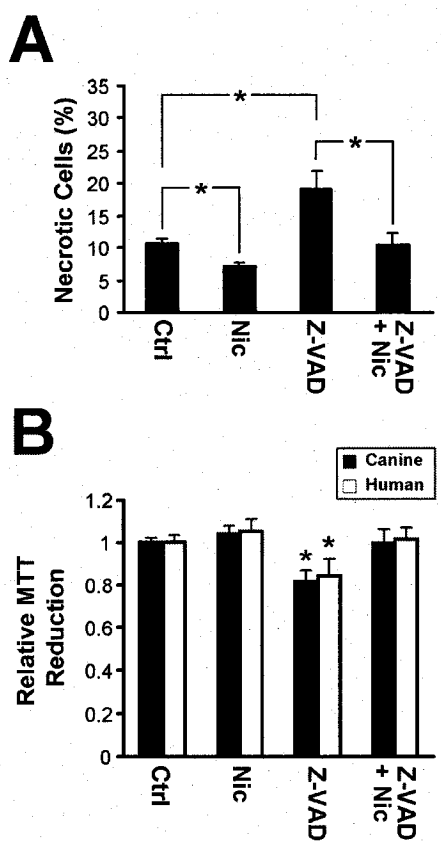
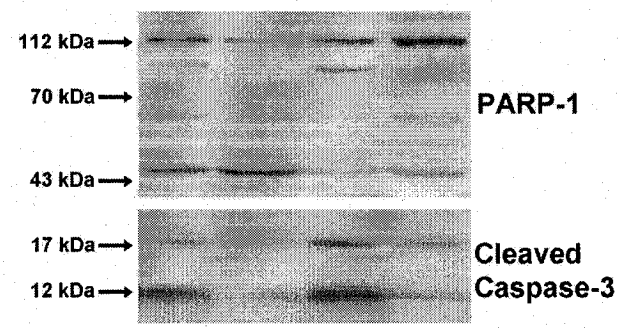

A
Necrotic Cells (%)
35
30
25
20
15
10
5
0
*
*
*
Ctrl
Nic
Z-VAD
Z-VAD + Nic
B
Canine
Human
Relative MTT Reduction
1.2
1
0.8
0.6
0.4
0.2
0
*
*
Ctrl
Nic
Z-VAD
Z-VAD + Nic

Figure 5.4. Effects of nicotinamide and Z-VAD-fmk on PARP-1 and caspase-3 processing in canine islets. Following isolation, canine islets were treated with either Z-VAD-fmk (50 μM) and/or nicotinamide (10 mM) for 72 hours. PARP-1 cleavage and caspase-3 activation were analyzed by Western blotting. The blot shown is representative of three independent experiments.



Z-VAD	-	+	-	+
Nic	-	-	+	+

Figure 5.5. Caspase-3 inhibitors induce necrosis in isolated canine islets. Following isolation, canine islets were treated with Z-VAD-fmk (50 μM), DEVD-fmk (50 μM), or YVAD-cmk (50 μM) for 72 hours. (**A**) Necrosis was assessed by PI/FDA staining of dispersed islets. The number of PI positive cells was expressed as a percentage of the total cells counted. Bars represent mean values ± SEM for three independent experiments. (**B**) Caspase-3 activity in islet cell lysates was assessed by measuring the cleavage of DEVD-*p*NA. The amount of *p*NA released was measured by analyzing the absorption at 405 nm. For each graph, bars represent the mean values ± SEM for three independent experiments. * $P<0.05$, ** $P<0.01$

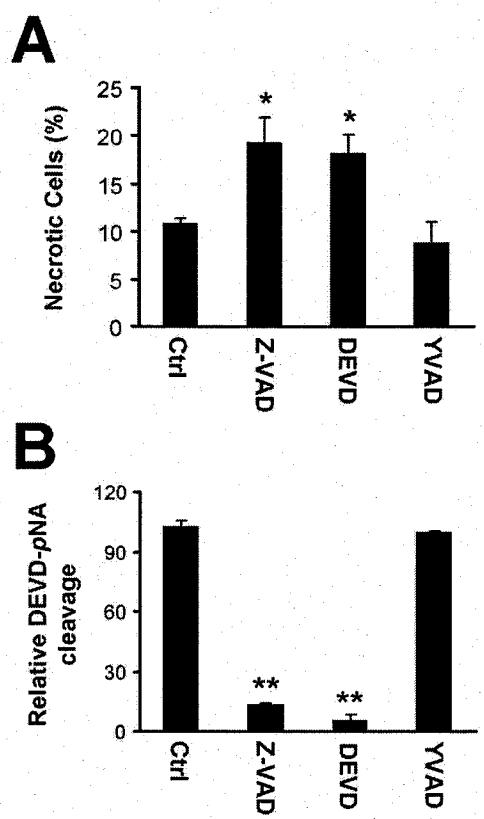

A
Necrotic Cells (%)
25
20
15
10
5
0
*
*
Ctrl
Z-VAD
DEVD
YVAD
B
Relative DEVD-pNA cleavage
120
90
60
30
0
**
**
Ctrl
Z-VAD
DEVD
YVAD

Figure 5.6. Canine islets express increasing levels of Bax over the first 72 hours following isolation. (**A**) Whole cell lysates were taken of canine islets at the indicated times following isolation and Bax expression was analyzed by Western blotting. The blot is representative of four independent experiments. Isolated canine islets were dispersed and stained with JC1 immediately following isolation (**B**) and following 72 hours in culture (**C**). (**D**) Isolated islets were also concomitantly treated with both Z-VAD-fmk (50 μM) and nicotinamide (10 mM) and stained with JC1.

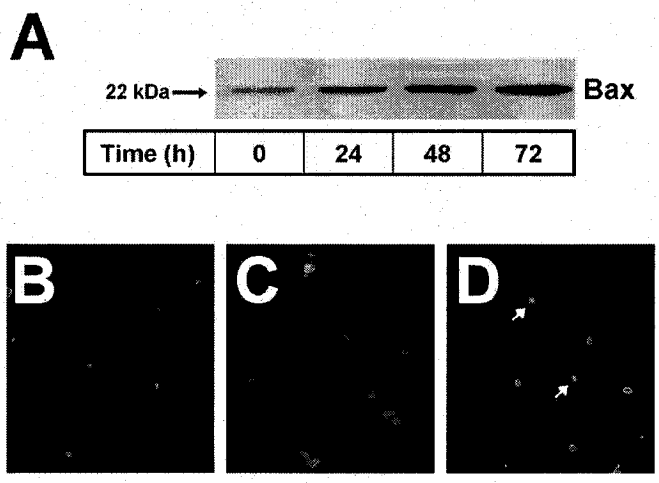
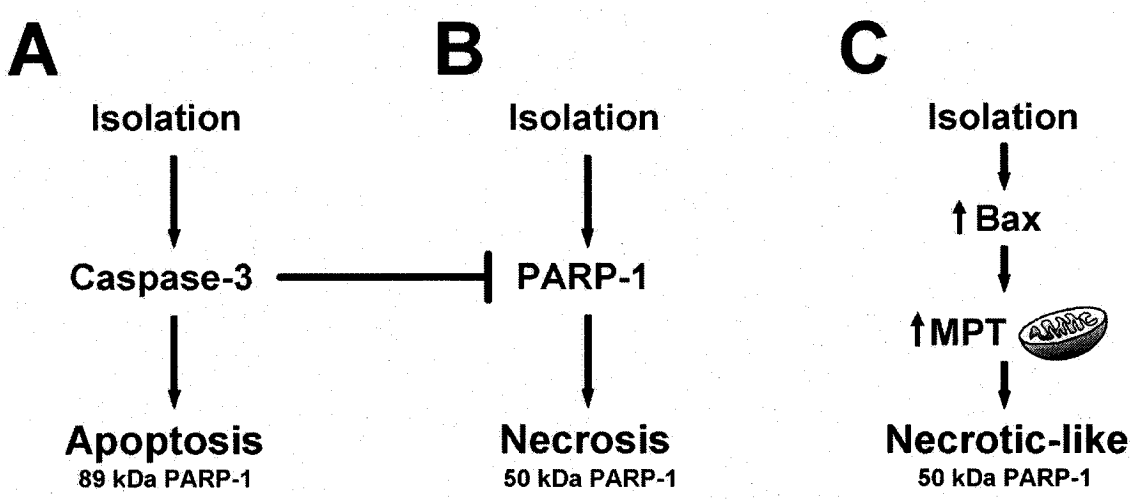

A
22 kDa
Bax
Time (h)
0
24
48
72
B
C
D

Figure 5.7. Proposed model of death pathways acting in isolated islets. (**A**) In the absence of caspase inhibition, caspase-3 is able to cleave and deactivate PARP-1, helping to assure an apoptotic fate. (**B**) In a damaged cell, PARP-1 overactivation can lead to necrosis. When caspase-mediated PARP-1 cleavage is blocked, PARP-1 is able to induce necrotic cell death in cells which may have otherwise undergone apoptosis. (**C**) Bax mediated disruption of the mitochondrial membrane could account for the islet cell death which occurs in the absence of caspase and PARP-1 activity.


A
Isolation
Caspase-3
Apoptosis
89 kDa PARP-1
B
Isolation
PARP-1
Necrosis
50 kDa PARP-1
C
Isolation
↑Bax
↑MPT
Necrotic-like
50 kDa PARP-1

5.5. DISCUSSION

In the present study we show that following isolation, islets appear to undergo progressive necrosis, displaying a PARP-1 cleavage pattern typical of necrotic cells, characterized by the appearance of a 50 kDa cleavage product. We have previously shown that AKT becomes highly activated within the first 24 hours following isolation of canine islets (270), a finding which was recently demonstrated in human islets as well (151). The anti-apoptotic role of AKT is well established (134;330). Though the underlying mechanism responsible for the activation of AKT in islets following isolation is not known, it could be responsible for the decrease in apoptosis which is evident following three days in culture. In addition, changes in ion concentrations, chronic decreased oxygen tension, and decreased ATP are all factors which could limit the availability of sufficient energy to insure the execution of the apoptotic program (189;216;217). Despite the possibility of late apoptotic cells appearing necrotic by Annexin-V/PI staining, the observed increase in necrotic PARP cleavage in combination with decreased oligonucleosomal fragmentation argues against such an interpretation. Additionally, the cells were cultured as intact islets, allowing for the potential clearing of apoptotic cells.

The DNA repair enzyme PARP catalyses the addition of long branched chains of poly-(ADP-ribose) to a variety of nuclear proteins using NAD^+ as a substrate (331). Under conditions which cause severe DNA damage, overactivation of PARP-1 leads to depletion of NAD^+, resulting in impaired ATP production and finally cell death (86). PARP-$1^{-/-}$ mice are resistant to

streptozotocin-induced diabetes, indicating β-cell protection from necrosis (332). Another PARP-1 inhibitor, PD128763, is effective at protecting islet cells from NO, oxygen radical generating compounds, and streptozotocin at concentrations 100 times less than those required for nicotinamide (333). It has been suggested, however, that the protection afforded by PARP-1 inhibitors against streptozotocin could be due to upregulation of the multidrug resistance transporter (MDR-1) that occurs in the absence of PARP-1 activity (334;335).

Cleavage of PARP-1 by caspase-3 to 89 kDa and 24 kDa fragments is a defining characteristic of classical apoptosis. One of the functions for cleavage of PARP-1 during apoptosis induced by insults such as alkylating agents is to prevent survival of the extensively damaged cells (336). PARP-1 cleavage also insures that energy stores are available for the execution of apoptosis (86). In the current study, we provide evidence that PARP-1 cleavage in isolated canine islets leads to a major fragment at 50 kDa, indicative of necrosis (325;326). Interestingly, the appearance of this 50 kDa fragment was increased by Z-VAD-fmk treatment, indicating that caspases are not responsible for this pattern of PARP-1 cleavage. A similar finding was reported previously, where the differential PARP-1 cleavage during necrosis was not blocked by Z-VAD-fmk, as opposed to apoptotic PARP-1 cleavage (337). The necrotic pattern of PARP-1 cleavage is likely due, at least in part, to lysosomal proteases which are released into the cytosol during necrosis (326). Granzyme B, a serine protease, has been demonstrated to cleave PARP-1 into a 64 kDa N-terminal fragment and a 54 kDa C-terminal fragment (338). The cysteine protease calpain can also cleave PARP-

1 to generate a 40 kDa fragment (339). However, since Z-VAD-fmk also inhibits calpain (340), other proteases are likely to be responsible for the 50 kDa fragment observed in isolated islets. We show that nicotinamide is able to prevent the appearance of the Z-VAD-fmk induced 50 kDa PARP-1 fragment, demonstrating that PARP-1 activation plays a role in determining its eventual fragmentation pattern.

Our data provide evidence that caspase inhibition leads to increased caspase-independent cell death. A similar effect was observed in cultured B lymphocytes, where caspase inhibition led to increased necrotic cell death (341). In L929 mouse fibroblasts, Z-VAD-fmk potentiated TNF-α-induced cell death (342). In this case, the increase in cell death was shown to be characterized by ATP depletion and was also suppressed by PARP-1 inhibition. Another study in L929 cells has shown that the caspase-3 inhibitor DEVD-fmk did not prevent the loss of viability in response to TNF-α treatment, but it did prevent caspase-3 activation, PARP-1 cleavage, and DNA fragmentation (343). Similarly, in NGF-deprived rat sympathetic neurons, the general caspase inhibitor boc-aspartyl(OMe)-fluoromethylketone (BAF) led to a delayed non-apoptotic cell death (344). Although Z-VAD-fmk could completely block DNA fragmentation and chromatin condensation, it was incapable of preventing delayed necrosis caused by the induction of mitochondrial permeability transition (345). Caspase inhibition was shown to block oligonucleosomal fragmentation in dissociated islet cell cultures, however neither necrosis nor overall viability were assessed (170). The inability of caspase inhibition to prevent islet cell death may be due to the fact

that the caspases lie downstream of any mitochondrial dysfunction, and thus their inhibition has no effect on mitochondria-mediated cell death (76). Indeed, we have demonstrated increasing levels of Bax in islets following isolation, suggestive of the involvement of the mitochondrial pathway (329). Overexpression of Bax alone has previously been shown to induce cell death, even in the presence of caspase inhibitors (328).

The current results also propose that by inhibiting mechanisms of necrotic cell death, it is possible to enhance islet cell apoptosis. It has been shown previously that PARP-1 inhibition prevented necrotic death while increasing caspase activity and DNA fragmentation in peroxynitrite treated macrophages (346). DNA methylation alone has been shown to induce necrosis; however, inhibition of PARP-1 in this particular model not only led to decreased necrosis, but to increased apoptosis as well (347). Similarly, fibroblasts from PARP-$1^{-/-}$ mice show apoptotic features when exposed to sulfur mustard, unlike the wild-type animal which displays necrotic cell death (348). PARP-1 inhibition in LLC-PK1 cells treated with H_2O_2 protected these cells from necrosis, but led to increased apoptosis (349). PARP-1 inhibitors attenuated necrotic but not apoptotic neuronal death in experimental models of cerebral ischemia (350). Nicotinamide also protected rat β-cells from necrosis induced by streptozotocin or H_2O_2, but this effect was associated with delayed apoptosis (351). The same study also demonstrated that nicotinamide can protect human β-cells against H_2O_2-induced necrosis without the occurrence of delayed apoptosis. Taken

together, these data indicate that PARP-1 inhibition may target cells for an apoptotic form of cell death, depending on the insult and cellular context.

In summary, it appears that apoptosis and necrosis are interdependent events which complement each other in order to assure the execution of a cell receiving a death signal. PARP-1 is a key mediator of apoptotic and necrotic cell death signals, a notion that is further supported by the recent discovery that PARP-1 mediates the release of apoptosis-inducing factor (AIF) from the mitochondria (352). Islet cell death following isolation is certainly not unprovoked since the isolation procedure is exceptionally harsh. However, unless a new method of islet isolation is developed, new strategies must be adopted to decrease islet cell death caused by the enzymatic digestion and purification steps which are central to the current protocol. In the present work, we demonstrate that inhibition of caspase-mediated PARP-1 cleavage in isolated islets can lead to increased necrosis, and that nicotinamide can increase caspase-3 activity under the same conditions. Based on these results, it appears that inhibiting caspase or PARP-1 activity could be too late as an intervention to improve islet survival. Indeed, islet isolation is a multifaceted insult which results in the activation of several cell death mechanisms (Figure 5.7). Therefore, therapies aimed at increasing islet survival following isolation should be designed to prevent multiple forms of death from occurring by targeting early events in cell death signalling.

5.6. ACKNOWLEDGMENTS

This work was supported by the Juvenile Diabetes Research Foundation, the Canadian Institutes for Health Research, and the Canadian Diabetes Association. L. Rosenberg is a National Scientist supported by the Fonds de la Recherches en Santé du Quebec. The authors would like to thank Despina Agapitos, Mark Lipsett, and Ali Hazrati for technical assistance with the islet isolation procedure.

CONNECTING TEXT

In Chapter 5, we demonstrated that caspase inhibition was unable to prevent islet cell death and suggested that the commitment to islet cell death occurs at, or upstream, of mitochondrial dysfunction. Indeed, in Chapter 3 we demonstrated that SP600125 treatment suppressed mitochondrial depolarization in isolated human islets and led to lasting protection from cell death. In the following chapter, we examined whether a novel Ku70-derived Bax-inhibitory peptide (V5) could protect human islets against cell death induced by isolation as well as by other known islet stressors.

CHAPTER 6

Bax inhibitory peptide protects human islets against insults which require Bax translocation to the mitochondria

Reid Aikin, Lawrence Rosenberg, and Dusica Maysinger

6.1. ABSTRACT

A major limitation to the success of islet cell transplantation as a therapy for type I diabetes is the cell loss induced by the islet isolation procedure. We have previously demonstrated a role for mitochondrial dysfunction in isolated islet cell death. The present study examined whether a novel Ku70-derived Bax-inhibitory peptide (V5) could protect human islets against cell death induced by isolation as well as by other known islet stressors. V5 efficiently penetrated to all cells within islets and allowed normal glucose-stimulated insulin release. Treatment of isolated islets with V5 reduced mitochondrial depolarization, Bax translocation, and cytochrome c release resulting in improved islet viability. V5 also suppressed cell death induced by a mixture of proinflammatory cytokines. However, V5 did not prevent cytochrome c release or cell death triggered by oxidative stress or thapsigargin, suggesting that these insults do not require Bax translocation to induce mitochondrial permeabilization. These findings demonstrate that V5 is selectively protective in islets against insults requiring Bax translocation to the mitochondria. In addition, V5 could be a beneficial constituent of islet culture media immediately following isolation, and perhaps during earlier steps of the islet isolation procedure.

6.2. INTRODUCTION

Programmed cell death can be triggered by an assortment of insults, which activate intracellular pathways that lead to the cell's demise. The ability of particular organelles to act as damage sensors ensures that cell death is triggered upon failure of key cellular functions (353). Members of the Bcl-2 family of proteins are critical regulators of cell death pathways and are found in the cytosol, the mitochondria, and the endoplasmic reticulum (ER) (78). The proapoptotic Bcl-2 proteins can be subdivided into "multidomain" and "BH3-only" proteins. Multidomain proapoptotic proteins, such as Bax and Bak, contain the conserved Bcl-2 homology domains (BH) 1-3. Upstream of the multidomain proteins are the BH3-only proapoptotic proteins, such as Bid and Bad, which have only the amphipathic α-helical BH3 domain. Antiapoptotic Bcl-2 family members contain all four BH domains (BH1-4) and are generally thought to prevent apoptosis by sequestering the proapoptotic Bcl-2 proteins, although the exact mechanisms remain elusive (79).

The proapoptotic multidomain proteins Bax and Bak play an essential role in mitochondrial dysfunction and cell death caused by a variety of stimuli (82). Bak is an integral membrane protein found mainly in the outer mitochondrial membrane, with a small fraction localized to the ER (83;84). In contrast, Bax normally resides in the cytosol of healthy cells, with a small percentage being localized to the ER, nuclear matrix, and the mitochondria (83;84). During apoptosis Bax changes conformation and translocates from the cytosol to the mitochondria and, along with Bak, mediates mitochondrial permeabilization and

the release of cytotoxic proteins such as cytochrome c (78). Though the precise mechanism of mitochondrial permeabilization by proapoptotic Bcl-2 proteins is unclear, this step represents a critical checkpoint for survival beyond which the cell is committed to death.

Recently, several proteins have been identified that can associate with Bax in the cytosol and inhibit its translocation to the mitochondria. Members of the 14-3-3 family can interact with Bax in healthy cells (354), and stress-induced phosphorylation of 14-3-3 proteins by JNK causes dissociation of 14-3-3 from Bax and translocation of Bax to the mitochondria (355). The antiapoptotic protein humanin has also been shown to bind Bax and prevent its activation (356). Sawada et al. recently identified the DNA repair protein Ku70 as a suppressor of Bax activation using a yeast-based functional screen (357). In unstimulated cells, cytosolic Bax is found associated with Ku70 (357;358). In response to a death stimulus, Ku70 is acetylated by PCAF and CBP, resulting in its dissociation from Bax (358). In healthy cells, Ku70 is maintained in a deacetylated state by SIRT1, and increased levels of SIRT1 are associated with decreased Bax-mediated apoptosis (359). Membrane-permeable peptides derived from the Bax-sequestering domain of Ku70 were found to inhibit apoptosis induced by UVC irradiation, staurosporine, and anti-cancer drugs (360). One particular Bax-inhibiting peptide (BIP), designated V5 (VPMLK), is effective in preventing cell death in human cell lines as well as primary porcine, mouse, and rat cell cultures (360;361). V5 has also been shown to prevent cell death caused by NGF-withdrawal in sympathetic neurons (362). The effectiveness, membrane-

permeability, and stability of these BIPs make them ideal therapeutics for preventing apoptosis in a clinical setting.

Transplantation of islets of Langerhans, which contain the insulin producing β-cells, has been shown to be an viable therapy for the treatment of type 1 diabetes (53). However, the harshness of the islet isolation procedure exposes these cells to significant stress, which could contribute to decreased viability following isolation (169;322). Consequently, multiple organ donors are often necessary to meet the tissue requirements of a single patient (53;55). Therapeutic strategies aimed at the inhibition of islet cell death could be expected to increase islet yield and improve cell viability, thereby making more efficient use of available donor tissue. We have previously demonstrated that the commonly employed method of islet isolation leads to the induction of islet apoptosis, with increasing levels of mitochondrial dysfunction and necrotic-like death becoming apparent after 72 hours in culture (61;275). Caspase inhibition is unable to prevent isolated islet cell death, instead targeting these cells towards a more necrotic-like death phenotype (275). In addition, the amount of mitochondrial depolarization occurring in isolated islets is unaffected by caspase inhibition, leading to the notion that the commitment to islet cell death could be occurring at the level of, or upstream of mitochondrial dysfunction (275). Human islets express high levels of Bax relative to Bcl-2 (329), and a decrease in Bcl-2 mRNA levels and Bcl-2 immunostaining has been observed in isolated human islets after 5 days in culture (363). Overexpression of Bcl-2 in murine (364) and human (365) islets has been shown to reduce apoptosis following isolation.

Additionally, transfection of human islets with Bcl-2 afforded them protection from cytokine-mediated cell death (253;254). We therefore examined whether the Bax-inhibitory peptide V5 could protect islets against cell death induced by isolation as well as by other known islet stressors.

6.3. MATERIALS AND METHODS

Human Islet Isolation. Pancreata were retrieved from heart-beating cadaveric donors at the time of multi-organ harvest for transplantation. Consent for donation of tissues for research was obtained by the local organ procurement organization. Warm ischemia time was approximately 5 minutes and cold ischemia time, using University of Wisconsin (UW) solution perfusion, was between 30 minutes and 4 hours. Islets were isolated using the method of Ricordi et al. (48). Briefly, the main pancreatic duct was canulated and a cold (6-8°C) solution of 1.43 mg/ml Liberase enzyme blend (Roche, Montreal, QC, Canada) in perfusion solution (Mediatech, Herndon, VA, USA) supplemented with 0.2 mg/mL DNAse I (Roche) was infused through the canula into the pancreas using a syringe. The distended pancreas was placed in a sterilized aluminium digestion chamber (Bio-Rep, Miami, FL, USA) through which the perfusion solution was recirculated at 37°C. The extent of tissue digestion was assessed by staining aliquots of digestate with dithizone (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) and visualizing the islets under an inverted light microscope (Nikon, Montreal, QC, Canada). The digestion process was terminated by cooling the circuit to between 5 and 10°C when the majority of the islets were seen to be free of surrounding acinar tissue. The digestate was collected, centrifuged, and washed three times with wash solution (Mediatech) and islets were purified on a continuous density gradient (Biocoll separating solution; Biochrom AG, Berlin, Germany) using a COBE 2991 Cell Processor (COBE BCT, Denver, CO, USA). The number of islet equivalents (IEQ) and islet

purity were assessed using dithizone (Sigma), a zinc chelater which stains the zinc rich β-cells. Glucose-stimulated insulin secretion was routinely assessed to ensure islet functionality.

Islet cell culture. Isolated islets (>90% purity) were cultured in CMRL 1066 medium (Gibco, Burlington, ON, Canada) containing 10% fetal bovine serum (FBS) (Wisent, St. Bruno, QC, Canada), at 37°C in a humidified atmosphere of 5% CO_2. Medium was changed every other day. For acute cytokine stimulation experiments, islets were treated with 100 ng/mL TNF-α, 2 ng/mL IL-1β, and 100 ng/mL INF-γ (R&D Systems Inc., Minneapolis, MN, USA). The biological activities of the cytokines are 10 U/ng (TNF-α), 50 U/ng (IL-1β), 10 U/ng (INF-γ). The general caspase inhibitor Z-VAD-fmk (Calbiochem, San Diego, CA, USA) was used at a final concentration of 50 μM. CsA (Sigma) was used at a final concentration of 1 μM, which we found to be non-toxic to islets over a 48 hour period (data not shown). The V5 Bax inhibitory peptide VPMLK (University of Calgary Peptide Synthesis, Calgary Canada; and Calbiochem) and the negative control peptide IPMIK (Calbiochem) were used at 100 μM, unless otherwise indicated. V5 fluorescently labelled with 5-carboxyfluorescein (5-FAM-V5; Sheldon Biotechnology Centre, Montreal, Canada) was used at 100 μM. The mitochondrial Ca^{2+} uniporter inhibitor RU360 (Calbiochem) was prepared fresh prior to use and was used at a final concentration of 5 μM. In experiments examining the potential protective effect of a compound, the compound was added 30-60 minutes prior to the stressing agent. For all compounds prepared in

DMSO, the final concentration of DMSO in the culture media was kept below 0.1%. Vehicle controls were prepared for all treatments.

DEVDase Assay. The islet samples (2000 IEQ) were spun down for 2 minutes at 900 rpm and 4°C. The pellet was washed twice with ice cold PBS and then dissolved in lysis buffer (50 mM Tris-HCl, pH 8.0, 1.37 mM NaCl, 1% (v/v) nonident P-40, 10% (v/v) glycerol). The samples were sonicated and spun down for 20 minutes at 14000 rpm, 4°C, and the supernatant was kept for analysis. The protein content was determined using BIO-RAD Protein Assay Dye Reagent (BIO-RAD, Mississauga, Ontario, Canada). In a 96-well plate, lysate containing 100 μg of protein was added to caspase buffer (50 mM HEPES, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, 10 mM dithiothreitol, 100 mM NaCl, pH 7.2) containing 200 μM Ac-DEVD-*p*NA. Samples were incubated at 37°C for 90 minutes and the release of *p*NA was analyzed by measuring the absorbance at 405 nm using a Benchmark Microplate Reader (BIO-RAD).

MTT Assay. Mitochondrial metabolic activity was used as an indicator of islet viability (235). Aliquots containing 500 IEQ in 500 μl of medium were placed in sterile eppendorf tubes and 50 μL of stock 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/mL) (Sigma) was added to each sample. The samples were incubated at 37°C for 2 hours, washed twice with cold PBS, and lysed with 200 μL of DMSO (Sigma). Two 100 μL aliquots from each sample were loaded onto a 96-well plate and the absorbance was measured at

595 nm using a Benchmark Microplate Reader (BIO-RAD). Four independent samples were analyzed per experiment and each experiment was performed at least three times.

Fluorescent Microscopy. In some cases, islets were dispersed by first washing with dispersion solution (Gibco) followed by a 10 minute incubation at 37°C with trypsin/EDTA (Gibco). Trypsinized islets were washed with cold CMRL 1066 with 10% FBS, pipetted gently, and resuspended in PBS. Whole islets or dispersed islets were resuspended in PBS containing 2 μM propidium iodide (PI) (Molecular Probes Inc., Eugene, OR, USA) and 0.67 μM fluorescein diacetate (FDA) (Molecular Probes). The percentage of PI-positive cells was determined by dividing the number of PI positive cells by the total number of cells counted. JC1 (Molecular Probes) was used according to manufacturers directions to identify cells with depolarized mitochondria. The stained islets were placed on glass slides with cover-slips and incubated for 15 minutes at room temperature in a foil-covered container with a moist paper-towel. Slides were visualized under a fluorescent Olympus BX60 microscope connected via a digital video camera to a PC. Five hundred cells were counted per slide, with three slides per group for at least three independent experiments. Images were analyzed using Image Pro Plus 4.0 software (Media Cybernetics Inc., Silver Spring, MD, USA). Confocal microscopy was performed using a Zeiss LSM 510 Confocal Laser Scanning Microscope (Carl Zeiss Canada Ltd., Toronto, Ontario, Canada).

Glucose-Stimulated Insulin Release. Cultured islets (100 IEQ per group in duplicate) were washed with CMRL 1066 and incubated in Hank's buffered saline containing 2.2 mM glucose for two consecutive periods of 60 minutes at 37°C. Next, islets were incubated for 30 minutes with 22 mM glucose and then another 30 minutes with 22 mM glucose with 50 μM 3-isobutyl-1-methylxanthine (IBMX), an inhibitor of cyclic-AMP (cAMP) phosphodiesterase. Finally, islets were washed with Hank's buffered saline and incubated for 1h in 2.2 mM glucose. The supernatants were kept following each incubation and analyzed for insulin content using a commercially available insulin ELISA kit (Crystal Chem Inc., Downers Grove, Il, USA). Insulin release was normalized to the protein content of the pellet determined using BIO-RAD Protein Assay Dye Reagent (BIO-RAD).

Subcellular Fractionation. Cytosolic and mitochondria-enriched fractions were prepared using an ApoAlert Cell Fractionation Kit according to the manufacturer's directions (BD Biosciences, San Jose, CA, USA).

Western Blotting. The islet samples (2500 IEQ) were spun down for 2 minutes at 900 rpm and 4°C. The pellet was washed twice with ice cold PBS and then dissolved in lysis buffer (50 mM Tris-HCl, pH 8.0, 1.37 mM NaCl, 1% (v/v) nonident P-40, 10% (v/v) glycerol, 0.1 mM sodium orthovanadate, complete protease inhibitor cocktail tablet (Roche)). The samples were sonicated and spun down for 20 minutes at 14000 rpm, 4°C, and the supernatant was kept for analysis. The protein content was determined using BIO-RAD Protein Assay Dye

Reagent (BIO-RAD). Lysates were diluted 6:1 with 6X Laemmli sample buffer (0.375 M Tris-HCl pH 6.8, 12% (w/v) SDS, 3% (v/v) glycerol, 0.2% (w/v) bromophenol blue, 12% (v/v) β-mercaptoethanol in double distilled water) and boiled for 5 minutes. An equal amount of protein (50 μg) was loaded for each sample into a 12% polyacrylamide gel run at 100 V for 90 minutes. Transfer onto nitrocellulose was conducted at 250 mA for 90 minutes. Membranes were blocked with 2% bovine serum albumin in washing buffer (25 mM Tris, 150 mM NaCl, 0.05% Tween 20 in double distilled water). Blocked membranes were then probed with primary antibodies. Anti-cytochrome c (BD Biosciences) was used at a dilution of 1:1000, anti-active caspase-3 (Cell Signalling Tech., Beverly, MA, USA) at 1:1000, anti-Bax (Santa Cruz Biotechnology, CA, USA) at 1:800, and anti-COX4 (BD Biosciences) was used at 1:500. Following primary antibody incubation, the membranes were washed for 30 minutes in washing buffer then incubated for 1 hour in anti-rabbit horseradish peroxidase-linked antibody (1:4000) (Amersham LifeSciences Inc., Buckinghamshire, England). Following another 30 minute wash, the blots were developed using the ECL chemiluminescence system (Amersham) and Kodak X-OMAT film (Kodak, Rochester, NY, U.S.A.). Membranes were stripped by incubating at 65°C for half an hour in stripping buffer (100 mM β-mercaptoethanol, 2% (w/v) SDS and 62.5 mM Tris-HCl pH 6.7) and reprobed with primary antibody.

Statistical Analysis. All results are expressed as mean ± standard error of the mean (SEM). Statistical significance was determined using a one-way *ANOVA*

with a post-hoc Bonferroni's test as well as the Student's t-test using SYSTAT 9 (SPSS Inc., Chicago, IL, USA). Differences were considered significant where $P < 0.05$.

6.4. RESULTS

5-FAM-labelled V5 is taken up by all cells within the islet and distributes mainly to the cytosol. Isolated human islets were treated with 5-FAM-labelled V5 to examine the ability of this peptide to permeate to the β-cell rich core of the islet. Confocal microscopy demonstrated that 5-FAM-V5 entered all cells within the islets and efficiently penetrate to the core of isolated islets (Figure 6.1A-B). V5 localization to the cytosol has previously been shown to depend on the presence of Bax, where Bax-deficient cells displayed high nuclear localization of V5 (360). Assessment of single cells from dispersed human islets demonstrated that 5-FAM-V5 distributes mainly to the cytosol, with some minor nuclear localization, consistent with the notion that V5 is interacting with cytosolic Bax (Figure 6.1C-D).

V5 improves isolated human islet survival following routine isolation. In order to determine the effective concentration of V5, freshly isolated islets were cultured for 48 hours in the presence of increasing concentrations of V5 and viability was assessed using the MTT assay. At a concentration of 100 μM, V5 caused a 21% increase in isolated islet survival, whereas a negative control peptide (IPMIK) had no effect (Figure 6.2A). No additional improvement in viability was observed with V5 at doses higher than 100 μM. Thus, V5 was used at 100 μM for the remaining experiments.

In order to determine whether islets treated with V5 maintained their enhanced viability when V5 was withdrawn, we cultured islets for 96 hours during which time V5 was administered either during the first 48 hours and then

withdrawn, during the last 48 hours only, or during the entire 96 hour incubation. Islets that received V5 during the first 48 hours showed a 19% increase in MTT reduction despite the removal of V5 after 48 hours (Figure 6.2B). Islets that received V5 throughout the entire 96 hour culture period exhibited a 22% increase in MTT reduction. No significant effect on islet viability was observed in islets treated with V5 during only the last 48 hours.

We have previously observed increasing levels of necrotic-like cell death in isolated islets that was associated with an increase in mitochondrial depolarization and was not preventable by caspase inhibition (275). Islets treated immediately following isolation with V5 had significantly less PI-positive cells than untreated islets following 48 hours in culture (Figure 6.2C). V5 also caused a significant decrease in the number of cells displaying mitochondrial depolarization, as assessed by JC1 staining (Figure 6.2D). Using cell fractionation, V5 treatment was shown to reduce the release of cytochrome c from the mitochondria into the cytosol (Figure 6.2E). As demonstrated previously (360), V5 was able to decrease Bax translocation from the cytosol to the mitochondria. Western blotting for cytochrome c oxidase subunit IV (COX4) was used to confirm that the cytosolic fractions were not contaminated with mitochondrial proteins. Caspase-3 processing was also reduced in V5 treated islets (Figure 6.2F). Therefore, treatment of islets immediately following isolation with V5 prevents Bax translocation to the mitochondria, cytochrome c release, and caspase-3 activation, leading to increased islet viability.

V5 does not affect glucose-stimulated insulin release. Glucose-stimulated insulin release is closely coupled to particular metabolic steps in which the mitochondria serves a critical role (366). Perturbations in mitochondrial function and integrity can lead to defective insulin secretion (366). Overexpression of Bcl-X_L has previously been shown to impair insulin secretion and was associated with altered mitochondrial polarization and Ca^{2+} signalling in response to glucose (367). We thus examined whether V5 affected glucose-simulated insulin release in isolated islets. Freshly isolated islets were cultured for 48 hours in the presence of 100 μM V5 and glucose-stimulated insulin release was assessed. V5 had no effect on basal insulin release at low glucose levels or on insulin release in response to high glucose (Figure 6.3). Addition of IBMX, an inhibitor of cyclic-AMP phosphodiesterase, causes elevated intracellular cAMP levels that lead to increased exocytosis of insulin (304). IBMX-induced insulin release was unaffected by V5 treatment (Figure 6.3). Therefore, V5 did not affect glucose-stimulated insulin-secretion from isolated human islets.

V5 suppresses cytokine-induced cell-death. Inflammatory cytokines are believed to mediate β-cell destruction in type I diabetes (192). In particular, IL-1β, INF-γ, and TNF-α are often elevated in type I diabetic patients, as well as in several animal models of diabetes, and induce β-cell death in vitro (192). Additionally, intra-islet cytokine production in isolated islets has been suggested to contribute to apoptosis in transplanted islets (195). We therefore examined whether V5 could prevent cytokine-induced human islet cell death. Isolated human islets were cultured for 48 hours following isolation and were pretreated

with V5 for 1 hour prior to treatment with TNF-α, IL-1β, and INF-γ for 24 hours. Cytokine treatment caused a 37% decrease in islet viability, an effect that was prevented by pretreatment with V5 (Figure 6.4A). Cytokine treatment also resulted in a nearly 4-fold increase in DEVDase activity, which is indicative of caspase-3-like protease activity (Figure 6.4B). V5 prevented the rise in DEVDase activity cause by cytokine treatment (Figure 6.4B). Cytokine-induced processing of caspase-3 to its cleaved (active) form was also prevented by V5 (Figure 6.4D). Due to the limited number of islets available, Western blot analysis was used to assess caspase-3 activation in the remaining experiments. Western blot analysis of cytosolic and mitochondria-enriched fractions showed that V5 decreased cytokine-induced cytochrome c release from the mitochondria into the cytosol (Figure 6.4C). Treatment with V5 also prevented cytokine-induced Bax translocation to the mitochondria, as seen by the increased cytoplasmic localization of Bax upon V5 treatment (Figure 6.4C). These results suggest a prominent role for Bax-mediated mitochondrial permeabilization in cytokine-induced islet cell death.

V5 does not protect against H_2O_2-induced cell death. The β-cells of the islet are particularly sensitive to oxidative stress due to low expression levels of antioxidant proteins (27). In addition, oxidative stress has been proposed as a mechanism of β-cell failure in the development of diabetes (207). Oxidative stress has also been suggested to play a role in isolated islet failure (208). We therefore examined the ability of V5 to protect isolated islets against H_2O_2-induced cell death. Isolated human islets displayed a dose-dependent decrease

in viability in response to treatment with H_2O_2 for 24 hours (Figure 6.5A). Treatment of islets with 500 μM H_2O_2 resulted in a 40% decrease in viability, an effect which was not prevented by V5 (Figure 6.5B). A significant protective effect was observed with cyclosporine A (CsA) (Figure 6.5B), which is a known inhibitor of the mitochondrial permeability transition pore (PTP) (368). In addition, the pan-caspase inhibitor Z-VAD-fmk was also able to reduce the H_2O_2-induced decrease in viability after 24 hours. We then examined the effects of V5 on H_2O_2-induced cytochrome c release and found that V5 was unable to prevent cytochrome c release into the cytosol following H_2O_2 treatment (Figure 6.5C). We did not observe increased Bax translocation to the mitochondria upon H_2O_2 treatment, indicating that either it was not occurring at the time point examined or it was not detectible above the amount of translocated Bax already present in isolated islets.

V5 does not protect against thapsigargin-induced cell death. The finding that V5 was unable to protect against H_2O_2-induced cytochrome c release and cell death suggested that perhaps Bax translocation was not implicated in activation of the mitochondrial (intrinsic) pathway in response to oxidative stress. Indeed, H_2O_2 has been shown to induce Ca^{2+}-mediated apoptosis independently of translocation of Bax to the mitochondria (84). We therefore examined whether V5 could protect against cell death induced by thapsigargin, which causes a rise in cytosolic Ca^{2+} levels by inhibiting the sarcoplasmic endoplasmic reticulum Ca^{2+} ATPase (SERCA) pump that transports Ca^{2+} from the cytosol into the lumen of the endoplasmic reticulum (ER). Isolated human islets displayed a dose-

dependent decrease in viability in response to treatment with thapsigargin for 24 hours (Figure 6.6A). Treatment with 1 μM thapsigargin for 24 hours resulted in a 40% decrease in islet viability, which was not prevented by V5 (Figure 6.6B). In contrast, both CsA and Z-VAD-fmk were able to suppress thapsigargin-induced cell death after 24 hours. Treatment with thapsigargin for 24 hours induced cytochrome c release into the cytosol which was unaffected by V5 (Figure 6.6C). No significant changes in Bax translocation were observed following thapsigargin treatment (Figure 6.6C).

Inhibition of mitochondrial Ca^{2+} uptake protects islets against thapsigargin but not H_2O_2. Ca^{2+} can induce opening of the mitochondrial permeability transition pore, which can lead to cytochrome c release (369;370). The close proximity of mitochondria and ER allows for rapid uptake of Ca^{2+} into the mitochondria following release from ER stores (371), and Ca^{2+} entry into mitochondria is required for certain forms of cell death (372). In particular, both thapsigargin and H_2O_2 are able to promote Ca^{2+}-dependent PTP opening (373). We therefore examined the effects of a specific inhibitor of the mitochondrial Ca^{2+} uniporter (RU360) on the loss of islet viability induced by thapsigargin or H_2O_2. RU360 partially prevented the decrease in islet viability caused by thapsigargin (Figure 6.7A). However, no protective effect was observed by RU360 against H_2O_2. Similar results were obtained by PI/FDA staining (data not shown).

Figure 6.1. 5-FAM-labelled V5 is taken up by all cells within the islet and distributes mainly to the cytosol. Freshly isolated islets were treated with 100 μM 5-FAM-V5 for 20 hours and examined by confocal microscopy. (**A**) Bright field image and (**B**) fluorescent image at a focal plane through the center of the islet, with 5-FAM-V5 appearing green. (**C**) Bright field and (**D**) fluorescent images from dispersed islets following treatment with 100 μM 5-FAM-V5 for 20 hours. The images shown are representative of three independent experiments.

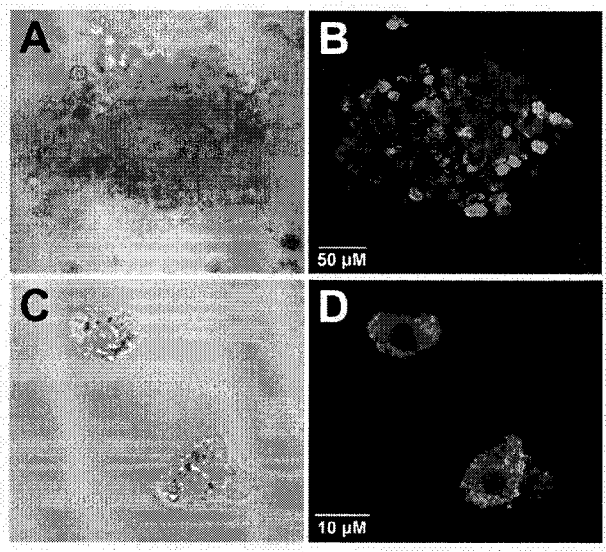

A
B
50 µM
C
D
10 µM

Figure 6.2. V5 improves isolated human islet survival following routine isolation. (**A**) Freshly isolated islets were treated with increasing concentrations of V5 (black bars) or the negative control peptide IPMIK (white bars). Following 48 hours in culture, islet viability was assessed by MTT assay. Bars represent the mean MTT reduction expressed as a percentage of the control ± SEM for quadruplicate samples from five independent experiments. (**B**) Freshly isolated islets were cultured with or without the addition of V5 (100 μM) to the culture media for the indicated periods. Following 96 hours in culture, islet viability was assessed by MTT assay. Bars represent the mean MTT reduction expressed as a percentage of the control ± SEM for quadruplicate samples from four independent experiments. (**C**) Immediately following isolation, islets were cultured with or without the addition of V5 (100 μM) to the culture media. Membrane permeability was assessed following 48 hours in culture using PI/FDA-staining. The number of PI-positive cells was expressed as a percentage of the total cells counted. Bars represent the mean values ± SEM for three independent experiments. (**D**) Immediately following isolation, islets were cultured with or without the addition of V5 (100 μM) to the culture media. Mitochondrial depolarization was assessed following 48 hours in culture by JC1 staining. The number of cells with depolarized mitochondria was expressed as a percentage of the total cells counted. Bars represent the mean values ± SEM for three independent experiments. (**E**) Following 48 hours in culture with or without V5 (100 μM), mitochondrial and cytosolic fractions were prepared and analyzed by Western blotting. (**F**) Following 48 hours in culture with or without V5 (100 μM), whole cell lysates were prepared. Caspase-3 processing was examined by Western blot analysis. The blots shown are representative of three independent experiments. *, $P<0.05$ compared with untreated controls (Ctrl).

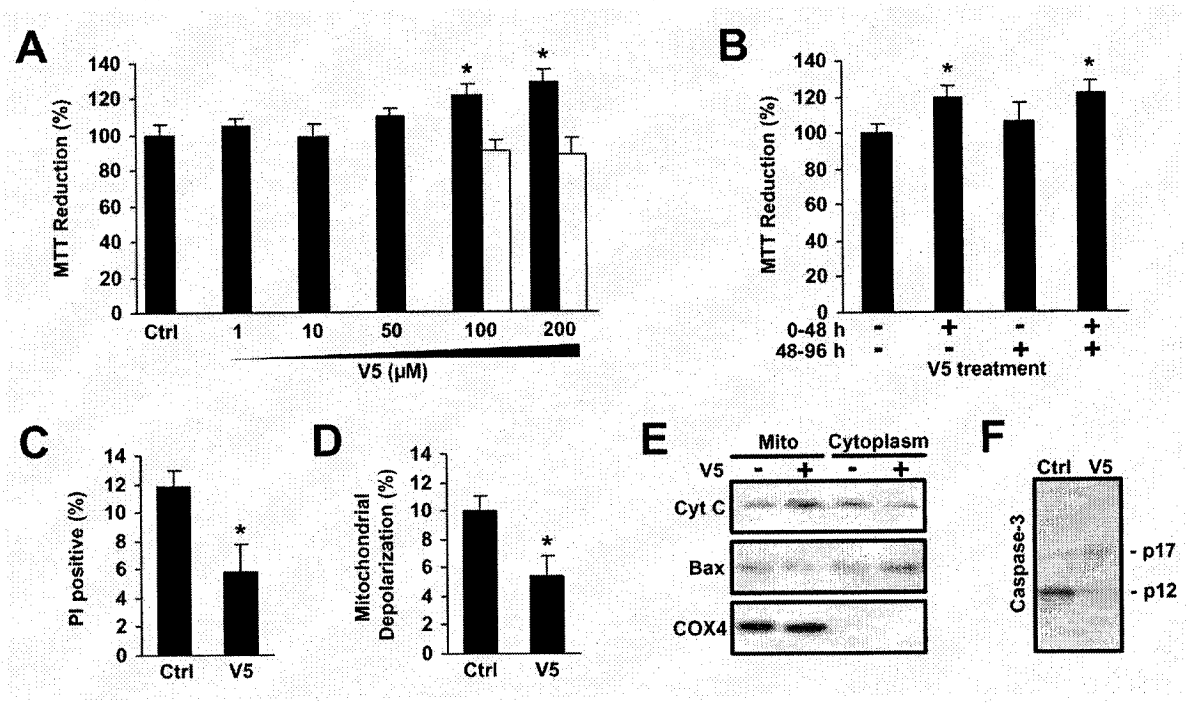

A
MTT Reduction (%)
140
120
100
80
60
40
20
0
Ctrl
1
10
50
100
200
V5 (μM)
B
MTT Reduction (%)
0-48 h
48-96 h
V5 treatment
C
PI positive (%)
Ctrl
V5
D
Mitochondrial Depolarization (%)
Ctrl
V5
E
Mito
Cytoplasm
V5
Cyt C
Bax
COX4
F
Ctrl
V5
Caspase-3
- p17
- p12

Figure 6.3. V5 does not affect glucose-stimulated insulin release. Immediately following isolation, islets were cultured with or without the addition of V5 (100 μM) to the culture media. Glucose-stimulated insulin release was examined following 48 hours in culture as described in the Materials and Methods. Bars represent mean values ± SEM for three independent experiments.

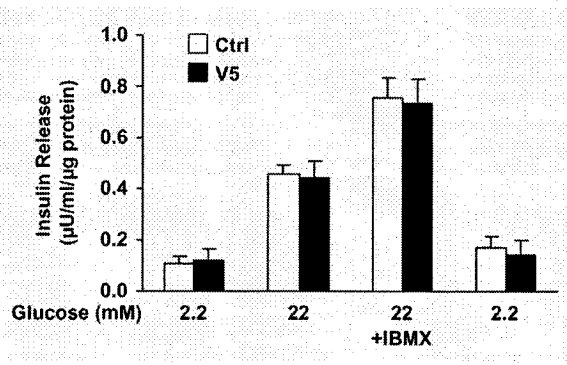

Ctrl
V5
Insulin Release
(µU/ml/µg protein)
1.0
0.8
0.6
0.4
0.2
0.0
Glucose (mM)
2.2
22
22
+IBMX
2.2

Figure 6.4. V5 suppresses cytokine-induced cell-death. Freshly isolated islets were cultured for 48 hours and then treated with or without cytokines (100 ng/ml TNF-α, 2 ng/mL IL-1β, 100 ng/ml INF-γ) and V5 (100 μM) for 24 hours. (**A**) Islet viability was assessed by MTT assay. Bars represent the mean MTT reduction expressed as a percentage of the control ± SEM for quadruplicate samples from three independent experiments. (**B**) Caspase-3 activity of islet cell lysates was assessed by measuring the cleavage of DEVD-*p*NA. The amount of *p*NA released was measured by analyzing the absorption at 405 nm. For each graph, bars represent the mean values ± SEM for three independent experiments. (**C**) Mitochondrial and cytosolic fractions were prepared and analyzed by Western blotting using antibodies against cytochrome c, Bax, and COX4. The blots are representative of three independent experiments. (**D**) Whole cell lysates were prepared and caspase-3 processing was examined by Western blot analysis. The blot is representative of three independent experiments. *, $P<0.05$; **, $P<0.01$ compared with untreated controls (Ctrl).

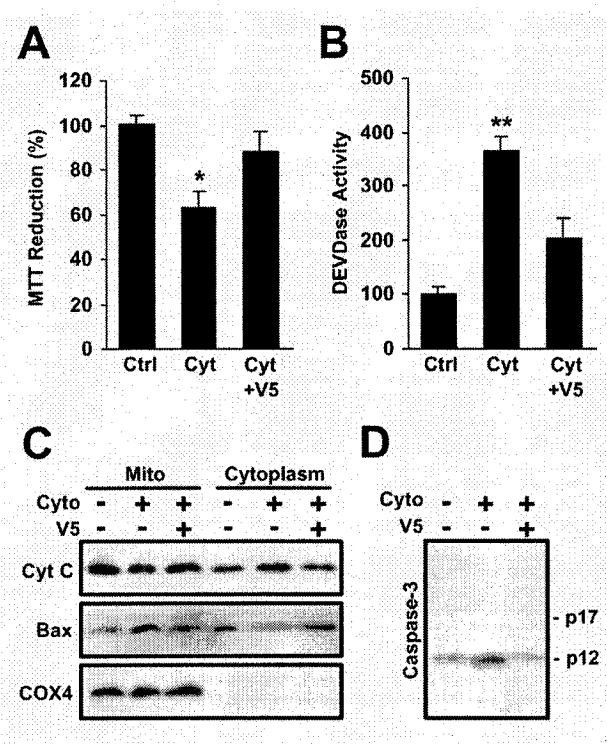

A
MTT Reduction (%)
120
100
80
60
40
20
0
*
Ctrl
Cyt
Cyt +V5
B
DEVDase Activity
500
400
300
200
100
0
**
Ctrl
Cyt
Cyt +V5
C
Mito
Cytoplasm
Cyto - + + - + +
V5 - - + - - +
Cyt C
Bax
COX4
D
Cyto - + +
V5 - - +
Caspase-3
- p17
- p12

Figure 6.5. V5 does not protect against H_2O_2-induced cell death. (**A**) Freshly isolated islets were cultured for 48 hours and then treated with increasing concentrations of H_2O_2 for 24 hours. Islet viability was assessed by MTT assay. Bars represent the mean MTT reduction expressed as a percentage of the control ± SEM for quadruplicate samples from three independent experiments. (**B**) Freshly isolated islets were cultured for 48 hours and then treated as indicated with H_2O_2 (500 μM), V5 (100 μM), CsA (1 μM), and Z-VAD (20 μM) for 24 hours. Islet viability was assessed by MTT assay. Bars represent the mean MTT reduction expressed as a percentage of the control ± SEM for quadruplicate samples from three independent experiments. (**C**) Freshly isolated islets were cultured for 48 hours and then treated as indicated with H_2O_2 (500 μM) and V5 (100 μM) for 24 hours. Mitochondrial and cytosolic fractions were prepared and analyzed by Western blotting using antibodies against cytochrome c, Bax, and COX4. The blots are representative of three independent experiments. (**D**) Freshly isolated islets were cultured for 48 hours and then treated as indicated with H_2O_2 (500 μM) and V5 (100 μM) for 24 hours. Whole cell lysates were prepared and caspase-3 processing was examined by Western blot analysis. The blot is representative of three independent experiments. *, $P<0.05$; **, $P<0.01$ compared with untreated controls (Ctrl) unless otherwise indicated.

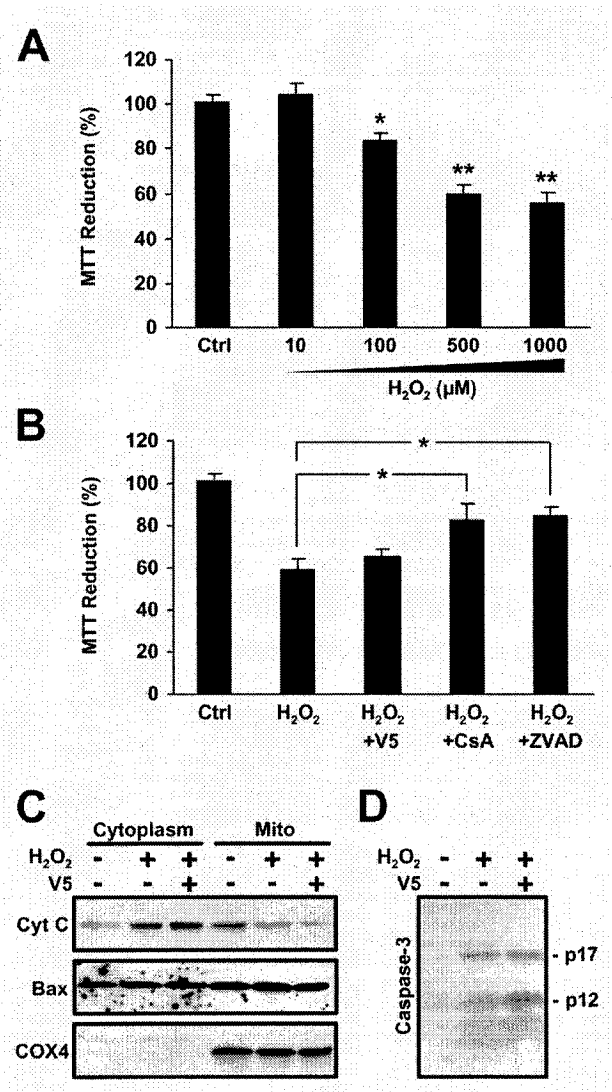
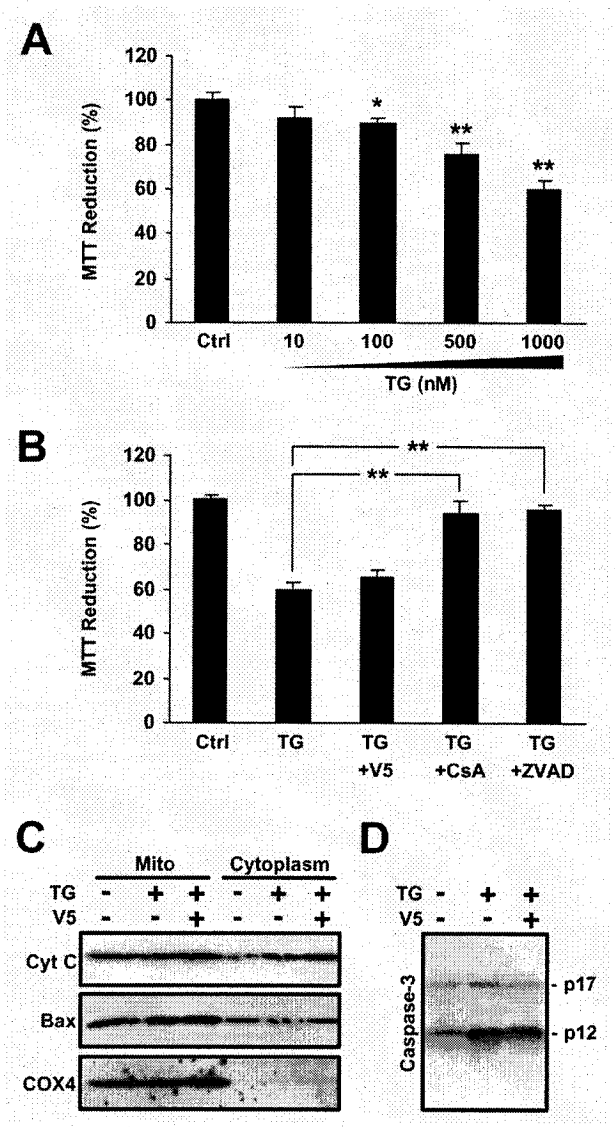

A
MTT Reduction (%)
120
100
80
60
40
20
0
*
**
**
Ctrl
10
100
500
1000
H2O2 (μM)
B
MTT Reduction (%)
120
100
80
60
40
20
0
*
*
Ctrl
H2O2
H2O2 +V5
H2O2 +CsA
H2O2 +ZVAD
C
Cytoplasm
Mito
H2O2
V5
Cyt C
Bax
COX4
D
H2O2
V5
Caspase-3
- p17
- p12

Figure 6.6. V5 does not protect against thapsigargin-induced cell death. (**A**) Freshly isolated islets were cultured for 48 hours and then treated with increasing concentrations of thapsigargin (TG) for 24 hours. Islet viability was assessed by MTT assay. Bars represent the mean MTT reduction expressed as a percentage of the control ± SEM for quadruplicate samples from three independent experiments. (**B**) Freshly isolated islets were cultured for 48 hours and then treated as indicated with thapsigargin (TG, 1 μM), V5 (100 μM), CsA (1 μM), and Z-VAD (20 μM) for 24 hours. Islet viability was assessed by MTT assay. Bars represent the mean MTT reduction expressed as a percentage of the control ± SEM for quadruplicate samples from three independent experiments. (**C**) Freshly isolated islets were cultured for 48 hours and then treated as indicated with thapsigargin (TG, 1 μM) and V5 (100 μM) for 24 hours. Mitochondrial and cytosolic fractions were prepared and analyzed by Western blotting using antibodies against cytochrome c, Bax, and COX4. The blots are representative of three independent experiments. (**D**) Freshly isolated islets were cultured for 48 hours and then treated as indicated with thapsigargin (TG, 1 μM) and V5 (100 μM) for 24 hours. Whole cell lysates were prepared and caspase-3 processing was examined by Western blot analysis. The blot is representative of three independent experiments. *, $P<0.05$; **, $P<0.01$ compared with untreated controls (Ctrl) unless otherwise indicated.


A
MTT Reduction (%)
120
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20
0
*
**
**
Ctrl
10
100
500
1000
TG (nM)
B
MTT Reduction (%)
**
**
Ctrl
TG
TG +V5
TG +CsA
TG +ZVAD
C
Mito
Cytoplasm
TG
V5
Cyt C
Bax
COX4
D
TG
V5
Caspase-3
p17
p12

Figure 6.7. Inhibition of mitochondrial Ca^{2+} uptake protects islets against thapsigargin but not H_2O_2. (**A**) Freshly isolated islets were cultured for 48 hours and then treated as indicated with thapsigargin (TG, 1 μM) and RU360 (RU; 5 μM) for 24 hours. Islet viability was assessed by MTT assay. Bars represent the mean MTT reduction expressed as a percentage of the control ± SEM for quadruplicate samples from three independent experiments. (**B**) Freshly isolated islets were cultured for 48 hours and then treated as indicated with H_2O_2 (500 μM) and RU360 (5 μM) for 24 hours. Islet viability was assessed by MTT assay. Bars represent the mean MTT reduction expressed as a percentage of the control ± SEM for quadruplicate samples from three independent experiments. *, $P<0.05$; **, $P<0.01$ compared with untreated controls (Ctrl) unless otherwise indicated.

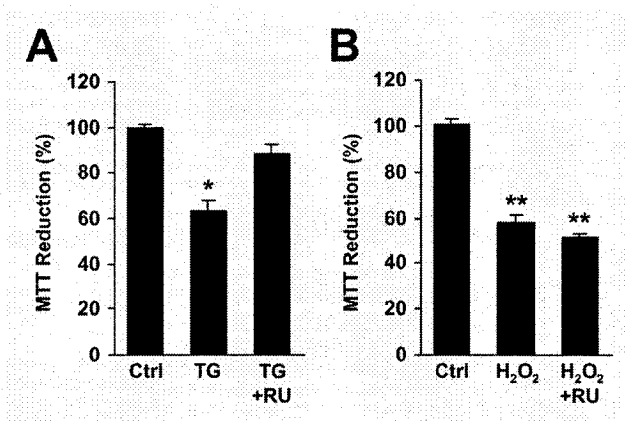
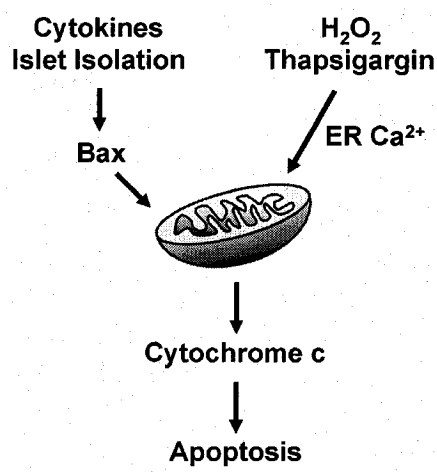

A
MTT Reduction (%)
120
100
80
60
40
20
0
*
Ctrl
TG
TG +RU
B
MTT Reduction (%)
120
100
80
60
40
20
0
**
**
Ctrl
H2O2
H2O2 +RU

Figure 6.8. Proposed model of Bax-mediated and Ca^{2+}-mediated cytochrome c release. See text for details.


Cytokines
Islet Isolation
H_2O_2
Thapsigargin
Bax
ER Ca^{2+}
Cytochrome c
Apoptosis

6.5. DISCUSSION

Our results suggest that islet cell death caused by isolation and culturing is dependent on the translocation of Bax to the mitochondria. Isolated islets are subject to growth factor withdrawal, detachment from the ECM, and hypoxia, among other insults. It has previously been demonstrated that Bax is required for cell death induced by trophic factor deprivation (82;374), detachment from the extracellular matrix (375), and hypoxia (182). Bax therefore plays a critical role in the execution of cell death triggered by many of the insults to which islets are exposed upon isolation from the pancreas.

Our previous work suggested that the inability of caspase inhibition to prevent islet cell death may be due to the fact that caspases lie downstream of mitochondrial permeabilization, and thus their inhibition has little effect on cell death caused by mitochondrial dysfunction (275). In the present study, we show that V5, administered immediately following isolation, can significantly reduce mitochondrial depolarization. The absence of an altered death phenotype, even 48 hours after the removal of V5, suggests that V5 can block the commitment to islet cell death following isolation. V5 was unable to improve isolated islet survival when administered 48 hours after isolation, underscoring the early and critical role of Bax translocation in islet cell death following isolation.

Bax translocation and cytochrome c release were still evident following V5 treatment (Figure 6.2E), indicating that intervention at an earlier time point could be more effective in reducing islet cell death. Although Bax is present at the mitochondria in V5-treated cells, V5 may shift the balance between proapoptotic

and antiapoptotic Bcl-2 proteins at the mitochondria in favor of the latter. Indeed, in some cell types Bax can be detected at the mitochondria in healthy cells (84), where other proteins may suppress its proapoptotic activity. Interestingly, epithelial cells detached from the ECM can be replated following Bax translocation and survive, indicating that Bax translocation may be a reversible event (376).

Our results show that V5 was able to protect against cell death induced by a combination of the proinflammatory cytokines TNF-α, IL-1β, and INF-γ, demonstrating that Bax translocation is necessary for cytokine-induced cell death in islets. Previously, transfection of human islets with Bcl-2 was shown to protect them from cytokine-mediated cell death (253;254). It has also been reported that non-viral transfection with Bcl-X_L fused to a protein transduction domain (PTD) protected the insulin secreting βTC-3 cell line from TNF-α-induced apoptosis (377). These findings suggest that islets contain type II cells, in which death receptor signalling requires mitochondrial amplification in order to cause cell death (378).

β-cells are electrically excitable cells capable of action potentials (304). Treatment of β-cells with a combination of IL-1β and INF-γ has been shown to increase cytosolic Ca^{2+} levels via low voltage-activated (LVA) Ca^{2+} channels (291). Antagonists of Ca^{2+} channels can prevent the rise in cytosolic Ca^{2+} and reduce cell death in cytokine treated β-cells (291;379). Cytokine-induced cell death in this case may be partially mediated by Ca^{2+}-activated proteins such as calpain or calcineurin (379). The Ca^{2+} rise observed in β-cells following cytokine

treatment may represent a parallel or secondary death signal to more classical death receptor (extrinsic) and mitochondrial (intrinsic) signalling pathways. The situation may be analogous to staurosporine, which can cause a rise in cytoplasmic Ca^{2+} level, but ultimately depends on Bax-mediated permeabilization of the mitochondria to cause cell death (84). Concordantly, V5 was able to protect islets staurosporine-induced cell death (unpublished observation).

Our findings support the existence of Ca^{2+}-mediated cytochrome c release which may not require Bax translocation to the mitochondria, but is CsA-sensitive. Thapsigargin can cause induction of the mitochondrial PTP by a CsA-sensitive mechanism (370). Similarly, H_2O_2 was also able to promote Ca^{2+}-dependent PTP opening (373). Ca^{2+} can also promote the dissociation of cytochrome c from cardiolipin (380), which is thought to be an important first step in cytochrome c release from the mitochondria (381). Evidence also suggests that ER Ca^{2+}-mediated cytochrome c release does not required Bax at the mitochondria (84). Importantly, inhibition of mitochondrial Ca^{2+} uptake using RU360 can prevent cytochrome c release and apoptosis (382-384). Our finding that inhibition of mitochondrial Ca^{2+} uptake protected islets against thapsigargin supports such a notion. It is unclear why RU360 had no protective effect against H_2O_2 in our system. It is possible that the stability of RU360, a redox-sensitive compound (385), was compromised by H_2O_2 treatment. RU360 was previously shown to have no protective effect against cell death or DVEDase activity induced by oxidative stress generated by photodamage (386).Taken together, these findings suggest that certain stimuli can initiate apoptosis by ER-to-

mitochondrial Ca^{2+} transport leading to PTP opening and cytochrome c release which does not require Bax targeting to the mitochondria.

The role of the Bcl-2 family members in regulating intracellular Ca^{2+} homeostasis is controversial (387). Bax and Bak are present at the ER and Bax can form oligomers at the ER upon treatment with thapsigargin, tunicamycin, or brefeldin A, all of which can induce ER-stress (83). In addition, cells lacking Bax and Bak at the ER are resistant to thapsigargin or H_2O_2-induced cell death (84). Whether or not endogenous Bax translocates to the ER following a death stimulus remains unclear. It is possible that resident ER Bax and Bak regulate Ca^{2+} release from the ER, which may explain why V5 was unable to prevent cell death induced by thapsigargin or H_2O_2, while Bax/Bak deficient cells are resistant to these insults. It is also possible that Bak may play a larger role in ER-mediated insults, and would thus be unaffected by V5 treatment (357;360). Overexpressed Bcl-2 can protect against thapsigargin-induced cell death (388), which may be due to the ability of Bcl-2 to sequester membrane inserted Bax at the ER. On the other hand, it is not know whether V5 can interact with membrane-inserted Bax. These findings suggest that V5 is only able to prevent cell death which is dependent on Bax translocation from the cytosol to the mitochondria.

Several issues remain to be addressed concerning the mechanism of action of V5. Though the interaction between Ku70 and Bax has been demonstrated (357;358), it remains to be established whether V5 interacts directly with Bax. It is also unclear whether V5 can suppress activated Bax, or whether V5 can only maintain Bax in an inactive form. Characterization of the

Bax-V5 interaction may shed light on the mechanism of Bax activation as well as lead to the development of novel Bax inhibiting drugs.

In summary, the current findings suggest that V5 could be a beneficial constituent of islet culture media immediately following isolation, and perhaps during earlier steps of the islet isolation procedure. However, our results suggest that V5 is only able to prevent cell death that is dependent on Bax translocation from the cytosol to the mitochondria. Therefore, the use of V5 in combination with other agents, such as antioxidants, may lead to improved islet survival.

6.6. ACKNOWLEDGMENTS

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CHAPTER 7

General Discussion

7.1. The Integrated Response to Isolation

The first objective of this thesis was to elucidate signal transduction events and related intracellular activities that are implicated in islet cell survival/death following islet isolation. The isolation of pancreatic islets imposes considerable stress on these cells, resulting in significant levels of cell death following isolation (61;169). Concordantly, islets displayed high activation of the stress-activated JNK pathway immediately following isolation (114) (Chapter 2). However, within 24 hours in culture, JNK activation was greatly reduced concomitant with an increase in AKT activation (Chapters 2 and 3). Inhibition of PI3K/AKT signalling resulted in sustained JNK phosphorylation and cell death, while activation of AKT suppressed JNK phosphorylation, indicating that the rise in AKT activity during the first 12 hours in culture suppressed JNK activation induced by islet isolation (Chapter 3). It has been suggested recently that a decrease in MKK7 expression may mediate the decreased JNK phosphorylation observed in isolated islets (115), however we did not observe any changes in MKK7 in isolated human islets (data no shown). Furthermore, MKK7 is required for cytokine-induced JNK phosphorylation (389) and cytokine-induced JNK phosphorylation is still observed in cultured human islets (113;115;390)(Chapter 3), suggesting the presence of MKK7. Thus, it remains to be confirmed whether changes in MKK7 expression play a significant role in regulating JNK activity in isolated islets.

One of the stimulus of the AKT pathway in cultured islets was found to be insulin secreted by the islets themselves, acting in an autocrine manner (Chapter 4). The delay in the onset of AKT phosphorylation could be due to the fact that a

significant proportion of the insulin content of islets is lost during the purification step of the isolation (391). Thus, immediately following isolation, these degranulated islets may have reduced levels of basal insulin release. The eventual result of this autocrine activation of the pro-survival AKT pathway, and subsequent suppression of JNK, was a decrease in the appearance of apoptotic cells in islets after 72 hours in culture (Chapter 5). However, cell death was still occurring, despite elevated AKT activation, which presented necrotic-like characteristics (Chapter 5). This could be due to the energy requirements of AKT-mediated survival (175). Indeed, AKT-mediated survival in the absence of growth factors is dependent on the presence of glucose (311). Furthermore, AKT cannot prevent apoptosis under hypoxic conditions (182). Therefore, efforts to improve oxygen delivery during culture should further improve islet survival.

7.2. Prevention of Isolated Islet Cell Death

The second objective of this thesis was to develop therapeutic interventions to promote β-cell survival by targeting the key signalling events. These results will now be discussed in context of their applicability to clinical islet transplantation.

Since AKT phosphorylation levels were initially low in islets following isolation, the potent AKT activator IGF-I was added to canine islet cultures in an attempt to increase cell survival (Chapter 2). Although IGF-I increased AKT activation, no significant effect was observed on islet viability after 48 hours in culture. This could be due to the fact that untreated islets also display increased

AKT phosphorylation as early as 1 hour following isolation and robust AKT phosphorylation 12 hours after isolation (Figure 2.1 and 3.1). Therefore, the additional stimulation of the AKT pathway by IGF-I for a few hours immediately following isolation would not be expected to cause a significant increase in islet survival. In addition, since IGF-I is known to suppress insulin secretion (392), it is also possible that treatment of islets with IGF-I decreases the autocrine insulin survival pathway. Treatment of human islets with simvastatin was shown to significantly reduce apoptosis in a PI3K-dependent manner after only 12 hours, and the protective effect was maintained for 72 hours (151). Furthermore, simvastatin-treated islets showed improved function when transplanted into streptozotocin-induced diabetic mice. Islets in this study were cultured in CMRL 1066 at 22°C, instead of 37°C as in our studies. In addition, the levels of cell death observed in this study were twice that observed in our system, thus a protective effect may have been more evident under these more stressful conditions. Therefore, activators of the PI3K/AKT pathway that do not interfere with insulin secretion may be useful in improving isolated islet survival.

Since high levels of JNK phosphorylation were observed immediately following isolation, we examined whether the JNK inhibitor SP600125 could improve isolated islets survival (Chapter 3). This early activation of JNK coincided with the peak of islet apoptosis which occurs 24 hours following isolation (113;114) (Chapter 5). We therefore examined the effects of SP600125 treatment immediately following isolation on islet survival and function after 48 hours in culture. SP600125 treatment increased MTT reduction, decreased the

number of PI-positive cells, decreased mitochondrial depolarization, and decreased caspase-3 activity (Figure 3.6). The increased islet viability was maintained even after withdrawal of SP600125 for 48 hours, indicating that this compound did not simply delay cell death; it blocked the commitment to cell death. These findings indicate that JNK inhibition by SP600125 may be a viable strategy for improving isolated islet survival.

Previous results indicated that elevated p38 phosphorylation in isolated islets correlated with decreased survival (114). We therefore explored the possibility that p38 was also contributing to isolated islet cell death. However, when administered immediately following isolation, SB203580 led to decreased viability (Figure 3.8B). p38 inhibition has previously been demonstrated to aggravate cytokine-mediated human islet cell death (295). In addition, inhibition of p38 potentiated IL-1β-induced cell death in a β-cell line (120). Recently, p38 was demonstrated to be required for hypoxia-induced activation of HIF-1 (393), and so p38 inhibition may prevent adaptation to hypoxic conditions. This finding may also explain our observation that islets with elevated p38 phosphorylation have lower survival rates (114;169), since these cell may have been exposed to more severe oxygen deprivation. Taken together, these findings suggest that p38 may act as a survival pathway in isolated islets. A recent study demonstrated that a 1 hour treatment with SB203580 improved islet graft survival, an effect that was claimed to be due to reduced local production of inflammatory cytokines at the graft site (196). Thus, despite the negative effect of p38 inhibition directly on islet

survival, it may indirectly improve islet graft survival by reducing the production of local inflammatory mediators which contribute to graft failure.

Our results indicate that general caspase inhibition is unable to prevent isolated islet cell death (Chapter 5). Particularly, it appears that inhibitors of caspase-3 activity led to increased islet cell necrosis, despite blocking DEVDase activity. Previously, caspase inhibition was shown to block oligonucleosomal fragmentation in dissociated islet cell cultures, however neither necrosis nor overall viability were assessed (170). Similarly, caspase-3 inhibition using DEVD-fmk was shown to decrease DNA fragmentation in human islets cultured for 48 hours (394). However, there was no measure of necrosis or total cell death, so it is not known whether DEVD-fmk also induced necrosis. Indeed, several studies using various cell types have demonstrated that caspase inhibition did not prevent cell death, despite suppressing DNA fragmentation (343-345). Nonetheless, islets cultured for 48 hours in the presence of DEVD-fmk were better at reversing hyperglycaemia in diabetic mice compared to untreated islets (394). It is unclear whether this finding relates to improved survival in culture or at the graft site, or if this was due to increase insulin-secretion, which was noted in DVED-fmk-treated islets (57). A more detailed examination of the fate of grafts pre-treated with caspase inhibitors would therefore be needed in order to determine the usefulness of these compounds in islet transplantation. However, based on our findings of increased cell death and decreased viability following treatment with either Z-VAD-fmk or DEVD-fmk would argue against their use. On the other hand, since the caspase-1-like inhibitor YVAD-cmk did not demonstrate

the negative effects associated with caspase-3 inhibition, and caspase-1 is involved in processing of IL-1β, a cytokine known to block β-cell function and induced cell death (247), this inhibitor could prove beneficial to islet graft survival. Indeed, caspase-1 inhibition improved the survival of embryonic nigral tissue transplanted into a rat model of Parkinson's disease (395).

Caspase inhibition was unable to prevent isolated islet cell death, and instead targeted these cells towards a necrotic-like death phenotype (Chapter 5). Since nicotinamide has previously been shown to decrease necrotic cell death through its inhibitory effect on PARP-1 (86), we investigated its potential to increase islet survival. The addition of nicotinamide alone to the islet culture media caused a mild, but significant, decrease in PI-positive cells (Figure 5.3A). Interestingly, nicotinamide treated islets displayed an increase in the 89 kDa PARP-1 fragment as well as an increase in active caspase-3 (Figure 5.4). Therefore, nicotinamide appears to inhibit necrosis while at the same time increase apoptosis. Therefore, further studies would be needed to examine the protective effect of nicotinamide against isolated islet cell death.

The fact that mitochondrial depolarization was still visible in islets treated with both Z-VAD-fmk and nicotinamide indicated that mitochondrial dysfunction may account for the necrotic-like death observed in the absence of PARP-1 and caspase activity. Therefore, the commitment to islet cell death could be occurring at the level of, or upstream of, mitochondrial dysfunction. We therefore examined whether a Bax-inhibitory peptide (V5) could protect human islets against cell death induced by isolation. Treatment of isolated islets with V5 reduced

mitochondrial depolarization, Bax translocation, and cytochrome c release resulting in improved islet viability. The beneficial effect of V5 was maintained even after withdrawal of V5 for 48 hours, indicating that this compound did not simply delay cell death; it blocked the commitment to cell death. These findings indicate that V5 could be a beneficial constituent of islet culture media immediately following isolation.

7.3. Combination Therapy: towards developing an islet recovery medium

The current protocol for clinical human islet transplantation does not attempt to specifically prevent islet cell death during the isolation and culture periods. In light of the considerable levels of cell death induced by the isolation, there would be significant benefit to designing a safe, efficient treatment to improve islet survival following isolation. The cell has many redundant signalling mechanisms to insure a proper response to external stimuli. Taking into consideration the plethora of insults experienced by islets during isolation and culture, it is unlikely that one treatment will be sufficient to prevent all the cell death mechanisms activated. Thus a combinatorial approach will most likely be required in order to obtain maximal protect of islets. We will now consider treatments, in addition to those examined in this thesis, which could be constituents of a combination therapy to prevent islet cell death.

The present results indicate that both SP600125 and V5 can improve islet survival following isolation. It remains to be examined whether V5 and SP600125 would have any additive/synergistic effects when administered in combination.

Though the transcriptionally-independent effects of JNK are mediated through BAX (99;100), JNK also mediates the transcription of several pro-apoptotic genes (92). Therefore, a combination of SP600125 and V5 could be expected to have some additive effect on islet survival.

Our findings indicate that insulin improves the survival of isolated islets and support the supplementation of islet culture medium with insulin immediately following isolation (Chapter 4) (113). Unlike IGF-I, which has a suppressive effect on insulin secretion (392), recent work indicates that insulin may promotes insulin expression and secretion (396-398). Since JNK is known to suppress insulin signalling (300), it is possible that insulin signalling pathway is partially suppressed immediately following isolation when JNK is highly active. Therefore, a combination of JNK inhibition (SP600125) and insulin treatment immediately following islet isolation may further increase AKT activation and survival.

Our lab has previously shown that ECM proteins can improve the survival of isolated islets (69). Both collagen and fibronectin were able to decrease apoptosis following isolation. A hydrogel containing collagen and laminin was shown to decrease isolated rat islet cell death (399). One of the drawbacks of culturing cells in a matrix is that the gel needs to be digested in order to retrieve the cells, which creates additional stress on the cells. This problem could be overcome by the use of soluble matrix proteins. It is noteworthy that Akt signalling is known to maintain survival of detached cells (318). Growth factor signalling through AKT is sufficient in many cases to overcome the loss of ECM contact (400), and so it may not be necessary to provide ECM proteins during a

short culture period. However, synergism between growth factor and integrin signalling has been reported (320), so the additional benefits of ECM proteins to islet survival should be explored.

As discussed in section 1.9.6, β-cells are particularly sensitive to oxidative stress due to low expression levels of antioxidant proteins (26;27), and many of the insults to which islet are exposed during isolation and culture can lead to oxidative stress (209;212). Treatment of isolated human islets with 17β-estradiol, which has antioxidant properties, was shown to decrease apoptosis in culture and improve graft function following transplantation into diabetic mice (60;295;401), though some of these effects were receptor-mediated. In addition, overexpression of antioxidant proteins has been shown to improve isolated islet survival (209;402;403). Treatment of isolated islet with a class of antioxidants, known as superoxide dismutase (SOD) mimics, preserved cell mass after 7 days in culture, but had little effect on islet viability (208). Further study is required to determine the usefulness of SOD mimics in islet transplantation. Finally, reduction of oxidative stress also improves islet function (404;405), thus maintaining the autocrine insulin-mediated survival signal. Therefore, further studies aimed at improving isolated islet survival should include antioxidant molecules.

Culture techniques that are suitable for dispersed cell cultures may not be adequate for proper oxygen delivery throughout the entire islet. As mentioned earlier, AKT cannot prevent apoptosis under hypoxic conditions (182). Therefore,

efforts to improve oxygen delivery during isolation and culture should provide additional or even synergistic protection with activators of AKT such as insulin.

7.4. The Question of Timing

The therapeutic window could be significantly increased by the addition of treatments prior to and/or during the isolation procedure. In theory, as long as there is no chemical incompatibility between the desired agent and the particular solution in question, therapeutic interventions could be added to all steps of the isolation. However, some steps are more attractive for interventions that others. Although the cold perfusion prior to the resection of the pancreas would be the earliest step to begin treatment with a particular anti-apoptotic agent, the agent would be circulated throughout the body and would have to be compatible with all harvested organs. Therefore, the organ preservation solution provides the first opportunity for pretreating the pancreas prior to the enzymatic digestion. The enzymatic digestion solution is a more difficult environment to introduce certain compounds due to the presence of collagenases. The Ficoll gradient may also provide difficulty due to the high osmolarity and the importance of maintaining the gradient properties.

In many cases, the protective effect afforded by a particular agent is only observed when it is administered prior to the stressing agent (230;246). However, due to the variable nature of the isolation procedure, it is difficult to ascertain the effects of treatments prior to the digestion because of the lack of an adequate control group. For example, if a pancreas were to be cut in two and

separate digestions performed so as to eliminate donor variability between groups, they would still not be identical since the digestion step could cause considerable variability between the two. The earliest point at which two groups can be separated and treated in an identical manner is following the digestion. Therefore, it is difficult to accurately assess the protective effect of a particular agent which may need to be added prior to the digestion in order to obtain a significant effect.

7.5. Implications of Autocrine Insulin Survival Signal

Our findings demonstrate that insulin acts in an autocrine manner to activate AKT and mediate the survival of isolated human islets. These findings provide new information on how culturing islets prior to transplantation may be beneficial to their survival by allowing for autocrine activation of the pro-survival AKT pathway. Indeed, treatments which activate AKT during islet culture can improve graft survival (151). Adenovirus-mediated overexpression of constitutively active AKT1 in isolated human islets decreased apoptosis induced by serum/glucose withdrawal or high glucose, and decreased the number of transplanted islets required to reverse streptozotocin-induced diabetes in mice (150). Therefore, it is plausible that AKT activation could render islets less susceptible to injury during the immediate post-transplantation period. Taken together, these findings suggest that culturing islets prior to transplantation allows for secreted insulin to activate AKT in an autocrine fashion, yielding islets

which are more likely to survive the insults encountered immediately following transplantation.

The finding that insulin acts as an autocrine survival signal provides a key link between islet function and survival. As such, treatments which improve insulin gene expression and function may improve islet survival by promoting this autocrine survival pathway. Conversely, suppressers of insulin secretion could lead to decreased islet cell viability by preventing this autocrine survival pathway. For example, it is plausible that the negative effects of calcineurin inhibitor-based immunosuppressants on islet survival is due to the fact that these compounds suppress insulin secretion (309;310), thus decreasing autocrine insulin-mediated islet survival.

7.6. CONCLUSIONS

Based on the objectives stated at the outset of this thesis, the present findings support the following conclusions:

1. Following isolation, isolated human islets display elevated JNK phosphorylation and high levels of apoptosis. Once in culture, secreted insulin acts in an autocrine manner to activate AKT, leading to the suppression of JNK and decreased apoptosis.
2. Treatments that prevent mitochondrial dysfunction, such as SP600125 and V5, are able to improve islet survival by blocking the commitment to cell death. Caspase inhibition, on the other hand, was unable to prevent isolated islet cell death since it did not prevent mitochondrial depolarization. These findings reveal the interdependence of necrosis and apoptosis in isolated islets, suggesting therapeutic strategies which target early events in cell death signalling in order to prevent multiple forms of islet cell death.

ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

"This requirement is mandatory for all McGill University doctoral theses. Elements of the thesis that are considered to constitute original scholarship and an advancement of knowledge in the domains in which the research was conducted must be clearly indicated."

1. In culture, insulin acts in an autocrine manner to activate AKT and mediate the survival of isolated human islets.

2. PI3K/AKT signalling suppresses the JNK pathway in isolated human islets and this cross-talk represents an important anti-apoptotic consequence of PI3K/AKT activation.

3. The JNK inhibitor, SP600125, improves isolated human islet survival and function.

4. Caspase inhibition using Z-VAD-fmk resulted in increased necrosis in both human and canine islets by a nicotinamide-sensitive mechanism, demonstrating the interdependence of necrosis and apoptosis in isolated islets

5. The Ku70-derived Bax-inhibitory peptide (V5) protects human islets against cell death following isolation.

6. V5 did not prevent cytochrome c release or cell death triggered by oxidative stress or thapsigargin, suggesting that these insults do not require Bax translocation to induce mitochondrial permeabilization.

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APPENDIX