

**THE ROLE OF FAS-MEDIATED APOPTOTIC PATHWAY IN
AMYLOID-INDUCED β -CELL DEATH**

by

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Abstract

In type 2 diabetes (T2D), progressive dysfunction and loss of β -cells in pancreatic islets eventually leads to hyperglycemia. The formation of toxic protein aggregates known as islet amyloid contributes to β -cell dysfunction and death in T2D. Islet amyloid is formed mainly by aggregation of the β -cell hormone named islet amyloid polypeptide (IAPP), which is produced and released along with insulin. Amyloid also forms in cultured and transplanted human islets, indicating that in addition to its role in pathogenesis of T2D, amyloid formation contributes to islet graft failure in type 1 diabetes (T1D). The mechanism(s) underlying amyloid-induced β -cell death are still unclear. Fas is a cell death receptor that has been implicated in the pathogenesis of both T1D and T2D. We hypothesised that amyloid-induced β -cell death is mediated, at least partially, by activation of the Fas apoptotic pathway and that blocking the key steps in this pathway may protect β -cells from amyloid toxicity in T2D and islet grafts in T1D.

Our studies showed that endogenously formed human IAPP aggregates induce β -cell Fas upregulation leading to interaction of Fas and its ligand FasL, thereby promoting activation of the Fas apoptotic pathway initiated by caspase-8. Blocking Fas/FasL interaction reduced amyloid-induced β -cell apoptosis, and β -cell specific deletion of Fas or caspase-8 in hIAPP-expressing mouse islets improved β -cell survival and function.

We further demonstrated that amyloid-induced β -cell Fas upregulation is mediated by interleukin-1 β (IL-1 β). Amyloid formation in cultured islets closely correlated with elevated IL-1 β production and blocking IL-1 β signalling reduced amyloid-induced β -cell Fas upregulation, caspase-8 activation and apoptosis. Moreover, IL-1 β -induced β -cell dysfunction caused impaired proIAPP processing and potentiated amyloid formation, which was restored by blocking IL-1 receptor. These findings support a dual role for IL-1 β in amyloid formation and its β -cell toxicity. Similarly, enhancing islet function by the GLP-1 receptor agonist exenatide improved impaired proIAPP processing and reduced amyloid formation.

In summary, our data show that the Fas apoptotic pathway plays a major role in amyloid-induced β -cell death and that blocking the key mediators of this pathway may provide a new therapeutic strategy to preserve β -cells in T2D and prolong islet graft survival in T1D.

Preface

All experimental work, data analysis, and preparation of the manuscripts for publication were conducted by YJ. Park with guidance from Dr. L. Marzban and technical assistance from N. Safikhan for the animal breeding and PCR genotyping, islet isolation, and optimizing parts of the immunolabelling studies (Chapters 3–6). All animal studies were reviewed and performed in accordance with the guidelines and principles of the laboratory animal care and the ethical protocols (breeding: #A13-0042; experimental: #A13-0338) approved by the Canadian Council on Animal Care and the University of British Columbia's Animal Policy and Welfare Committee. Human islets used for the studies presented in this thesis were provided by the Ike Barber Human Islet Transplant Laboratory (Vancouver, BC, Canada) in accordance with approved procedures and guidelines (#H14-00442) of the Clinical Research Ethics Board of the University of British Columbia.

A version of studies presented in Chapter 3 has been published as **Park YJ**, Lee S, Kieffer TJ, Warnock GL, Safikhan N, Speck M, Hao Z, Woo M, Marzban L. (2012) Deletion of Fas protects islet β -cells from cytotoxic effects of human islet amyloid polypeptide. *Diabetologia* 55(4):1035-1047. These studies showed, for the first time, that activation of Fas apoptotic pathway is one of the major mechanisms mediating amyloid-induced β -cell apoptosis. YJ. Park planned and performed the experiments, prepared and analyzed the data, and wrote the manuscript for publication in consultation with her supervisor Dr. L. Marzban. Drs. M. Woo and Z. Hao (University of Toronto, Toronto, ON, Canada) provided transgenic mice with β -cell specific Fas deletion and provided technical advice on breeding and genotyping them. Dr. G.L. Warnock provided human islets and advice on human islet studies. Dr. T.J. Kieffer provided technical support as well as advice on the experimental design and manuscript revisions. N. Safikhan and M. Speck (Kieffer lab) provided technical support for islet isolation and preparation. S. Lee (co-op student in Marzban lab) contributed to quantitative immunolabelling studies.

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caspase-8 in amyloid-induced β -cell death in human and mouse islets. *Diabetologia* 57(4):765-775. Studies in this chapter showed that amyloid-induced β -cell Fas upregulation leads to activation of the Fas apoptotic pathway initiated by caspase 8. YJ. Park designed the study plan, conducted majority of the experiments, and prepared the original and revised manuscript drafts for publication with guidance from Dr. L. Marzban. Drs. M. Woo and R. Hakem (University of Toronto) provided transgenic mice with β -cell specific caspase-8 deletion and provided technical advice on breeding and genotyping them. Dr. G.L. Warnock provided human islets as well as advice on human islet studies. Dr. T.J. Kieffer provided technical support as well as advice on the experimental design and manuscript revisions. Z. Ao and N. Safikhan provided technical support for islet isolation and preparation. F. Yang (co-op student in Marzban lab) contributed to quantitative immunolabelling studies.

A manuscript containing a part of the data presented in Chapter 5 is currently in preparation for submission. **Park YJ**, Warnock GL, Ao Z, Safikhan N, Meloche M, Asadi A, Kieffer TJ, Marzban L. Dual role of IL-1 β in islet amyloid formation and β -cell toxicity. Also, a portion of data from Chapter 5 has been published in **Park YJ**, Zhang Y, Ao Z, Meloche RM, Warnock GL, Marzban L. (2012) The IL-1 receptor antagonist Anakinra enhances survival and function of human islets during culture: implications in clinical islet transplantation. *Canadian Journal of Diabetes* 36: 244-250. These studies provided proof-of-principle of the new concept that the pro-inflammatory cytokine IL-1 β produced by the islets during culture mediates amyloid-induced β -cell Fas upregulation and that blocking IL-1 β signalling protects islet β -cells from amyloid toxicity. YJ. Park designed and performed the studies in both manuscripts as well as wrote and revised the manuscript drafts with feedback from her supervisor Dr. L. Marzban. Drs. G.L. Warnock and M. Meloche provided human islets as well as advice on human islet studies. Dr. T.J. Kieffer provided technical support as well as advice on the experimental design and manuscript revisions. Z. Ao and N. Safikhan provided technical support for islet isolation and preparation. A. Asadi (Kieffer lab) provided technical assistance for optimizing a part of the immunolabelling studies.

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(2013) The glucagon-like peptide-1 receptor agonist Exenatide restores impaired pro-islet amyloid polypeptide processing in cultured human islets: implications in type 2 diabetes and islet transplantation. *Diabetologia* 56(3):508-519. This study demonstrated that treatment with the glucagon-like peptide-1 receptor agonist exenatide can restore impaired proIAPP processing and reduce amyloid formation in cultured human islets. This new strategy may potentially be used to reduce amyloid formation and toxicity in patients with type 2 diabetes and human islet grafts transplanted into patients with type 1 diabetes. YJ. Park contributed to the experimental designs, analysis and interpretation of data, and drafting the manuscript and revising it with guidance from L. Marzban. G.L. Warnock and M. Meloche provided human islets as well as advice on human islet studies. D.M. Thompson provided exenatide and advice on optimizing exenatide treatment studies. T.J. Kieffer provided technical support as well as advice on the experimental design and manuscript revisions. Z. Ao and N. Safikhan provided technical support for islet isolation and preparation. H. Chen (physiology honours student in Marzban lab) contributed to optimizing the exenatide islet treatment studies.

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List of abbreviations

Ad-Cont-siRNA	An adenovirus that delivers a random non-specific small interfering RNA
Ad-ProhIAPP-siRNA	An adenovirus that delivers a proIAPP-specific small interfering RNA
Amyloid β	A β
ANOVA	Analysis of variance
Apaf-1	Apoptotic protease activation factor-1
ApoE	Apolipoprotein E
ATF6	Activating transcription factor 6
β 2M	β -2-microglobulin
BB-DP	BioBreeding diabetes-prone
BB-DR	BioBreeding diabetes-resistant
BMI	Body mass index
BSA	Bovine serum albumin
cAMP	Cyclic-adenosine monophosphate
CDA	Canadian Diabetes Association
CGRP	Calcitonin gene-related peptide
CHOP	C/EBP homologous protein
CPE	Carboxypeptidase E
CTL	CD8 T lymphocytes
CTLA-4	Cytotoxic T lymphocyte associated-4
DISC	Death-induced signalling complex
DMSO	Dimethyl sulfoxide
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated protein degradation
ERK	Extracellular signal-regulated kinase
FasL	Fas ligand
FBS	Fetal bovine serum
FFA	Free fatty acid
FLIP	FLICE inhibitory protein
GADAs	Glutamic acid decarboxylases

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GIP	Gastric inhibitory polypeptide
GLP-1	Glucagon-like peptide-1
GLP-1R	Glucagon-like peptide-1 receptor
GSIS	Glucose-stimulated insulin secretion
HbA1C	Glycated hemoglobin A1C
HFIP	1,1,1,3,3,3-Hexafluoro-2-propanol
hIAPP	Human islet amyloid polypeptide
HIP rats	Human islet amyloid polypeptide rats
HLA	Human leukocyte antigen
HSPG	Heparan sulphate proteoglycans
IAPP	Islet amyloid polypeptide
IDE	Insulin degrading enzyme
IFG	Impaired fasting glucose
IFN- γ	Interferon- γ
IGT	Impaired glucose tolerance
IL-1 β	Interleukin-1 β
iNOS	Inducible nitric oxide synthase
IRE1	Inositol-requiring enzyme 1
JNK	c-Jun N-terminal kinase
LC	Light chain
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MODY	Maturity onset diabetes of the young
MOI	Multiplicity of infection
NF- κ B	Nuclear factor kappa B
NLRP3	NLR family pyrin domain containing 3
NO	Nitric oxide
NOD	Non-obese diabetic
OGTT	Oral glucose tolerance test

PAM	Peptidyl amidating monooxygenase complex
PC1/3	Prohormone convertase 1/3
PC2	Prohormone convertase 2
PCNA	Proliferating cell nuclear antigen
PDX-1	Pancreatic duodenal homeobox 1
PERK	(PKR)-like endoplasmic reticulum kinase
PKA	Protein kinase A
PKB	Protein kinase B
PMSF	Phenylmethylsulfonyl fluoride
PTPN22	Protein tyrosine phosphatase non-receptor type 22
RAMPs	Receptor activity-modifying proteins
rIAPP	Rat islet amyloid polypeptide
ROS	Reactive oxygen species
SAP	Serum amyloid P component
STAT3	Signal transducer and activator of transcription 3
tBid	Truncated Bid
TCF7L2	Transcription factor 7-like 2
TNF- α	Tumour necrosis factor- α
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UPR	Unfolded protein response
WHO	World Health Organization

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To my loving family...

Chapter 1: Introduction

1.1 Diabetes mellitus

1.1.1 Definition and classification of diabetes mellitus

Diabetes mellitus is a metabolic disorder characterised by the presence of hyperglycemia due to insulin deficiency and/or defective insulin action (1,2). The term diabetes mellitus was first used by the Greek physician Aretaeus, which means ‘a siphon’ or ‘to pass through’ (diabetes) and ‘like honey’ (mellitus) to describe the excessive urination associated with the disease and the sweet smell and taste of the patients’ urine (3). The term ‘pre-diabetes’ refers to impaired fasting glucose (IFG), impaired glucose tolerance (IGT), or increased glycated hemoglobin A1C (HbA1C) levels which puts individuals at high risk of developing diabetes and diabetes-related complications (1,4).

As detailed in the recent American Diabetes Association report (2013), there are four clinical classes of diabetes: type 1 diabetes, type 2 diabetes, gestational diabetes, and other types of diabetes (4). However, the clinical phenotype and progression of disease may not be typical in a small portion of patients with diabetes. For instance, some patients cannot be clearly classified as having type 1 or type 2 diabetes. Occasionally, patients with type 2 diabetes may present with diabetic ketoacidosis, which is a common acute complication seen in type 1 diabetes (4). Similarly, some patients with type 1 diabetes may have a late onset of the disease with slow disease progression as seen in type 2 diabetes despite having features of autoimmunity (4).

1.1.2 Global diabetes prevalence and burden

Today, more than 382 million people worldwide are suffering from diabetes, a staggering 8.3 percent of adult population, and this number is expected to rise beyond 592 million by 2035 (2). Further, 316 million people have pre-diabetes and are therefore at high risk of developing the disease (2). In Canada, an estimated 2.4 million Canadians (6.8% of the population) had been diagnosed with either type 1 or type 2 diabetes in 2009, and this number is expected to increase to 3.7 million by 2019 (1). Significant concern has been raised about the global epidemic of diabetes and its harmful effects on both individual and national productivity. The personal costs

of diabetes include reduced quality of life, increased likelihood of diabetes-related complications, and even death (5). Moreover, the economic consequences of diabetes is high due to the combined burden of healthcare costs estimated close to \$548 billion (global) or \$12.2 billion (Canada) annually and loss of economic productivity from illness and premature deaths (1,2).

World Health Organization (WHO) has examined the global patterns of glycemia and diabetes prevalence in different parts of the world in a study expanding over 370 country-years and including 2.7 million participants (6). This study has reported that the global age-standardised mean of fasting plasma glucose has increased significantly over the past decade (6). The mean fasting plasma glucose and diabetes prevalence are on the rise in regions such as Latin America and the Caribbean, South Asia, Africa and the Middle East, with the largest rise seen in Oceania (6,7). Additionally, recent epidemiological data have shown that more than 80% of people with diabetes are living in the economically developing (low- and middle-income) countries (2,6).

1.1.3 Diagnosis of diabetes

According to the 2013 Canadian Diabetes Association report, a clinical diagnosis of diabetes and pre-diabetes in patients can be made based on the criteria summarised in Table 1-1 (1). For decades, the diagnosis of diabetes mellitus has been largely dependent on the plasma glucose criteria, either by measuring fasting plasma glucose or 2-hour plasma glucose in a 75 g oral glucose tolerance test (OGTT) (8). However, the major disadvantage of these glucose-based tests is day-to-day variability in a person's plasma glucose levels (4). On the other hand, HbA1C level reflects the average plasma glucose over a period of time (8 to 12 weeks) prior to the date of testing (9,10). The HbA1C test is a convenient method of assessing glycemic control in diabetic patients as it can be performed at any time of the day and more importantly, it does not require any special preparations such as fasting or diet changes prior to testing (1). However, HbA1C test is not recommended for monitoring glycemic control in individuals with abnormal red blood cell turnover due to pregnancy, a recent blood loss or transfusion, or diseases such as hemolytic anemia. In these individuals, the diagnosis of diabetes has to be made exclusively

based on the glucose-based criteria. Moreover, age-dependent differences in HbA1C levels have been reported (11); thus HbA1C test is not recommended for use as a diagnostic tool for diabetes mellitus in children and adolescents (1).

1.1.4 Diabetes-associated symptoms and complications

People with diabetes are at risk of developing debilitating and life-threatening health problems. Individuals with pre-diabetes or recent onset of the disease can remain undiagnosed for many years. They are often diagnosed only when the complications of diabetes have already developed. Some of the classic clinical symptoms of diabetes include polydipsia (excessive thirst), polyphagia (increased appetite), and polyuria (abnormally large passage of urine) along with overt hyperglycemia at the clinical presentation of the disease in these patients (1).

Chronic hyperglycemia leads to both microvascular and macrovascular complications in these patients. Microvascular (involving small vessels such as capillaries) complications include progressive retinopathy, neuropathy and nephropathy (12). In many countries, these diabetes-related microvascular complications are the leading cause for blindness, kidney failure, and lower-limb amputation (12). The mechanisms underlying microvascular complications include intracellular formation of advanced glycation end products, impaired metabolic control leading to increased oxidative stress and reactive oxygen species (ROS) production, chronic inflammation, abnormal stimulation of hemodynamic regulation systems, and activation of other pathogenic mediators (12,13).

Moreover, exposure to chronic hyperglycemia can significantly increase the risk for macrovascular (involving large vessels such as arteries) complications (13). Notably, as much as 70-80% of people with diabetes may die from diabetes-associated cardiovascular diseases (14). Several studies have shown that diabetes is associated with up to 4-fold increase in the risk for coronary heart disease (15). Moreover, hyperlipidemia, increased cholesterol levels, and systemic inflammation under diabetic conditions can dramatically accelerate atherosclerotic plaque formation, which can lead to stroke and myocardial infarction (12). Also, diabetic patients with previous history of myocardial infarction are at higher risk for another episode of myocardial infarction compared to non-diabetic patients with similar medical history (15).

1.1.5 Type 1 diabetes

Type 1 diabetes is characterised by the autoimmune-mediated destruction of insulin-producing pancreatic islet β -cells, which leads to insulin deficiency and hyperglycemia in those patients (16,17). However, it should be noted that although most cases of type 1 diabetes are attributable to the selective destruction of β -cells by the cellular immune system (type1A), a small number of cases arise due to idiopathic destruction or failure of β -cells (type1B) through a process that is still unclear (18,19). Type 1 diabetes accounts for approximately 5-10% of all patients with diabetes (20).

The incidence of type 1 diabetes has increased dramatically during the past few decades with large variation across both geographic populations and different racial groups (19). In 1990, WHO initiated the Multinational Project for Childhood Diabetes known as the DIAMOND project to better understand the global incident and prevalence of type 1 diabetes (21,22). The initial report published in 2000 has shown an annual increase in the incidence of type 1 diabetes essentially worldwide but to a varying degree among different geographical regions (22). The highest incident rates have been reported from Finland and Sardinia and the lowest rates from Venezuela and China (21,23). Moreover, in the United States, the SEARCH for Diabetes in Youth Study (SEARCH) has been designed to find correlations between incidence/prevalence of type 1 diabetes and factors such as race/ethnicity, age and sex (24-26). This multiethnic, population-based study (performed in 10 study locations covering a population of more than 10 million person-years) has reported the highest incidence rate in non-Hispanic white subjects and the lowest incidence rate among Asians and Pacific Islanders (26). Moreover, SEARCH has reported that the incidence rate of type 1 diabetes is associated with age (24). Type 1 diabetes is also referred to as juvenile- or childhood-onset diabetes due to its high prevalence in people under the age of 30 (1). According to SEARCH, it appears that the incidence of type 1 diabetes peaks during childhood (5-9 years of age) and near puberty in adolescents (10-14 years of age) (27). Lastly, SEARCH has shown that the age of clinical-onset of type 1 diabetes as well as the incidence rate for type 1 diabetes is slightly higher in girls than in boys (26). These data indicate that the person's race/ethnicity, age, and sex may be used as indicators for estimating the incidence and prevalence of type 1 diabetes.

1.1.5.1 Risk factors for type 1 diabetes

1.1.5.1.1 Genetic factors

Autoimmune diabetes is rarely caused by a single gene defect (28). Rather, development of type 1 diabetes is strongly influenced by multiple genetic factors involving defects in various susceptibility genes (23,29,30). Studies have shown that the disease concordance rate for type 1 diabetes in monozygotic twins is higher (30-50%) than disease concordance rate (6-10%) in dizygotic twins (31,32). Moreover, studies have reported that there is a positive correlation between the presence of a first-degree relative with type 1 diabetes and the likelihood of developing the disease (23). For example, in the United States, individuals with a family history of type 1 diabetes have a 1 in 20 lifetime risk of developing the disease compared to 1 in 300 lifetime risk seen in general population (33). These studies support the notion that genetic factors are important risk factors associated with type 1 diabetes.

To date, nearly 50 genetic loci have been identified to contribute to the development and progression of type 1 diabetes (19,34). From the long list of genes associated with type 1 diabetes susceptibility, genetic variation in human leukocyte antigen (HLA) region has been identified as an important risk factor for type 1 diabetes (35-37). The HLA region is located on chromosome 6p21, which is commonly referred to as the insulin-dependent diabetes mellitus locus 1 (IDDM1) (28). Among the three classes of HLA genes, class II HLA genes, which are responsible for encoding molecules involved in antigen presentation, remain the strongest genetic contributor for type 1 diabetes susceptibility (28,38-40). Moreover, according to multiple genome-wide association studies and meta-analyses performed on them, other confirmed risk loci associated with type 1 diabetes include the CTLA-4 (cytotoxic T lymphocyte associated-4), PTPN22 (protein tyrosine phosphatase non-receptor type 22), and CD25 (IL-2 receptor alpha chain) genes, all of which are thought to influence the immune responsiveness (28,41-44).

1.1.5.1.2 Environmental factors

The significant contribution of environmental factors to the etiology of type 1 diabetes is supported by evidence such as the variation in diabetes incidence rates among different race/ethnicities and geographical regions and rapid convergence of local disease incidence rates

in the migrating population from low- to high-incidence countries (30). Moreover, the epidemiological data suggest that the rise in occurrence of type 1 diabetes in populations with previously identified low-risk genotype (eg. Asians) has to be attributed to environmental changes in the region rather than genetic factors as the most cases of type 1 diabetes (85%) occurs in individuals without the family history for the disease (45).

Multiple environmental factors have been implicated as potential factors that may trigger production of diabetes-associated autoimmune antigens, which include (but are not limited to) viral infections, childhood vaccines, dietary factors, insufficient production of vitamin D, and environmental toxins and contaminants (23,30,46-49). Viral and infectious agents have been most frequently noted as environmental triggers for islet autoimmunity in type 1 diabetes. Based on the reports showing higher serum anti-enteroviral antibody titers (50,51), immunohistochemical detection of enterovirus in the pancreas, and increased enteroviral RNA in the peripheral blood from recent-onset patients with type 1 diabetes compared to healthy controls indicate that enteroviruses (eg. type B coxsackievirus) might be the prime viral candidates (52,53). Other viral candidates that have been reported to be associated with type 1 diabetes include rotavirus, cytomegalovirus, parvovirus, and encephalomyocarditis virus (54-57). Their association with induction of autoimmunity in type 1 diabetes is based on potential molecular mimicry between the viral proteins and T-cell epitopes (28). However, it is not yet validated whether there is a causal relationship between these viral candidates and type 1 diabetes (28).

Furthermore, a number of studies examined if dietary factors can promote islet autoimmunity in type 1 diabetes. The most predominant nutritional factor associated with incidence of type 1 diabetes is the exposure to cow's milk during early infancy (before 3-4 months of age) due to shortened period of breastfeeding (58-60). In support, a recent study has shown cross-reactivity between insulin and bovine α -casein, the main protein present in cow's milk, which suggests a potential for molecular mimicry that triggers autoimmunity against insulin-producing β -cells (61).

However, it is important to note that no single environmental factor identified to date has been conclusively linked to the increased risk of type 1 diabetes, suggesting that a combination

of these factors may influence the pathogenesis of type 1 diabetes in an individual-specific manner.

1.1.5.1.3 Gut microbiota

The gut microbiota (bacterial composition of the intestine) plays an important role in development and maintenance of the immune system (62). Studies suggest that alterations in the gut microbiota are associated with an overly activated immune system that can lead to autoimmunity and type 1 diabetes (62-64).

Hypothetical models have been proposed to explain how autoimmune diseases such as type 1 diabetes are linked to microbial factors affecting the immune system. The ‘hygiene hypothesis’ which describes the rising incidence of autoimmune diseases in general is attributable to increased overall hygiene which limits exposures to infectious agents resulting in reduced or altered stimulation of the immune system during development (65,66). Conversely, the ‘fertile field hypothesis’ suggests that microbial infections during early childhood promote the interaction with different antigens through which autoreactive T cells may arise (67). The ‘old friends hypothesis’ implicates the role of gut microbiota as direct inducers or regulators of the body’s immune system and self-tolerance (68). Lastly, the ‘perfect storm hypothesis’ supports the role of three combining factors in development of type 1 diabetes: the gut microbiota, increased gut permeability, and genetic alterations of mucosal immunity (69).

Moreover, these hypothetical models have been tested in studies performed on germ-free (sterile) and gnotobiotic (populated with selected microbes) animal models of type 1 diabetes (70-74). In non-obese diabetic (NOD) mice and BioBreeding diabetes-prone (BB-DP) rats, modulation of gut microbiota can prevent or slow down the development of diabetes (75,76). In one study, germ-free NOD mice that had been contaminated with spore-forming bacteria *Bacillus cereus* exhibited delayed onset of diabetes (75). Moreover, NOD mice lacking MyD88 (NOD.MyD88KO), an essential adaptor protein for multiple innate immune receptors (eg. toll-like receptors that recognise bacterial ligands), did not develop diabetes indicating that the interactions between the host innate immune system and the gut microbiota may be important for disease development (77). Furthermore, examination of stool samples from BB-DP and

BioBreeding diabetes-resistant (BB-DR) rats has shown that probiotic bacterial species are more abundant in BB-DR than in BB-DP rats (78). Based on these findings, it has been suggested that the modulation of bacterial composition in the gut may be used as a potential therapeutic strategy to prevent type 1 diabetes. Accordingly, it has been demonstrated that antibiotic selection of microbial lineages or artificial colonization of probiotic bacterial strains can help prevent or delay the onset of autoimmune diabetes (70,74,76,79,80). Results from these experiments on animal models suggest that gut microbiota plays an important role in the etiology of type 1 diabetes (62,81,82).

1.1.5.2 Pathophysiology of type 1 diabetes

1.1.5.2.1 Islet autoimmunity

Type 1 diabetes-associated autoimmunity has typically been identified by the presence of autoantibodies to islet and/or β -cell autoantigens (83). The cellular-mediated immune response to one or more islet antigens has been first characterised histologically by the presence of insulinitis (or islet inflammation) in the pancreas of patients with type 1 diabetes during post-mortem examination (84). Some of the autoantigens implicated for the etiology of type 1 diabetes include but are not limited to (pro)insulin, zinc transporter 8, chromogranin, glutamic acid decarboxylases, and transmembrane tyrosine phosphatases like insulinoma-associated antigen-2 (19,85-87). Type 1 diabetes-associated autoantibodies can be detected in the majority (70-80%) of newly diagnosed patients with type 1 diabetes (88). Moreover, these autoantibodies can be often detected long (months or years) before the clinical onset of type 1 diabetes, indicating that the autoimmune process is likely triggered early in life (89-91). In several studies including the NIH TrialNet, TEDDY, and German BABYDIAB, detection of islet autoantibodies provided an accurate prediction of type 1 diabetes risk (91-94). The presence of multiple islet autoantibodies (or in different combinations) has been associated with increased risk for developing type 1 diabetes (19,95). For example, results from Diabetes Prevention Trial – Type 1 showed that the 5-year risk of developing type 1 diabetes is higher in subjects positive for four autoantibodies (50%) compared to those with only one autoantibody (1.3%) (94,95).

It is still not clear how β -cell specific autoimmunity develops (including production of islet autoantibodies), but different theories have been proposed including: 1) molecular mimicry between β -cells and environmental agents; 2) inappropriate major histocompatibility complex (MHC) expression on lymphoid cells; 3) defects in central tolerance; and 4) aberrant T-cell activation (28,96-99).

1.1.5.2.2 Mechanisms of β -cell destruction in type 1 diabetes

At the clinical onset of type 1 diabetes, it has been a widely accepted notion that the majority of β -cells are lost (19). Similarly, in NOD mice, a linear loss of β -cell mass has been observed following initiation of insulinitis, which eventually leads to hyperglycemia and depletion of β -cells prior to the onset of the disease (100). However, growing data suggest that some pancreatic β -cells may persist in patients with type 1 diabetes for an extended period of time and may not be completely destroyed even in patients with long-standing type 1 diabetes (101-103). Accordingly, recent studies have shown that in some individuals, as much as 40 to 50 percent of the pancreatic β -cells may still be present at the onset of the disease (101).

Increased islet autoantigens and autoantibodies can trigger abnormal activation of immune responses leading to β -cell destruction. During immune-mediated attack on β -cells, apoptosis is the main form of β -cell death observed in human islets from type 1 diabetic patients and in animal models of type 1 diabetes (16,104). Accumulated evidence suggests that the cytotoxic CD8 T lymphocytes (CTL) play a dominant role in autoimmune β -cell destruction. The direct interaction between autoreactive CTL and antigen-presenting MHC class I expressed on β -cells is critical in mediating β -cell death in type 1 diabetes (28). For example, autoreactive CTLs that recognise epitopes derived from β -cell antigens such as (pro)insulin can selectively destroy islet β -cells (28). Moreover, the NOD mice lacking expression of MHC class I antigens required for the activation of CTL are resistant to autoimmune diabetes (105). Also, when CTL clones from NOD mice are isolated and transferred to the healthy recipient mice, it results in transfer of diabetic phenotype and autoimmune-mediated β -cell death (83). Lastly, it has been demonstrated that the presence of autoreactive CTL in the peripheral blood may be used as a predictive marker for type 1 diabetes in mice (106).

Two mechanisms have been proposed for CTL-mediated β -cell death. First, release of cytolytic granules containing serine proteases granzymes and perforin by the activated CTL leads to β -cell apoptosis (107). Specifically, perforin/granzyme pathway triggers activation of the mitochondria-mediated (intrinsic) apoptotic pathway in β -cells (108). In addition, the incidence of type 1 diabetes is significantly lower in transgenic NOD mice lacking perforin (109), and perforin-deficient CTL exhibits reduced cytotoxicity against β -cells compared to wild-type CTL (110). Next, the interaction between the upregulated Fas cell death receptor in β -cells with Fas ligand (FasL) present on the surface of activated CTL has been shown to activate Fas-mediated apoptotic pathway in β -cells (28). Fas may be upregulated in β -cells by the pro-inflammatory cytokines such as interleukin-1 β (IL-1 β), tumour necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) by the infiltrating immune cells such as macrophages and CD4⁺ T cells (28,110). Increasing evidence indicates that the Fas-dependent pathway may play a less dominant role in CTL-induced β -cell death (111). However, in the absence of perforin, the Fas-mediated apoptotic pathway may become the major effector mechanism utilised by the activated CTLs (110).

1.1.5.3 Current treatments for type 1 diabetes

1.1.5.3.1 Insulin therapy

Several therapeutic options are available for patients with type 1 diabetes. Insulin therapy is the most widely used treatment option for type 1 diabetes, which involves administration of exogenous insulin (112). Continuous efforts have been geared towards the development of insulin analogues, which are recombinant human insulin receptor ligands with improved pharmacokinetic properties compared to traditional biosynthetic human insulin (113-117). For instance, insulin lispro has demonstrated improved subcutaneous absorption characteristics and faster onset of action (115,118). Combinations of insulin analogues are currently used as an integral part of the insulin therapy. Patients with type 1 diabetes take daily long-acting (basal) insulin injections along with multiple injections of rapid-acting insulin with meals (112,119). Other insulin regimens such as pre-mixed insulin, which contains specific proportions of short- or rapid-acting insulin, are also used for certain clinical cases (23). Insulin can be administered via different delivery methods. Subcutaneous injections of insulin using insulin syringe is the most common mode of administration. Alternatively, the use of insulin pumps that deliver a

continuous subcutaneous insulin infusion has become increasingly popular over the past decade (120-122).

However, maintaining glycemic control using the conventional method of self-monitoring of blood glucose and multiple daily injections can be very challenging for patients with type 1 diabetes. Moreover, the major disadvantage of conventional insulin therapy is that it can potentially cause life-threatening episodes of hypoglycemia and hypoglycemia unawareness (119). Recent innovative advancement in technology led to development of an ‘artificial pancreas system,’ which is a closed-loop system that consists of continuous glucose monitor, a device that can sense glucose levels via a needle inserted under the skin, paired with an insulin pump that can automatically deliver insulin in response (123,124). The artificial pancreas system can mimic the biological function of the pancreas through continuous monitoring of glycemic variability and maintaining blood glucose level within the target range at all times in patients with type 1 diabetes (124). Moreover, the artificial pancreas system has the potential to automatically deliver additional hormones or drugs along with insulin, which may further improve glycemic control in patients using this system (125).

1.1.5.3.2 Whole pancreas or islet transplantation

β -cell replacement strategy by means of whole-pancreas or islet transplantation has been shown a promising approach for treatment of type 1 diabetes (126). Since the first pancreas transplantation in 1966, the success rate of whole-pancreas transplantation has continued to improve with graft survival up to 85% one year (and three years with tacrolimus-based maintenance therapy) after transplantation (127,128). In addition, whole-pancreas transplantation has been shown to reverse secondary complications of type 1 diabetes that were already present in the transplant recipient (128). However, the whole-pancreas transplantation requires an invasive surgical procedure that can lead to major open-surgery related complications including graft thrombosis, graft pancreatitis, pancreatic fistulae and pseudocyst formation, and even death (mortality rate up to 4%) (127). Therefore, whole-pancreas transplantation is often restricted to patients with severe glycemic lability that cannot be controlled by exogenous insulin or those

that are in need of simultaneous kidney-pancreas transplants or those already on immunosuppressive therapy after successful kidney transplantation (127).

In comparison to whole-pancreas transplantation, islet transplantation provides a safe alternative β -cell replacement therapy, which is minimally invasive with negligible mortality rate (129). According to the recent Collaborative Islet Transplant Registry report, islet transplantation has almost 20-fold lower peri-operational morbidity risk compared with whole-pancreas transplantation (130). Moreover, other advantages of islet transplantation include: 1) reversal of the diabetic phenotype using only endocrine component of the pancreas (~2% of pancreas mass); 2) utilization of islets isolated from deceased donor unsuitable for whole-pancreas transplantation; and 3) potential for *ex vivo* manipulation of isolated islets to improve β -cell survival and function prior to transplantation (131). However, current limitations include loss of islet β -cells during pre-transplant culture and following transplantation, which results in requirement of multiple donors per recipient, adding to the burden of shortage of human pancreas (17,131,132). Moreover, long-term insulin independence in islet recipients has not yet been achieved. A follow-up study from the Edmonton group showed that only about 15% of allogeneic islet transplant recipients with type 1 diabetes remained insulin-independent 5 years after transplantation (129,133). However, improvements to the current protocol of islet transplantation is well underway as shown by the recent report which indicates that changes in immunosuppressive regime can increase insulin-independence rate up to 62% at 5 years post-transplantation (129).

1.1.6 Type 2 diabetes

Type 2 diabetes is characterised by peripheral insulin resistance and a decrease in insulin secretion associated with β -cell dysfunction, leading to glucose intolerance and eventually hyperglycemia in these patients (134). It is the predominant form of diabetes that accounts for up to 90 percent of all diabetes cases (2,20). The incidence rate of type 2 diabetes varies significantly in different geographical regions of the world where type 2 diabetes has become an epidemic in economically developing and newly industrialised nations (7,135). For example, in 1980, the prevalence of type 2 diabetes was less than 1% in China, but a recent report suggests

that China is among the top ten countries projected to have largest number of people with type 2 diabetes by 2025 (136). Between 2010 and 2030, it is predicted that there will be a higher increase in the incidence rate for type 2 diabetes in developing countries (62%) compared to developed countries (20%) (7).

Type 2 diabetes has been traditionally known as adult-onset diabetes due to higher prevalence in adult population. There is a positive correlation between age and the proportion of people diagnosed with diabetes, likely because of the age-associated decline in the body's ability to produce insulin (137). According to the Public Health Agency of Canada (2011), the sharpest increase in the prevalence of diabetes is reported in the population over the age of 40 (138). Moreover, highest prevalence of diabetes is reported in the population between the ages 75 to 79 years old (138). However, a recent Canadian National Surveillance Study has reported that there is a trend of increasing disease incidence in adolescents under the age of 18 (139). Similarly, a recent epidemiology report indicates a dramatic increase in the incidence of type 2 diabetes in adolescents from developing countries (7). However, according to this study, the proportion of adolescents with type 2 diabetes is much higher in developing countries compared to developed countries where older populations are more affected (7). Finally, the incidence of type 2 diabetes may be associated with sex as shown by slightly higher prevalence in men (7.2%) compared to women (6.4%) (138).

1.1.6.1 Risk factors for type 2 diabetes

Exposure to diabetes-associated environmental factors in genetically predisposed individuals can increase the risk for developing type 2 diabetes. There is a strong inheritable genetic connection to type 2 diabetes in individuals with familiar history of diabetes, especially in first-degree relatives (140-142). In support, the concordance rate of type 2 diabetes between monozygotic twins is close to 100 percent and about 25 percent in those individuals with first-degree relatives with type 2 diabetes, which also indicate a strong correlation between genetic susceptibility and risk for type 2 diabetes (135,143,144). Genome-wide association studies identified more than 50 gene loci associated with risk of type 2 diabetes (145,146). Although the gene product and function of many of these loci are not yet known, identified genes to date are

associated with β -cell dysfunction, obesity, and impaired insulin sensitivity (147). Interestingly, a greater number of these loci are associated with β -cell dysfunction than obesity or impaired insulin sensitivity (134,148,149). Notably, TCF7L2 (transcription factor 7-like 2) has been shown to be the strongest susceptibility locus for type 2 diabetes among the genes identified to date, which is associated with impaired insulin secretion and incretin effects (134,148,150-152). Furthermore, scientists have calculated the genotype risk score of 18 of these type 2 diabetes susceptibility loci and tested if genotype risk scores can be used to predict the incidence of type 2 diabetes (153). Results from this study indicate that the genotype risk scores based on 18 risk alleles can only provide a slightly improved prediction of risk for type 2 diabetes compared to common clinical risk factors (eg. familiar history of type 2 diabetes) (153).

The lifestyle factors associated with increased risk for type 2 diabetes include high fat diet, large consumption of alcohol, smoking, and physical inactivity (154). For instance, the westernised lifestyle characterised by diet change (from unprocessed low-energy high-fiber foods to processed energy-dense foods) and reduced physical activity has been closely associated with increased prevalence of type 2 diabetes in migrating population from traditionally rural to urban environments (155).

Furthermore, the imbalance between total energy intake and expenditure leads to obesity (156). Obesity, defined by BMI of 30 kg/m² or higher, is one of the most important predictors of type 2 diabetes (156). According to the report from the Center for Disease Control and Prevention in 2004 (United States), the prevalence of obesity among adults with diagnosed type 2 diabetes is high (close to 55%) (135,157). Moreover, in a large cohort study in Swedish and Finnish subjects, obesity was a strong risk factor associated with type 2 diabetes (150). In addition, the recent dramatic increase in childhood obesity has been proposed to contribute to higher incidence of type 2 diabetes in children and young adults (139,158). The increased prevalence of type 2 diabetes in obese individuals is attributable to metabolic defects associated with obesity, which include β -cell failure to compensate for the excess nutrients, increased glucagon secretion and gluconeogenesis, and reduced incretin response (134).

Additional factors that have been found to increase the risk for type 2 diabetes include vitamin D and B12 deficiencies (159-162), exposure to environmental toxins such as air pollutants and pesticides (163), advancing age (164,165), and previous history of gestational diabetes (166-168).

1.1.6.2 Pathophysiology of type 2 diabetes

1.1.6.2.1 Insulin resistance

Since the early 1970s, insulin resistance has been recognised as the characteristic feature of type 2 diabetes (169). Under normal metabolic conditions, insulin promotes glucose uptake at the key storage sites such as muscle, fat, and liver (169). However, insulin resistance occurs when cells in these tissues fail to respond to the normal actions of insulin (169). The effects of insulin resistance in these insulin-sensitive tissues include: 1) inhibition of glucose uptake by skeletal muscle and adipose tissue likely due to defective glucose transport system; 2) promoting lipolysis in adipocytes leading to the increased release of free fatty acids (FFA) into the circulation; and 3) increasing gluconeogenesis and reduced glycogen synthesis in the liver (15,170).

Insulin resistance typically predates the clinical onset of type 2 diabetes (169). Moreover, development of insulin resistance is closely associated with obesity, disturbances to lipid metabolism and inflammation, and endoplasmic reticulum (ER) stress and unfolded protein response (UPR) (170). One of the most critical factors in the development of insulin resistance is obesity (171). As discussed in Chapter 1.1.6.1, obesity is an important risk factor for type 2 diabetes. In obese individuals, increased metabolic demand stimulates β -cells to expand and hypersecrete insulin in order to maintain glucose homeostasis (169). During this compensatory phase, hyperinsulinemia develops and blood glucose levels are maintained within normal range (169). However, when β -cells can no longer compensate for the increased insulin demand, hyperglycemia can develop in these individuals (169). Moreover, hypersecretion of insulin as well as hyperglycemia place an enormous burden on β -cells, resulting in progressive β -cell exhaustion and dysfunction, which further contribute to worsening hyperglycemia and insulin resistance (171). Moreover, in obese individuals, increased FFA and cytokines (adipokines)

produced by adipocytes have been associated with inflammation and insulin resistance in adipose tissue (172-174). Accordingly, the importance of lipid regulation for the development of insulin resistance has been demonstrated by earlier studies which showed that acute lipid infusion can lead to lower glucose utilization in the insulin sensitive tissues (15,175).

Previous studies have shown that ER stress and UPR contribute to development of insulin resistance in insulin-sensitive tissues. In hepatocytes, several mechanisms have been suggested for ER stress and UPR-induced insulin resistance. First, ER stress and UPR can activate the transcriptional factors that directly induce expression of hepatic enzymes responsible for regulation of gluconeogenesis or lipogenesis, leading to abnormal activation of these mechanisms and insulin resistance (170,176). Second, UPR activation can trigger activation of the c-Jun N-terminal kinase (JNK) pathway that can directly interfere with insulin signalling and promote insulin resistance (170,177,178). Third, ER stress can indirectly disrupt insulin signalling by inducing lipid accumulation in hepatocytes (170,179). Moreover, ER stress can induce insulin resistance in adipose tissue by impairing insulin signalling (180), increasing lipolysis (181,182), and promoting dysregulation of adipokine secretion (180). Although the role of ER stress in inducing insulin resistance in muscle cells is still unclear, some studies have shown that ER stress can decrease insulin signalling in muscle cells by activation of the JNK pathway (183,184).

Furthermore, insulin resistance can also be caused by the defects in insulin receptor, insulin molecule itself, or glucose transport system that prevent utilization of insulin (169,185). For example, hypersecretion of insulin in response to hyperglycemia may lead to inadequate time for insulin production and processing which can result in the release of immature insulin molecules (185). Finally, it has been shown that disturbances in insulin signalling in β -cells can lead to substantial decrease in β -cell mass and function (impaired insulin secretory response to glucose) which can further contribute to increased insulin resistance (186).

1.1.6.2.2 Mechanisms of β -cell death in type 2 diabetes

Although insulin resistance in peripheral insulin-sensitive tissues is important in the

development of type 2 diabetes, overt type 2 diabetes does not occur in the absence of β -cell dysfunction and loss (187). Individuals at the pre-diabetic stage (IGT and/or IFG) may only have approximately half of their β -cell mass (188). The decrease in β -cell mass in type 2 diabetes is mainly due to an increased rate of β -cell apoptosis (188).

Multiple mechanisms have been shown to contribute to β -cell apoptosis in type 2 diabetes. During development of type 2 diabetes, hyperglycemia leads to increased insulin production in β -cells by upregulation of insulin transcription and translation, which can result in accumulation of unfolded or misfolded proteins in the β -cell ER lumen (187,189). Moreover, a heavy load of misfolded or unfolded proteins can induce activation of UPR in β -cells, which is mediated by PKR-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6) pathways to decrease the global protein synthesis, promote proper protein folding, and degrade terminally misfolded proteins (190). However, when UPR can no longer attenuate the increasing protein load on the ER, it leads to ER stress resulting in β -cell dysfunction and death (189). Additionally, during this process, ER stress and UPR may crosstalk with inflammatory signals to further increase β -cell death (191).

In type 2 diabetes, increased metabolic stress due to hyperglycemia and insulin resistance can induce mitochondrial dysfunction, leading to production of reactive oxygen species (ROS) such as superoxide radicals, hydrogen peroxide, and hydroxyl radicals (192). Accumulation of ROS can lead to oxidative stress and activation of mitochondria-mediated apoptotic pathway in islet β -cells (193,194). β -cells are particularly susceptible to ROS-mediated damage because they contain low levels of antioxidant enzymes such as superoxide dismutase, catalase, and glutathione dismutase (195-197). In isolated rat islets, exposure to hydrogen peroxide results in oxidative stress-mediated activation of JNK, p38, and protein kinase C pathways that can reduce insulin gene expression, likely through suppression of PDX-1 and/or MafA binding to the insulin gene promoter (198). Moreover, transient oxidative stress can downregulate respiratory chain proteins and reduce mitochondrial ATP production, which can impair insulin secretory mechanism (199,200).

Chronic exposure to hyperglycemia and hyperlipidemia in type 2 diabetes can induce β -cell damage (201). Studies have shown that exposure to elevated glucose and/or free fatty acids induce Fas-, oxidative- and ER-stress mediated β -cell apoptosis (202-204), promote islet inflammation (205), increase islet amyloid formation (193), impair insulin gene expression by downregulating insulin gene promoter activity (206,207), and induce β -cell dysfunction (207,208). Moreover, recent studies have shown that the deleterious effects of elevated free fatty acids is potentiated by hyperglycemia (a phenomenon called glucolipotoxicity) which prevents fatty acid oxidation resulting in increased synthesis of β -cell toxic lipid metabolites such as ceramides (209,210).

1.1.6.2.3 Islet inflammation

The role of islet inflammation in the pathogenesis of type 2 diabetes is now well established. Islet inflammation is characterised by immune cell infiltration and local release of cytokines and chemokines, which result in β -cell dysfunction and death (211). Increased islet inflammation and islet-associated macrophages have been detected in pancreatic sections from type 2 diabetic individuals as well as animal models of type 2 diabetes (212-215). Islets themselves may be the source of cytokines and chemokines, which are released in response to metabolic stress associated with type 2 diabetes (211,213). In support, chronic exposure to hyperglycemia, increased levels of saturated FFAs, and increased ROS can trigger the production of intra-islet inflammatory mediators such as IL-1 β , IFN- γ , and TNF- α leading to β -cell dysfunction and death (205). Importantly, these pro-inflammatory signals can in turn activate other apoptotic mechanisms including ER stress and UPR in β -cells (216). There is emerging evidence that islet-derived cytokines and chemokines modulate immune cell (eg. macrophages) infiltration to exacerbate islet inflammation (211). Therefore, islet cytokines and chemokines may provoke immune response in an autocrine/paracrine manner, developing a vicious cycle of immune response. In addition, pro-inflammatory signals from other organs may contribute to islet inflammation. The adipokines released from the adipose tissues such as leptin have been implicated in islet inflammation leading to β -cell dysfunction and apoptosis (217,218).

1.1.6.3 Current treatments for type 2 diabetes

The long-term maintenance of glycemic control in patients with type 2 diabetes requires a multidisciplinary approach that involves lifestyle adjustments, pharmacotherapy, and even surgical approaches (146,219,220).

1.1.6.3.1 Lifestyle management

Newly diagnosed patients with type 2 diabetes usually do not require insulin administration and can manage their diabetic condition through lifestyle interventions, which include changes to their diet (rich in fiber/whole grains) and exercise regimen (220,221). Following a careful evaluation of the patient's physical and functional ability, adoption of healthier lifestyle can reduce the metabolic burden on β -cells and help reduce overt hyperglycemia (220). However, when lifestyle modifications fail to achieve desired glycemic control in some patients, hypoglycemic agents may be used in combination and/or along with insulin therapy (1,222).

1.1.6.3.2 Pharmacotherapy

Glycemic management in type 2 diabetes requires appropriate pharmacological intervention which can complement the changes in lifestyle and diet (219). The pharmacological agents included in the 2013 Canadian Diabetes Association guidelines can be categorised into different groups depending on their effects in the body (1). One group of anti-diabetic agents, which includes biguanides and thiazolidinediones, works to improve insulin sensitivity in the body (135,219,222). The second group of pharmacological agents including insulin analogues, sulfonylureas, meglitinides and incretin mimetics, reduce glycemia by increasing circulating insulin levels (135,219,222). However, because these agents are potent stimulants of endogenous insulin secretion, they potentially increase the risk of hypoglycemic episodes with the exception for incretin-based therapies (219,222,223). Other agents that do not fall into either of the above categories include α -glucosidase inhibitors that prevent digestion of carbohydrates thereby reduce glucose uptake from the gastrointestinal tract and amylin mimics that suppress glucagon secretion, delay gastric emptying and reduce appetite (135,219).

1.1.6.3.3 Bariatric surgery

For morbidly obese individuals (BMI >35 kg/m²) with type 2 diabetes whose physical movement is impaired, bariatric surgery may be an appropriate treatment option (219,224). Bariatric surgery requires removal of a portion of gastrointestinal tract, which results in drastic weight loss, decreased caloric intake, and a substantial decrease in fasting glycemia (225,226). In some patients, bariatric surgery dramatically reduced hyperglycemia and improved insulin sensitivity as early as one month post-surgery (227). In the Swedish Obese Subjects study, which compared the effects of bariatric surgery with those of BMI-matched, non-operated patients (control group), the surgical manipulation of the gastrointestinal tract resulted in decreased (~3-fold) relative risk of type 2 diabetes incidence compared to the control group at 10 years post-surgery (225,228). Moreover, other studies have reported that patients who received bariatric surgery show increased long-term survival compared to non-operated control patients, with reduced mortality rate by 33-89% (229-233). Importantly, although bariatric surgery is an invasive surgical procedure, the surgical mortality rate is relatively low (0.25-0.5%) (225,234,235).

1.1.7 Other types of diabetes

The third classification of diabetes is gestational diabetes, which can affect 3-20% of women during pregnancy (1). It is a temporary condition during pregnancy characterised by hyperglycemia due to the body's inability to produce enough insulin in response to the needs of the growing fetus and changing hormone levels (1,236). The risk factors for gestational diabetes include age greater than 35 years, ethnicity from a high-risk group (Aboriginal, Hispanic, South Asian, Asian, and African), obesity (BMI of 30 kg/m² or higher), treatment with corticosteroids, pre-diabetes, family history of type 2 diabetes, and previous diagnosis with gestational diabetes (1). Although gestational diabetes may be resolved after pregnancy, it has been shown that significant hyperglycemia during pregnancy results in increased risk of subsequent development of type 2 diabetes in both mother and the new-born (1,236).

The last classification of diabetes includes those with all other specific etiologies (237). Maturity onset diabetes of the young (MODY) is the most common form of diabetes in this

category; MODY refers to any of several hereditary forms of diabetes caused by mutations in an autosomal dominant sex-independent gene, which results in disruption of the insulin production in the body (238,239). MODY 2 and MODY 3 are the most common forms of MODY due to mutations in the hepatic nuclear factor 1 alpha and glucokinase genes, respectively (238). Typically, patients diagnosed with MODY have a strong family history of either type 1 or type 2 diabetes (240). Interestingly, some people with MODY may not show clinical signs or symptoms of diabetes such as hyperglycemia therefore may go unnoticed (240). Furthermore, patients with MODY may make up as much as 5% of the clinically diagnosed patients with either type 1 or type 2 diabetes (238).

1.2 Islet amyloid

1.2.1 Amyloid diseases

Amyloid is a generic term used to describe the state of protein aggregation (241). It is not clear why some proteins aggregate but there are many small peptides that are able to aggregate and create fibrillar structures (241,242). To date, 27 different human peptides with propensity to form amyloid *in vivo* have been identified (241). These peptides are converted from their soluble form into misfolded aggregates that display pathological behaviors and are linked to many diseases (242).

Amyloid diseases may arise due to either localised or systemic amyloidosis (241). Localised amyloidosis is characterised by the presence of intracellular and/or extracellular amyloid deposits formed only in the organ or tissue of precursor protein synthesis (243). Some examples of local amyloidosis include type 2 diabetes and neurodegenerative disorders such as Alzheimer's, Huntington's and Parkinson's disease. Formation of islet amyloid contributes to the pathogenesis of type 2 diabetes, which will be discussed in detail in the following sections. In Alzheimer's disease, cerebral plaque formation due to intracellular Tau protein fibrils and extracellular amyloid β ($A\beta$) proteins causes oxidative and inflammatory damage to the neurons leading to neuronal death and dysfunction (244). The neuronal damage will affect brain regions, which will eventually result in progressive loss of memory and other cognitive functions in patients with Alzheimer's disease (245).

In contrast, all systemic amyloidosis are typically caused by extracellular amyloid where the precursor protein responsible amyloid formation is expressed and secreted from a distinct organ/tissue different from the major site of amyloid deposition (243). To date, 15 different misfolded proteins have been shown to cause systemic amyloidosis (241). In systemic amyloidosis, amyloidogenic proteins are released and spread to other organs affecting their function (246). Thus, patients with systemic amyloidosis often suffer from multiple organ failure and share many non-specific symptoms such as fatigue, weakness, and weight loss (243). The most commonly diagnosed systemic amyloidosis is the light-chain amyloidosis (247). The light-chain amyloidosis is caused by extracellular deposition of immunoglobulin light chain (LC), which is the first amyloid protein identified and biochemically characterised in humans, or the fragment of a LC that is produced by abnormally proliferating monoclonal plasma cells in the bone marrow (247). Hyper-secreted LCs then deposit to various organs, causing organ failure and death (247).

1.2.2 Definition and morphology of islet amyloid

Islet amyloid was first discovered in 1901 by two independent groups, initially described as ‘islet hyalinization’ found in association with diabetes mellitus yet without much knowledge for its function (248,249). Interestingly, these hyaline substances have been also observed in non-diabetic individuals (especially in elderly population), although the extent of amyloid formation was much less than that found in patients with type 2 diabetes (250,251). Later, these hyaline substances found in non-diabetic and diabetic individuals were recognised as a form of localised amyloidosis (252).

Since then, several histological approaches have been used to detect islet amyloid formation and to describe the morphology of islet amyloid deposits. Congo red is a commonly used amyloid-binding dye that serves as a gold standard for the diagnosis of amyloid (253). Unstained amyloid normally exhibits very weak diachronic birefringence. Once stained with Congo red, amyloid exhibits distinctive apple green (or yellow green) birefringence under the polarised light (254). Other stains such as thioflavin T and S, methyl violet, and sulphonated Alcian blue have also been used to detect amyloid deposits in the islets (253). Using various

histological methods, researchers have shown that islet amyloid forms within the endocrine islet area and is not found systemically or in the exocrine portion of the pancreas (255). Smaller amyloid deposits are typically found between the capillary walls and islet cells whereas larger deposits have been shown to form large masses that affect the islet structure (255).

1.2.3 Islet amyloid polypeptide (IAPP)

1.2.3.1 Discovery of IAPP

The major component of islet amyloid has been identified as islet amyloid polypeptide (IAPP or amylin), which is a normal secretory product of the pancreatic insulin-producing β -cells (256,257). Initially, insulin and proinsulin molecules, which are the major islet secretory peptides, were the prime candidates for the core constituent of the islet amyloid. However, intensive purification of amyloid extracted from an insulin-producing pancreatic human tumour using concentrated formic acid followed by NH_2 -terminal amino acid sequence analysis led to a partial identification of a novel peptide which did not resemble insulin or its precursors but shared 50% homology with the neuropeptide calcitonin gene-related peptide (258). During that period, another independent group confirmed this finding through extraction of amyloid from three type 2 diabetic patients and purification of the peptide by gel filtration and reverse-phase high-performance liquid chromatography (257). Further characterization of the peptide purified from insulinoma as well as human and feline islet amyloid deposits showed that this peptide is secreted from insulin-producing β -cells (256-260). This purified peptide was first introduced as 'insulinoma amyloid peptide' (258), later was called 'diabetes-associated peptide' (257), and finally was named 'IAPP' (256) or 'amylin' (261). The term IAPP will be used throughout this thesis.

1.2.3.2 IAPP gene expression and post-translational modifications

IAPP is mainly expressed in the pancreatic β -cells. Moreover, IAPP expression has been also observed in the gastrointestinal tract in rodents, humans, and cats, as well as in the sensory neurons of rats (262-264). The gene encoding IAPP is located on the short arm of chromosome 12, whereas insulin and other members of the calcitonin family are encoded by a gene on

chromosome 11 (265). However, IAPP and insulin genes contain similar promoter elements that are shown to share the same transcriptional regulators (266). For instance, the transcription factor pancreatic and duodenal homeobox 1 (PDX-1) has been shown to regulate both IAPP and insulin gene expression in response to glucose in β -cells (267).

Upon expression, unprocessed IAPP (proIAPP) undergoes similar prohormone processing as proinsulin in β -cells (193). Figure 1-1 illustrates the series of proteolytic and post-translational modifications that proIAPP undergoes to produce mature IAPP. Figure 1-2 depicts the process by which proIAPP is processed and secreted from β -cells. Briefly, IAPP is produced in the ER as an 89 amino acid pre-proIAPP form containing a 22 amino acid NH₂-terminal signal sequence and two short flanking peptides (268,269). When the signal sequence is removed, it forms a 67 amino acid proIAPP, which is cleaved at the dibasic sites near the carboxy-terminus in the Golgi and amino-terminus in the secretory granules by prohormone convertase 1/3 (PC1/3) and prohormone convertase 2 (PC2), respectively, to produce the mature 37 amino acid IAPP (270,271). It has been shown that PC2 can process proIAPP at both its NH₂- and COOH-terminal cleavage sites in the absence of PC1/3 (271). Finally, mature IAPP undergoes post-translational modifications such as formation of the disulfide bridge between the cysteine residues at positions 2 and 7 and amidation of the COOH-terminal glycine residue prior to release from the β -cell granules for its full biological activity (272).

1.2.3.3 IAPP secretion and degradation

Mature IAPP is co-localised with insulin in the islet β -cells as demonstrated by the presence of both IAPP and insulin immunoreactivity in the β -cell secretory granules (273-275). The hexameric zinc-bound insulin molecules are stored in the core of the mature secretory granules while IAPP along with other components such as C-peptides are found in the halo region of the granule (273). The molar ratio between IAPP and insulin in the secretory granules is about 1-2:50 (193). Once released, the human plasma IAPP concentration is about 1-2% of insulin in healthy individuals within the picomolar range (4-25 pmol/l) (193,276). Similarly, the circulating levels of IAPP reported in rats range between 3-5 pmol/l at fasting and 15-20 pmol/l upon glucose stimulation (277).

Despite intensive investigation, the specific receptor for IAPP is not yet known. It has been suggested that IAPP may bind to the receptor activity-modifying proteins (RAMPs), which are single domain proteins that bind to the calcitonin receptor, to mediate its action (278,279). It is likely that the specific combinations of RAMPs with calcitonin receptors can produce high-affinity IAPP receptors as demonstrated by Zumpe *et al.* (280). Accordingly, expression of RAMPs and calcitonin receptors has been observed in IAPP-binding sites in the pancreatic β -cells and some areas of the brain (281,282).

Although the majority of secreted insulin is degraded in the liver, IAPP is normally eliminated from the body through the kidney as with the C-peptides (283). Alternatively, insulin degrading enzyme (IDE), a zinc metalloprotease that breaks down insulin, may be also involved in IAPP degradation (284). In support, treatment with IDE inhibitor bacitracin has shown to reduce IAPP degradation, which leads to increased amyloid formation and toxicity (285). Moreover, another zinc-containing metalloprotease called neprilysin has also been shown to degrade IAPP (286). Both IDE and neprilysin can also degrade the A β -peptide, another amyloidogenic protein that plays a key role in the pathological development of Alzheimer's disease (287,288).

1.2.3.4 Physiological role of IAPP

Although the physiological role of insulin in our body is well understood, the function of IAPP in humans is still not clear. The sequence of IAPP is highly conserved among different mammalian species with only a few amino acid substitution differences, indicating that it may have an important physiological function in the body (193,289). As IAPP is co-produced and co-released in parallel with insulin in response to food intake, it is reasonable to believe that IAPP may have a function in glucose regulation and metabolism. Several studies have reported that IAPP is involved in maintaining the body's glycemic control through the mechanisms affecting the gastrointestinal tract, central nervous system and islet cells (193,290,291).

In the gastrointestinal tract, IAPP has an inhibitory effect on gastric emptying (291-293). Slower gastric motility means reduced increments of glucose absorption during digestion and increased satiety, both of which can reduce the amount of food intake thereby decrease

postprandial blood glucose levels (291,294,295). Moreover, IAPP may exert its effects on gastric motility and satiation by affecting the action of other hormones such as cholecystokinin, leptin, and incretins that are known to inhibit gastric rate and regulate nutrient intake (296). Since pathologically increased gastric emptying is one of the characteristics of patients with type 1 or type 2 diabetes (297,298), it has been suggested that the lack of IAPP production and/or diminished physiological effects of IAPP in controlling gastric motility may contribute to the postprandial glucose excursions seen in these patients (193).

Previous studies have shown that the anorectic effects of IAPP are also mediated through the central nervous system (290,299). Unlike other peptide hormones such as cholecystokinin, which inhibit gastric emptying via their effects on the afferent vagus nerve fibers, IAPP likely elicits its anorectic effects by directly binding to the brain. In support, performing afferent vagotomy in rats resulted in no significant change in their gastric emptying rate as compared to control rats (294,300). Moreover, the binding sites for IAPP have been reported at several brain regions such as the nucleus accumbens and area postrema (301,302). Also, IAPP immunoreactivity has been observed in the hypothalamus and basal ganglia of rats and monkeys (303-305).

Lastly, physiological level of IAPP may affect the secretion of other islet cell hormones, namely insulin secretion from β -cells and glucagon secretion from α -cells (193). Some studies have shown that IAPP significantly inhibits insulin release from the β -cells even at low concentrations (75 pmol/l) (306-308). However, other studies have reported no inhibitory effect of IAPP on insulin response to glucose at different concentrations (10 pmol/l to 1 μ mol/l) (309-311). Therefore, it is still debatable whether IAPP has an inhibitory effect on glucose-stimulated insulin secretion from islet β -cells. Similarly, there are contrasting reports regarding the role of IAPP on glucagon secretion in response to glucose. Previous studies have shown that IAPP has no effect on hypoglycemia-stimulated glucagon secretion *in vivo* (308,312) whereas another study performed on isolated mouse islets *in vitro* has reported that low concentrations of IAPP (10^{-10} - 10^{-8} mol/l range) can suppress both basal and hypoglycemia-stimulated glucagon secretion (313). However, studies have shown that IAPP can inhibit arginine-stimulated

glucagon secretion as demonstrated *in vivo* in rats as well as *in vitro* in isolated mouse islets (308,313).

IAPP analogs such as pramlintide (Symlin) have been developed and are currently used as a treatment option for patients with type 1 and type 2 diabetes (314,315). Patients treated with pramlintide show sustained improvement in glycemic control with slower gastric emptying, reduced food intake, inhibition of inappropriate glucagon secretion, and increased acute first-phase postprandial insulin response, which led to significantly reduced HbA1C levels and body weight in these patients (315,316). These studies suggest that restoring the physiological effects of IAPP may be used as a feasible approach for treatment of type 1 and type 2 diabetes.

1.2.3.5 Fibrillogenesis of IAPP

Despite high similarity observed between the amino acid sequences of IAPP and calcitonin gene-related peptide (CGRP) (258,265), which is a 37 amino acid long neuropeptide primarily released by both peripheral and central neurons that has been implicated in the underlying pathology of pain migration and inflammation (317,318), IAPP can be easily induced to form amyloid fibrils *in vitro* but CGRP typically does not form fibrils (289). Comparison of the amino acid sequences of IAPP and CGRP shows that highest sequence homology can be found in the amino- and carboxy-terminal regions of two peptides with greatest sequence differences found between the amino acid residues 20 to 29 (IAPP₂₀₋₂₉) (319). This finding led to the hypothesis that the amino acid composition in the IAPP₂₀₋₂₉ region may be an important factor in IAPP fibrillogenesis (319). This hypothesis has been tested in studies comparing the amino acid sequences of IAPP in the mammalian species that form amyloid with those that do not form amyloid. In rodents, IAPP does not form fibrils due to the presence of three proline residues (at positions 25, 28 and 29) in the IAPP₂₀₋₂₉ region which disrupts the β -sheet formation required for IAPP fibrillogenesis while no proline residues can be found in the IAPP₂₀₋₂₉ region of the fibrillogenic IAPP in humans (Figure 1-3a) (289,320-322). Based on these findings, the IAPP₂₀₋₂₉ region has been termed ‘amyloidogenic region,’ which is important for IAPP fibril formation. Furthermore, subsequent studies have shown that other regions of IAPP may also contribute to the amyloidogenic potential of IAPP in humans. In particular, amino acid residues 14-20 have been suggested to facilitate IAPP fibrillogenesis (323).

Using thioflavin T assay, the kinetics of IAPP fibril formation has been characterised (Figure 1-3b). The initial lag phase which represents the nucleation process where IAPP monomers aggregate to form IAPP oligomers that will serve as a template for fibrillar IAPP, followed by a sigmoidal transition phase where elongation of protofibrils into mature fibrils occur (322,324,325). Then, progressive fibril formation leads to a steady state where a stable equilibrium exists between fibrillar IAPP and residual monomeric IAPP (322,325). Once formed, amyloid fibrils have unbranching structures varying in diameter (between 5 - 10 nm) and of indefinite length (193).

Spontaneous aggregation of fibrillogenic IAPP *in vitro* occurs within hours, depending on the peptide concentration and presence of lipids (325). It has been demonstrated that in the presence of phospholipids, hIAPP fibrillogenesis is accelerated by a reduction in the lag phase resulting in earlier fibril formation (326-328). Accordingly, an *in vitro* analysis showed a ten-fold increase in the rate of hIAPP fibril formation in the presence of synthetic and human tissue-derived phospholipids (328). Furthermore, in the presence of anionic lipid-rich membranes, IAPP fibrillogenesis occurs within minutes as compared to hours in the absence of the membrane (328,329). Thus, it has been suggested that the presence of negatively charged lipids in the cell membrane can enhance the nucleation process that is required for initiating the growth of stable fibrils (322,326,328,329).

Finally, other components of islet amyloid deposits such as serum amyloid P component (SAP), apolipoprotein E (ApoE) and heparan sulphate proteoglycans (HSPG) accumulate near the site of IAPP fibrillogenesis (330). Previous studies have shown that these proteins may also contribute to formation and/or stabilization of IAPP fibrils (331-334).

1.2.4 Association between islet amyloid formation and diabetes

1.2.4.1 Islet amyloid formation in type 2 diabetes

Islet amyloid is a pathological lesion in the pancreas of more than 90% of patients with type 2 diabetes (330,335-337). Amyloid deposits have also been observed in the post-mortem

pancreas of normal individuals, although the degree of amyloid formation and the number of affected islets were much lower than those from type 2 diabetic patients (193,250,251,338).

Despite intensive studies in the past decade, it is not clear why normally soluble human IAPP (hIAPP) molecules form toxic amyloid aggregates in type 2 diabetes. Several factors have been proposed to contribute to islet amyloid formation in type 2 diabetes. First, destabilization of IAPP due to insufficient proinsulin processing has been implicated in IAPP aggregation and subsequent amyloid formation (273). Previous studies have shown that insulin is a potent inhibitor of IAPP fibrillogenesis (193,339,340). Insulin prevents formation of IAPP fibrils *in vitro* by forming heteromolecular complexes with IAPP that can stabilise the IAPP molecules (273,340-342). One study has shown that the inhibitory action of insulin on IAPP fibril formation is dependent on the B-chain, which contains the interacting domain that specifically binds to IAPP (343). Conversely, proinsulin does not bind to IAPP (340). These findings suggest that increased proinsulin to insulin ratio (or decreased insulin production) due to improper processing of proinsulin as a consequence of high insulin demand in type 2 diabetes may reduce the stabilizing effects on IAPP by insulin and therefore contribute to IAPP fibril formation (340).

Second, inappropriate processing of proIAPP may be an important factor in hIAPP fibrillogenesis as the enzymes involved in the proinsulin processing are also responsible for processing proIAPP into mature IAPP (270,271,344,345). Thus, conditions such as hyperglycemia associated with impaired proinsulin processing will also likely contribute to impaired proIAPP processing. In support of this notion, increased proIAPP and its partially processed intermediate forms have been detected in the amyloid deposits from patients with type 2 diabetes and in the islets cultured with elevated glucose (346,347). Interestingly, the amino-terminal cleavage fragment of proIAPP contains a heparin-binding domain that can bind to HSPG (348). As heparan sulphate is present in the islet capillary basement membrane where islet amyloid first forms *in vivo*, binding of proIAPP to heparan sulphate has been proposed to induce formation of amyloid-like fibrils or act as a template for subsequent IAPP accumulation (330,348).

Third, increased concentration of IAPP in type 2 diabetes associated with elevated co-synthesis and release of IAPP with insulin may lead to increased IAPP fibril formation. In transgenic mouse models of type 2 diabetes, extensive amyloid deposits can be detected in the homozygous transgenic mice overexpressing hIAPP as compared to hemizygous hIAPP transgenic mice (expressing only one copy of hIAPP transgene), indicating that overexpression of IAPP itself may be an important factor in amyloid formation (349,350). Lastly, previous studies have demonstrated that hemizygous hIAPP-expressing transgenic mice do not normally form amyloid *in vivo*; however, the presence of predisposing factors such as a high fat diet can initiate and/or potentiate islet amyloid formation in these animals (351-353).

Notably, islet amyloid formation is associated with a decrease in β -cell number and insulin production in human patients with type 2 diabetes (336,354) and diabetic non-human primates (355). Studies performed on diabetic non-human primates and cats have shown that islet amyloid formation preceded the development of hyperglycemia (355-357). Moreover, several studies have demonstrated that islet amyloid formation in transgenic rodents expressing hIAPP is associated with decreased β -cell mass and development of hyperglycemia (350,358-363). Furthermore, increased amyloid deposition has been correlated with decreased β -cell area and increased β -cell apoptosis in human pancreas sections from type 2 diabetic patients (354). Similarly, formation of hIAPP aggregates in cultured human and hIAPP-expressing transgenic mouse islets leads to increased caspase-3 activation (364) and β -cell death (365,366). Conversely, prevention of amyloid formation enhances both survival and function of islet β -cells (366). Taken together, these findings indicate that amyloid formation is an important factor contributing to progressive β -cell death and dysfunction in type 2 diabetes.

1.2.4.2 Islet amyloid formation in islet transplantation

A high percentage of β -cells in islet grafts undergoes apoptosis during pre-transplant islet culture and in the first days following transplantation prior to islet re-vascularization (367). Loss of β -cells during transplantation process due to both immune and non-immune factors is currently a major factor limiting the success of clinical islet transplantation. Immune-mediated β -cell death associated with allograft rejection (368,369) and autoimmune attack on transplanted β -

cells (369,370), play a major role in islet graft destruction. However, decreased graft function has also been demonstrated in auto-islet transplants in human recipients (371,372), indicating that the decrease in islet graft function cannot be explained solely by immune mechanisms and that non-immune factors also play an important role in islet graft failure. More than 50% of the grafted islet mass (mainly β -cells) is rapidly lost due to non-immune factors at early stages after transplantation (373-375). Non-immune-mediated causes of β -cell death in islet grafts include disrupted microenvironment and vasculature in newly grafted islets (374,376-380), β -cell toxic effects of administered immunosuppressive drugs (374,381-383), and islet amyloid formation associated with β -cell dysfunction (366,367).

Widespread formation of amyloid has been detected in cultured human islets as well as in islet grafts transplanted into patients with type 1 diabetes (367,384,385). Unlike in type 2 diabetes where the process of amyloid formation is slow (years), hIAPP aggregation occurs rapidly in human islets during culture (days) and following transplantation into animal models of type 1 diabetes (weeks) (193,364,386,387). Moreover, previous *in vitro* studies have shown that formation of hIAPP aggregates in human islets during culture is associated with β -cell death and that prevention of amyloid formation and/or its toxicity enhances both survival and function of human islets (364,366). Similarly, amyloid deposition has been detected in islets from hIAPP-expressing mice transplanted into diabetic mice (388), which is associated with β -cell dysfunction and death *in vitro* and graft failure *in vivo* (365,389,390). Taken together, these findings suggest that amyloid formation is an important non-immune factor contributing to selective loss of β -cells in conditions associated with islet amyloid formation not only in type 2 diabetes but also in cultured and transplanted human islets.

1.2.4.3 Mechanisms of amyloid-induced β -cell death

The association between amyloid formation and cell death has been shown in pathogenesis of different types of amyloid diseases (391). In the context of type 2 diabetes, studies have shown that both intracellular and extracellular accumulation of islet amyloid is associated with β -cell death and dysfunction (366,392,393), although the site of initial amyloid formation is still under debate (289,394,395). Islet amyloid fibrils are mainly found extracellular

adjacent to islet β -cells (396,397), whereas intracellular amyloid-like aggregates have also been reported in β -cells (392,393). Although several studies have demonstrated that fibrillar hIAPP is toxic to β -cells (365,397,398), recent studies suggest that smaller forms of hIAPP aggregates (oligomers and protofibrils) are more toxic to β -cells than larger hIAPP fibrils and play a major role in islet amyloid-induced β -cell death (393,394). However, the molecular mechanisms that underlie hIAPP-induced β -cell death and the forms of hIAPP that contribute to this process are still under investigation. To date, several mechanisms have been proposed to contribute to hIAPP-induced β -cell death as described below and summarised in Figure 1-4.

1.2.4.3.1 Membrane disruption

The discovery of the neurotoxic mechanism of amyloidogenic A β peptide in Alzheimer's disease, which showed that A β forms ion-selective membrane channels in lipid bilayers to induce neuronal cell death (399,400), led to the idea that the cell membrane might be a target of hIAPP toxicity in β -cells. In support of this notion, histological studies have shown that extracellular hIAPP fibrils are deposited near the islet cell membranes, which is accompanied by disrupted membrane morphology (395). In addition, electron microscopic data have revealed that the extracellular hIAPP aggregates interact with the plasma membrane of MIN-6 mouse insulinoma β -cells (401). Moreover, extracellular hIAPP aggregates can cause defects in plasma membrane morphology as demonstrated by the overlap between the areas where extracellular hIAPP interact with the β -cell membrane and the areas of irregularly shaped membranes with deep invaginations (401).

In 1996, Mirzabekov *et al.* showed that different concentrations of synthetic hIAPP peptide (1-10 $\mu\text{mol/l}$) can form voltage-dependent non-selective ion-permeable channels in the planar phospholipid bilayer membranes whereas non-amyloidogenic rat IAPP (rIAPP) does not (402). Moreover, using various microscopic techniques such as atomic force microscopy, researchers have shown that hIAPP forms membrane pores, which consist of five-subunits each representing a hIAPP monomer (403). Importantly, these hIAPP membrane pores allow non-selective movement of ions, which causes destabilization of the intramembrane ionic environment and β -cell death (322,403).

Studies have demonstrated that monomeric hIAPP displays a strong tendency to insert itself into phospholipid membranes (404,405). Based on these findings, studies have elucidated the hIAPP residues that are important for interaction with the lipid membranes (405,406). It has been demonstrated that the N-terminal fragment of hIAPP (hIAPP₁₋₁₉), where positively charged residues are located, has a pronounced ability to insert itself into the phospholipid monolayers than fragments of the hIAPP amyloidogenic region (hIAPP₂₀₋₂₉) (405). It has been suggested that while hIAPP₁₋₁₉ fragment inserts itself into the membrane, the fibrillogenic hIAPP₂₀₋₂₉ fragment exposed to the extracellular matrix can potentiate aggregation with other hIAPP molecules (405,406). These findings suggest that the N-terminal residues of hIAPP may have a role in mediating the membrane toxic effects of hIAPP.

hIAPP oligomers (prefibrillar aggregates) can also form ion channels, which allow movement of ions such as calcium ions and thereby perturb the cellular ionic homeostasis (407,408). Trimeric and hexameric IAPP oligomers have been suggested as the major oligomeric forms of IAPP found in the membrane pore complexes (403). In addition to forming membrane pores, hIAPP oligomers can induce membrane disruption by perforating the membrane directly (193).

In contrast, recent studies have shown that the hIAPP fibril growth at the cell membrane can cause membrane damage rather than its specific forms (oligomers or fibrils) (322). Using the model membranes called large unilamellar vesicles, studies have demonstrated that hIAPP aggregates interact with the membrane in β -sheet conformation, which is characteristic of hIAPP fibril formation (329). This is further supported by the finding that the kinetics of synthetic hIAPP fibril formation show close resemblance to the kinetics of membrane damage (322). Similarly, A β fibril formation (and not a particular A β species) has been shown to induce membrane damage in neuronal cells (409). In this proposed mechanism, the growth of rigid hIAPP fibrils at the membrane leads to deformation of the membrane structure and cell death. Signs of membrane disruption such as membrane blebbing and vesicle budding have been observed in cells treated with fibrillogenic synthetic hIAPP (410,411).

Finally, it has been shown that polyanion-like HSPG present on the cell membranes of

both islet β -cells and capillary cells can bind to hIAPP. Therefore, it has been proposed that binding of hIAPP to HSPG, and in particular the basement membrane proteoglycan perlecan, may initiate nucleation and aggregation of hIAPP as well as block nutrient flow, leading to β -cell death (348,412).

1.2.4.3.2 ROS production and oxidative stress

It has been proposed that the ability of hIAPP oligomers to perforate membranes can also disrupt the membranes of intracellular organelles such as the mitochondria (193). Loss of mitochondrial membrane integrity can cause mitochondrial dysfunction and production of ROS (413). Increased ROS production induces oxidative stress mechanisms and eventually β -cell demise that involves activation of multiple cellular pathways as described in Chapter 1.1.6.2.2.

In transformed rat insulinoma β -cells, increased oxidative stress responses have been reported when treated with synthetic hIAPP (414). Specifically, this study has shown that treatment with hIAPP results in accumulation of intracellular ROS, which leads to increased lipid peroxidation (oxidative degradation of lipids) in β -cells (414). Similarly, in the isolated islets from hIAPP-expressing transgenic mice, formation of amyloid is associated with increased ROS levels and β -cell apoptosis in a time-dependent manner (415). Moreover, it has been shown that during short-term culture (48 h), antioxidant treatment reduces amyloid-induced ROS production, but does not prevent β -cell apoptosis in the islets from hIAPP-expressing mice (415). However, under long-term culture conditions (144 h), antioxidant treatment has been shown to reduce intracellular ROS levels and β -cell apoptosis in these islets (415). Overall, this study indicates that short-term exposure to amyloid-induced ROS and oxidative stress does not mediate β -cell apoptosis, but chronic exposure to ROS and oxidative stress may induce β -cell apoptosis that can be prevented by antioxidant treatments (415).

Similarly, the mechanism of $A\beta$ -induced neurotoxicity in Alzheimer's disease involves mitochondrial dysfunction, which promotes ROS production and oxidative stress (416-418). For example, in isolated mitochondria, $A\beta$ has been shown to alter mitochondrial enzyme activity, damage the electron transport chain components, and induce mitochondrial permeability

transition pore opening, which lead to cytochrome c release and apoptosis in neurons (417,419-422).

1.2.4.3.3 Endoplasmic reticulum stress and unfolded protein response

ER stress and defective UPR have been shown to mediate hIAPP-induced β -cell death. ER stress is caused mainly due to accumulation of proteins, particularly unfolded or misfolded proteins in the ER lumen, and/or disruption of intracellular calcium ion balance as detailed in Chapter 1.1.3.4 (423). The UPR serves as a protective mechanism to compensate for the effects of ER stress on cells. However, severe or prolonged ER stress eventually leads to impairment of UPR and activation of ER stress-mediated β -cell apoptosis (423).

Recent studies suggest that extracellular hIAPP aggregates have toxic effects on the ER, leading to ER dysfunction and ER stress-mediated β -cell death (322,424). In MIN6 β -cells, exogenously applied hIAPP has been shown to induce ER-stress and UPR as demonstrated by: 1) morphological evidence of ER dysfunction such as increased ER size likely due to accumulation of protein aggregates as well as sustained elevation of intracellular calcium ions, which supports the notion that hIAPP can deregulate intracellular calcium ion homeostasis; 2) increased gene and protein expression of HSP90 family of molecular chaperones; and 3) impairment of the ubiquitin-proteasome pathway (401). Importantly, over time, extracellular hIAPP-induced activation of the ER stress response has been shown to contribute to increased β -cell apoptosis in MIN6 cells (401). Similarly, disruption of ER homeostasis and UPR followed by increased β -cell apoptosis has been observed in isolated human islets in the presence of synthetic hIAPP aggregates (401).

The role of ER stress mechanism in mediating β -cell toxic effects of endogenously produced hIAPP is still under investigation. A study by Huang *et al.* (425) showed that adenoviral-mediated endogenous expression of hIAPP (but not non-fibrillogenic rIAPP) increased expression of ER stress marker C/EBP homologous protein (CHOP) in INS-1 β -cells. As CHOP mediates ER-stress induced apoptosis, higher expression of CHOP was associated with increased β -cell apoptosis in hIAPP-transduced INS-1 cells (425). Consistent with the findings in transduced INS-1 cells, amyloid formation by aggregation of biosynthetic hIAPP in

islets from the HIP rats (a transgenic rat model expressing endogenous hIAPP) and pancreas of type 2 diabetic patients was associated with increased CHOP expression as compared to islets from non-transgenic control rats and pancreas from non-diabetic patients, respectively (425). Moreover, ER stress-mediated defects in endoplasmic reticulum associated protein degradation (ERAD) and UPR have been proposed to initiate and/or potentiate amyloid formation, which could further mediate β -cell toxic effects of biosynthetic hIAPP aggregates. It has been suggested that elevated production of hIAPP, in parallel with increased insulin biosynthesis and impaired processing of proIAPP in the ER-Golgi system due to ER dysfunction in type 2 diabetes, may contribute to hIAPP aggregation and fibril formation (425,426). In support, studies have shown that the accumulation of hIAPP aggregates in the ER due to ER dysfunction can further exacerbate hIAPP fibril formation (424,425).

However, Hull *et al.* (427) have suggested a contrasting view on the role of ER stress in hIAPP-induced β -cell death. In this study, amyloid formation in the pancreas of both type 2 diabetic human and hIAPP-expressing transgenic mice was associated with increased β -cell death but not expression of ER stress markers such immunoglobulin heavy chain-binding protein, CHOP, and X-box binding protein 1. Moreover, expression of ER stress markers did not significantly change in cultured islets from hIAPP-expressing mice as compared to control mice (427). Based on these data, Hull *et al.* concluded that ER stress may not play a significant role in mediating hIAPP-induced β -cell death (427).

1.2.4.3.4 Impaired autophagy-lysosomal degradation pathway

Impaired autophagy has been proposed to be a factor in hIAPP β -cell toxicity. Autophagy (autophagocytosis) is the basic catabolic mechanism that involves degradation of dysfunctional or misfolded proteins as well as defective cellular components (428). Autophagy allows the recycling of degraded cellular components thereby playing an important role in maintaining cellular energy levels (428). During this process, targeted cellular constituents are isolated from the rest of cell within a double-membraned vesicle known as phagophore to form an autophagosome (429,430). The autophagosome then fuses with an enzyme-rich lysosome to form an autolysosome, where the engulfed materials become degraded and recycled (430).

Importantly, the autophagosome provides for a specialised type of autophagy that is used to degrade intracellular protein aggregates such as hIAPP aggregates (protofibrils), since they are too large to be degraded by the ubiquitin-proteasome pathway (430). Furthermore, both phagophore and autophagosome formation have been linked to ER stress in response to formation of hIAPP aggregates (431).

In HIP rats, overexpression of hIAPP is associated with defects in autophagy and lysosomal degradation manifested as increased number of autophagosomes paired with increased accumulation of p62, a marker for impaired autophagy and/or lysosomal degradation (432). Moreover, disruption of autophagy has been associated with increased intracellular accumulation of hIAPP aggregates and hIAPP-induced β -cell apoptosis (431-435). Conversely, in hIAPP-transduced INS-1 cells and human islets, stimulation of autophagy has been shown to reduce cellular IAPP content and protect β -cells against the toxic effects of biosynthetic hIAPP aggregates (432,433). Taken together, these findings suggest that aggregation of hIAPP may promote impaired autophagy in β -cells and contribute to hIAPP-induced β -cell death.

1.2.4.3.5 Caspase-mediated apoptotic pathways

Despite intensive studies in the past decade, the apoptotic signalling pathways that mediate amyloid-induced β -cell death in primary islets are still not fully understood. There are two major cell apoptotic pathways as illustrated in Figure 1-5: the Fas cell death receptor-mediated (extrinsic) pathway and the mitochondrial-mediated (intrinsic) pathway. Regardless of origin of the death stimulus, both apoptotic signalling pathways converge on a common set of machinery that is activated by a family of caspases (cysteine-aspartate specific proteases), also known as the interleukin-1 β converting enzyme family proteases (436,437). Importantly, *in vitro* studies have reported activation of caspases in β -cells following exposure to hIAPP aggregates associated with increased β -cell death. A previous study has shown that exposure to supra-physiological concentrations of synthetic hIAPP aggregates can induce activation of caspases in transformed islet β -cells (438). Moreover, an earlier study from our research group has demonstrated the critical role of caspase-3, the terminal effector enzyme in caspase cascade, in β -cell toxicity mediated by endogenously formed hIAPP aggregates in human and hIAPP-

expressing transgenic mouse islets (364). This study further showed that prevention of caspase-3 activation reduces hIAPP-induced β -cell death, resulting in preservation of β -cell mass and improved β -cell function (364).

Furthermore, the interaction between caspases with other apoptotic pathways including stress-activated protein kinase pathways (JNK and p38) may be also important in mediating amyloid-induced β -cell death (439-442). Previous studies suggest that JNK pathway may be partially activated by caspase-8 and that activation of JNK likely activates caspase-3 and -1 indirectly in hIAPP-treated islet β -cells (439,440). Moreover, selective inhibition of p38 signalling pathway has been shown to suppress hIAPP-induced caspase-3 activation and β -cell death (442). In line with these findings, multiple caspases have been shown to mediate A β -induced apoptotic neuronal death pathways in the pathogenesis of Alzheimer's disease (443-446).

Taken together, while growing evidence indicates that caspases play a significant role in amyloid-induced β -cell toxicity, the apoptotic signalling pathways and key caspases involved in this process have yet to be identified.

1.2.4.4 Transgenic mouse models of human islet amyloid formation

Deposition of islet amyloid has been reported in pancreatic islets from humans, non-human primates, cats and degu (193). Islet amyloid formation in these mammalian species has been linked to spontaneous development of type 2 diabetes (193,354,447,448). Using humans as a model to study islet amyloid is restrictive due to ethical considerations. Also, studies in non-human primates and other aforementioned mammalian species can be costly and impractical. Rodents such as rats and mice are laboratory animals commonly used for research in diabetes to circumvent the problems associated with pancreas availability and cost. However, the rodent models of diabetes do not form amyloid as rodent IAPP is non-fibrillogenic due to proline residue substitutions in the amyloidogenic region of the hIAPP (Figure 1-3a) (193). Therefore, in order to study the pathological characteristics of islet amyloid and its implications in type 2 diabetes, several transgenic rodent models have been developed.

Different research labs have created various transgenic mouse and rat models expressing hIAPP and have characterised these rodent models (350,351,353,359-363,392,449-454). Table 1-2 lists the hIAPP-expressing transgenic rodent models and summarises their respective phenotypes. In these transgenic rodent models, hIAPP expression has been specifically targeted to β -cells through fusion of the hIAPP transgene to the rat insulin II promoter (455). These transgenic rodent models have provided valuable experimental tools to investigate the role of islet amyloid formation and its β -cell toxicity in type 2 diabetes.

1.3 Rationale, hypothesis and objectives

Type 2 diabetes is characterised by progressive β -cell dysfunction and reduced β -cell mass. Islet amyloid, mainly formed by aggregation of hIAPP, contributes to impaired β -cell function and increased β -cell death in type 2 diabetes. Islet amyloid also forms in cultured and transplanted human islets during transplantation process associated with progressive loss of β -cells which may eventually lead to islet graft failure. Despite extensive efforts in the past decade, the molecular mechanisms underlying islet amyloid-induced β -cell dysfunction and death remain elusive. Extensive *in vitro* studies using supraphysiological concentrations ($\mu\text{mol/l}$) of synthetic hIAPP have revealed different mechanisms for how hIAPP aggregates destroy β -cells. However, it appears that mechanisms by which endogenously formed hIAPP aggregates at physiological concentrations (pmol/l) mediate β -cell dysfunction and apoptosis are far more complicated than those demonstrated *in vitro* and likely involve the activation of both non-immune and immune cellular pathways. Moreover, this gap of knowledge in our understanding of the mechanisms behind islet amyloid-induced β -cell toxicity has been a rate-limiting step in development of therapeutic approaches to prevent this process in patients with type 2 diabetes and human islet recipients with type 1 diabetes.

The overall goal of this project was to identify the molecular mechanisms by which endogenous formation of islet amyloid exerts toxic effects on primary islet β -cells, and ultimately develop new ways to prevent islet amyloid-induced β -cell death and dysfunction in conditions associated with islet amyloid formation. In particular, we hypothesised that ***islet amyloid-induced β -cell death is mediated, at least partially, by activation of the Fas apoptotic***

pathway and that blocking the key steps in this pathway may provide a new approach to protect β -cells from amyloid toxicity in type 2 diabetes and islet grafts in type 1 diabetes. In order to address this hypothesis, we have proposed the following four objectives:

Objective 1: To examine if hIAPP aggregates can induce Fas expression in islet β -cells and whether deletion of Fas can protect β -cells from amyloid toxicity (Chapter 3).

We first examined if exogenously applied hIAPP aggregates can induce Fas expression in transformed and dispersed primary islet β -cells. Next, we used two *ex vivo* models of islet amyloid formation, cultured human and hIAPP-expressing transgenic mouse islets, to determine if endogenously formed hIAPP aggregates can upregulate Fas in β -cells. To further confirm the correlation between amyloid formation and β -cell Fas expression, we examined if inhibition of amyloid formation can prevent β -cell Fas upregulation in cultured islets. Finally, we generated a new transgenic mouse model with β -cell specific hIAPP expression and Fas deletion to test if deletion of Fas can protect islet β -cells from amyloid toxicity.

Objective 2: To test if amyloid-induced Fas upregulation can promote Fas and FasL interaction, leading to the activation of Fas apoptotic pathway mediated by caspase-8 (Chapter 4).

In this objective, we used dispersed islet cells to examine if Fas upregulation mediated by exogenously applied hIAPP aggregates can induce activation of caspase-8, the key upstream enzyme in the Fas-mediated apoptotic pathway. Next, we used cultured human and hIAPP-expressing transgenic mouse islets to determine if endogenously formed hIAPP aggregates can induce activation of caspase-8, leading to β -cell apoptosis. Finally, we generated a new transgenic mouse model expressing hIAPP but lacking caspase-8 in β -cells to examine if blocking caspase-8 signalling can prevent the cytotoxic effects of islet amyloid.

Objective 3: To investigate the role of IL-1 β signalling in amyloid formation and amyloid-induced Fas upregulation in β -cells (Chapter 5).

Studies in Chapters 3 and 4 suggested that the pro-inflammatory cytokine IL-1 β may play an important role in mediating amyloid-induced Fas upregulation. Thus, in this objective, we examined if IL-1 β mediates amyloid-induced Fas upregulation in human islet β -cells. We next tested if blocking the IL-1 β signalling by treatment with IL-1 receptor antagonist anakinra can prevent amyloid-induced β -cell Fas upregulation and apoptosis in cultured human islets. Finally, we examined if IL-1 β induced β -cell dysfunction can potentiate amyloid formation and if enhancing β -cell function by blocking IL-1 β signalling reduces amyloid formation.

Objective 4: To develop a new approach to preserve β -cells by reducing amyloid formation and its β -cell toxicity (Chapter 6).

Impaired proIAPP processing due to β -cell dysfunction is an important contributing factor to hIAPP misfolding and aggregation. In this objective, we used a GLP-1 receptor agonist exenatide as an independent approach to test if improving β -cell function can restore impaired proIAPP processing in cultured human islets. Next, we examined if restored proIAPP processing by exenatide can reduce islet amyloid formation and protect islet β -cells from amyloid β -cell toxicity.

1.4 Significance

The overall purpose of the studies proposed in this thesis is to enhance our understanding on the role of islet amyloid in the pathogenesis of type 2 diabetes and islet graft failure in type 1 diabetes. Our findings from these studies may lead to development of new therapeutic strategies to preserve β -cells in conditions associated with islet amyloid formation thereby slow down progression of type 2 diabetes and improve long-term survival of islet grafts in clinical islet transplantation.

Table 1-1. Current diagnostic criteria for pre-diabetes and diabetes.

Condition	Criteria
Normal	Fasting plasma glucose between 3.8 – 5.5 mmol/l or Plasma glucose of ≤ 7.7 mmol/l 2 hours after 75 g oral glucose challenge or HbA1C $< 5.7\%$
Pre-diabetes	IFG: Fasting plasma glucose between 6.1 – 6.9 mmol/l or IGT: Plasma glucose between 7.8 – 11.1 mmol/l after 75 g oral glucose challenge or HbA1C between 6.0 – 6.4%
Diabetes	IFG: Fasting plasma glucose ≥ 7.0 mmol/l or IGT: Plasma glucose of ≥ 11.1 mmol/l 2 hours after 75 g oral glucose challenge or HbA1C $\geq 6.5\%$ or Random plasma glucose level ≥ 11.1 mmol/l

IFG: impaired fasting glucose; IGT: impaired glucose tolerance; HbA1C: Glycated hemoglobin A1C

Table 1-2. Transgenic rodent models expressing hIAPP and their characteristics.

Species	Transgene	Background	Characteristics
Mouse	hIAPP ^{+/-}	FVB/N	This mouse model normally do not develop diabetes unless in the presence of growth hormones and dexamethasone. Small intracellular and extracellular amyloid deposits are detected in this model, which are associated with hyperglycemia, β -cell degeneration and dysfunction. (359)
Mouse	hIAPP ^{+/+}	FVB/N	hIAPP homozygous mice spontaneously develop hyperglycemia and diabetes (by 10-14 weeks of age) due to increased hIAPP gene expression compared to hemizygous hIAPP ^{+/-} mice. Islet amyloid forms in these mice along with a rapid decline in β -cell mass and insulin secretory capacity. (350)
Mouse	hIAPP ^{+/-}	A ^{vy} /A	These mice were developed by cross-breeding hemizygous hIAPP transgenic mice with A ^{vy} /Agouti mice (a mouse model of obesity). Only obese (but not lean) mice develop diabetes by 15 weeks of age. These mice are characterised by significantly reduced β -cell mass (~80%) and increased β -cell apoptosis (~10-fold) compared to wild-type mice. (360,362)
Mouse	hIAPP ^{+/-}	C57BL/6J	These mice do not spontaneously develop diabetes but exhibit impaired glucose stimulated insulin secretion <i>in vitro</i> (451) and <i>in vivo</i> (449). Accumulation of amyloid fibrils has been detected in the β -cell secretory granules of this model by electron microscopy (392,450).
Mouse	hIAPP ^{+/-}	C57BL/6J x DBA	These mice do not spontaneously develop diabetes but demonstrate impaired glucose tolerance (452,456). Extensive amyloid deposits are observed in male mice but are rarely found in female mice (351). In these mice, the presence of diabetogenic factors such as elevated dietary fat (353) and obesity is associated with increased islet amyloid formation (454).
Mouse	hIAPP ^{+/-}	ob/ob	This mouse model spontaneously develops hyperglycemia and diabetes, which is associated with substantial decrease in β -cell mass. The presence of extensive extracellular amyloid deposits has been reported in these mice compared to the non-transgenic ob/ob mice. (361)

Species	Transgene	Background	Characteristics
Rat	hIAPP ^{+/-}	CD	Hemizygous hIAPP (HIP) rats develop diabetes between 5 and 10 months of age along with extensive islet amyloid formation. Increased β -cell apoptosis (~10 fold) and decreased β -cell mass (~50-80%) have been reported in these rats compared to wild-type rats. Increased β -cell proliferation with no change to β -cell neogenesis has been observed in these mice. (363)
Rat	hIAPP ^{+/+}	CD	Homozygous HIP rats form amyloid and spontaneously develop diabetes within the first 2 months of life along with a marked reduction in β -cell mass due to increased β -cell apoptosis. (363)

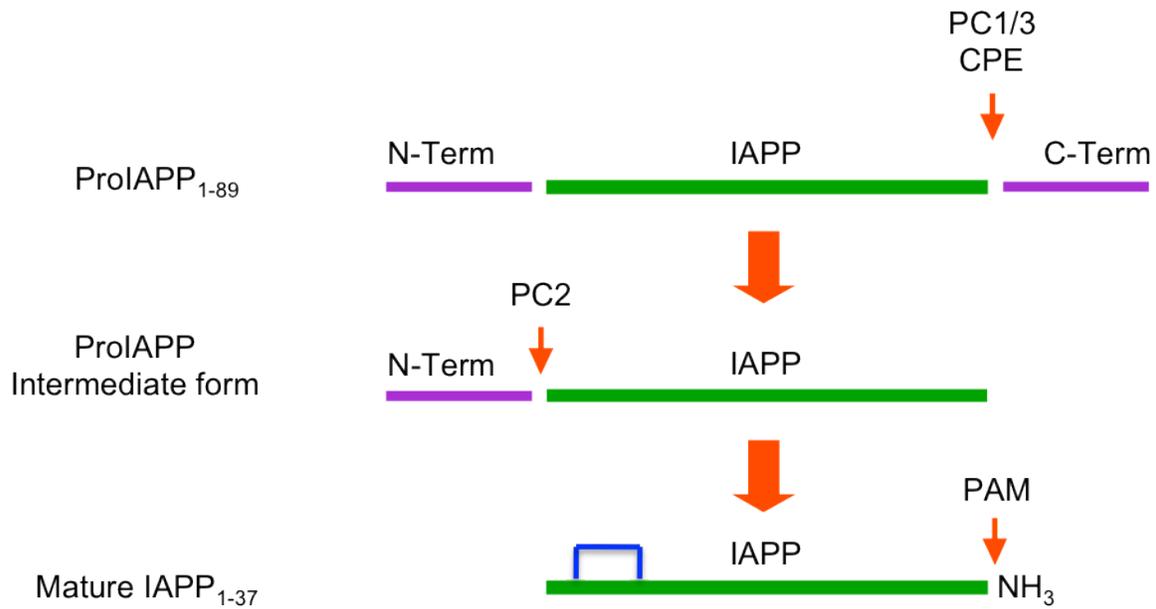


Figure 1-1. ProIAPP undergoes a series of proteolytic and post-translational modifications to form mature IAPP.

Briefly, proIAPP is cleaved by prohormone convertase 1/3 (PC1/3) and prohormone convertase 2 (PC2) at the C-terminal and N-terminal ends, respectively. Carboxypeptidase E (CPE) removes the dibasic residues that remain at the C-terminal end following PC1/3 cleavage. Lastly, the C-terminal glycine residue amidation by the peptidyl amidating monooxygenase complex (PAM) and formation of a disulfide bridge between residues 2 and 7 (blue) result in the production of biologically active (mature) IAPP.

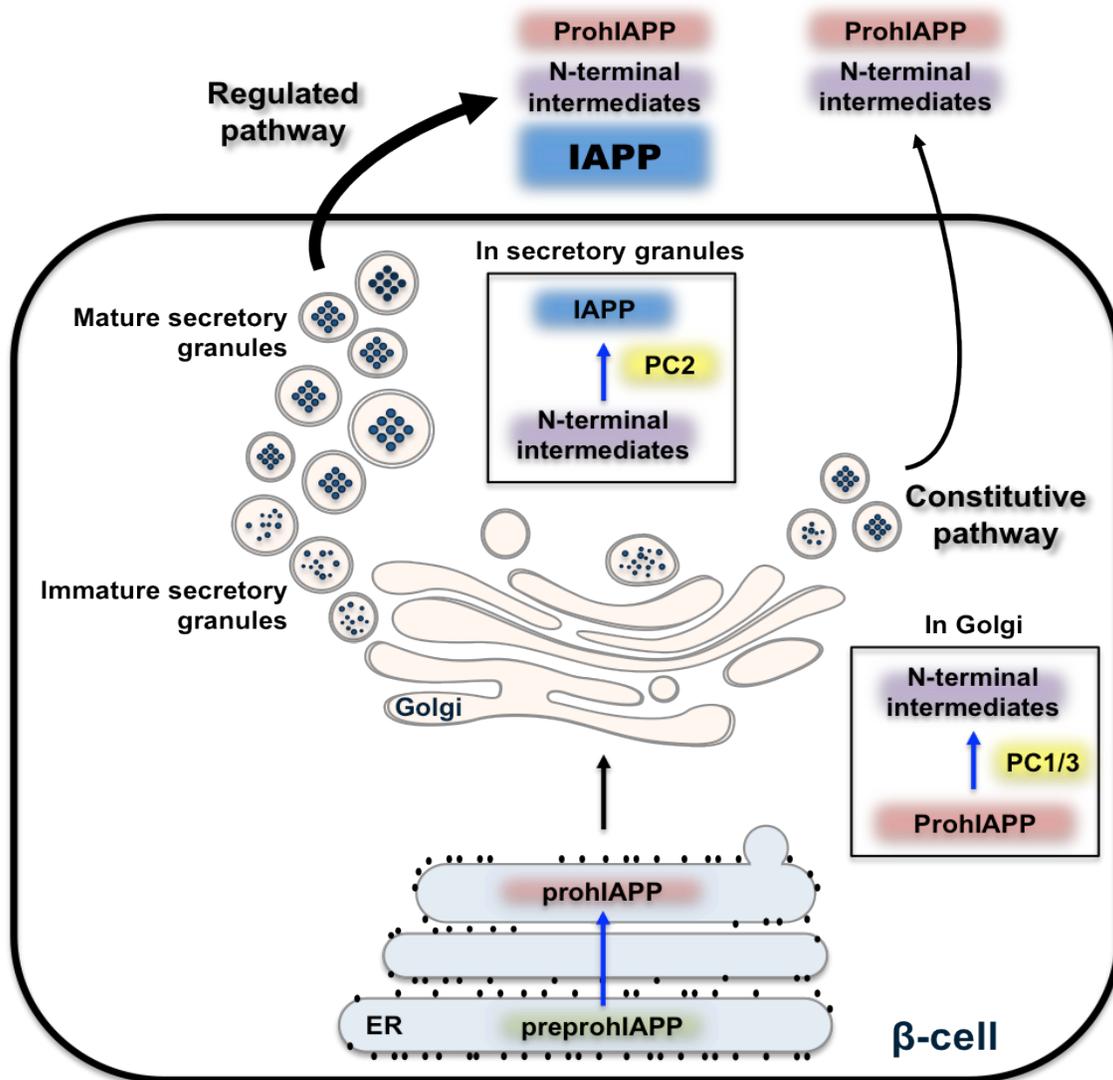


Figure 1-2. IAPP processing and secretion from β -cells.

IAPP is produced in the ER as preproIAPP. Then, the signal sequence is removed from preproIAPP to form proIAPP, which is cleaved by PC1/3 in the Golgi and by PC2 in the secretory granules to produce mature IAPP. Mature IAPP and low levels of its precursors are released from β -cells via regulated (major) secretory pathway. ProIAPP and its intermediates can also be released via constitutive (minor) secretory pathway.

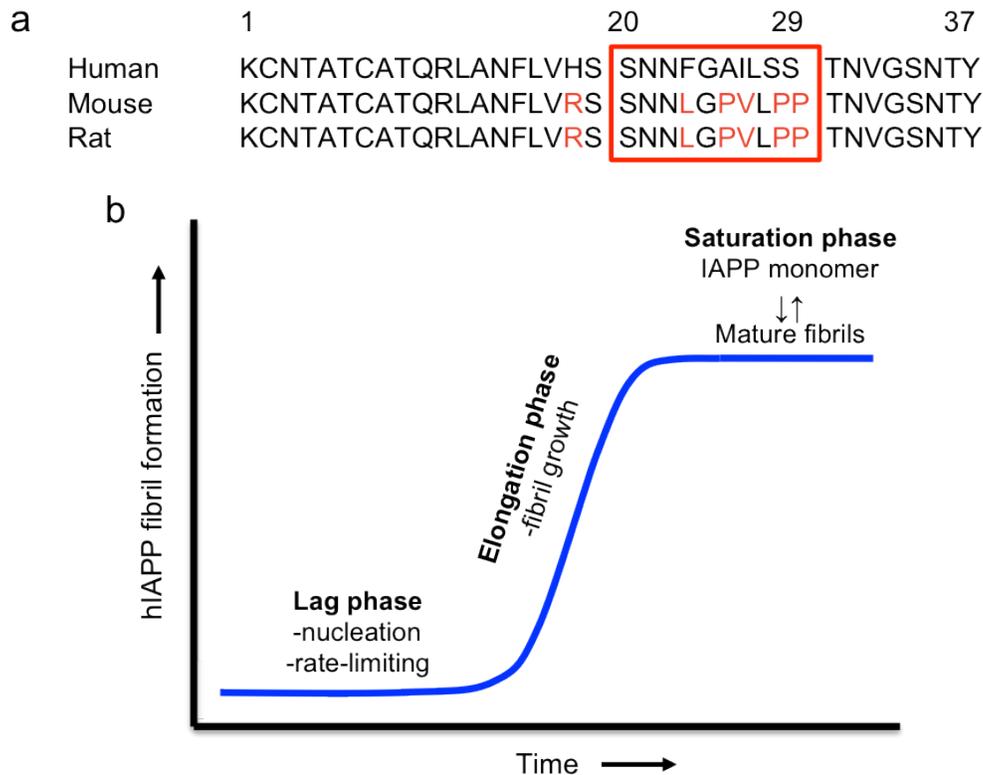


Figure 1-3. Characteristics of IAPP and sequence of events during hIAPP fibril formation. (a) The amino acid sequence of mature IAPP in human, mouse and rat. IAPP is strongly conserved among mammalian species but with notable variation in the amyloidogenic region (IAPP₂₀₋₂₉) highlighted in red. (b) The kinetics of hIAPP fibril formation. During the lag phase, rate-limiting nucleation process occurs where hIAPP monomers associate to form hIAPP protofibrils (oligomers). Then, hIAPP protofibrils continue to elongate by addition of peptides to their ends in the exponential fibril growth phase. Fibrils continue to grow until it finally reaches a saturation phase (a ‘steady-state’) where soluble hIAPP monomers are at equilibrium with mature fibrils (adapted from (193)).

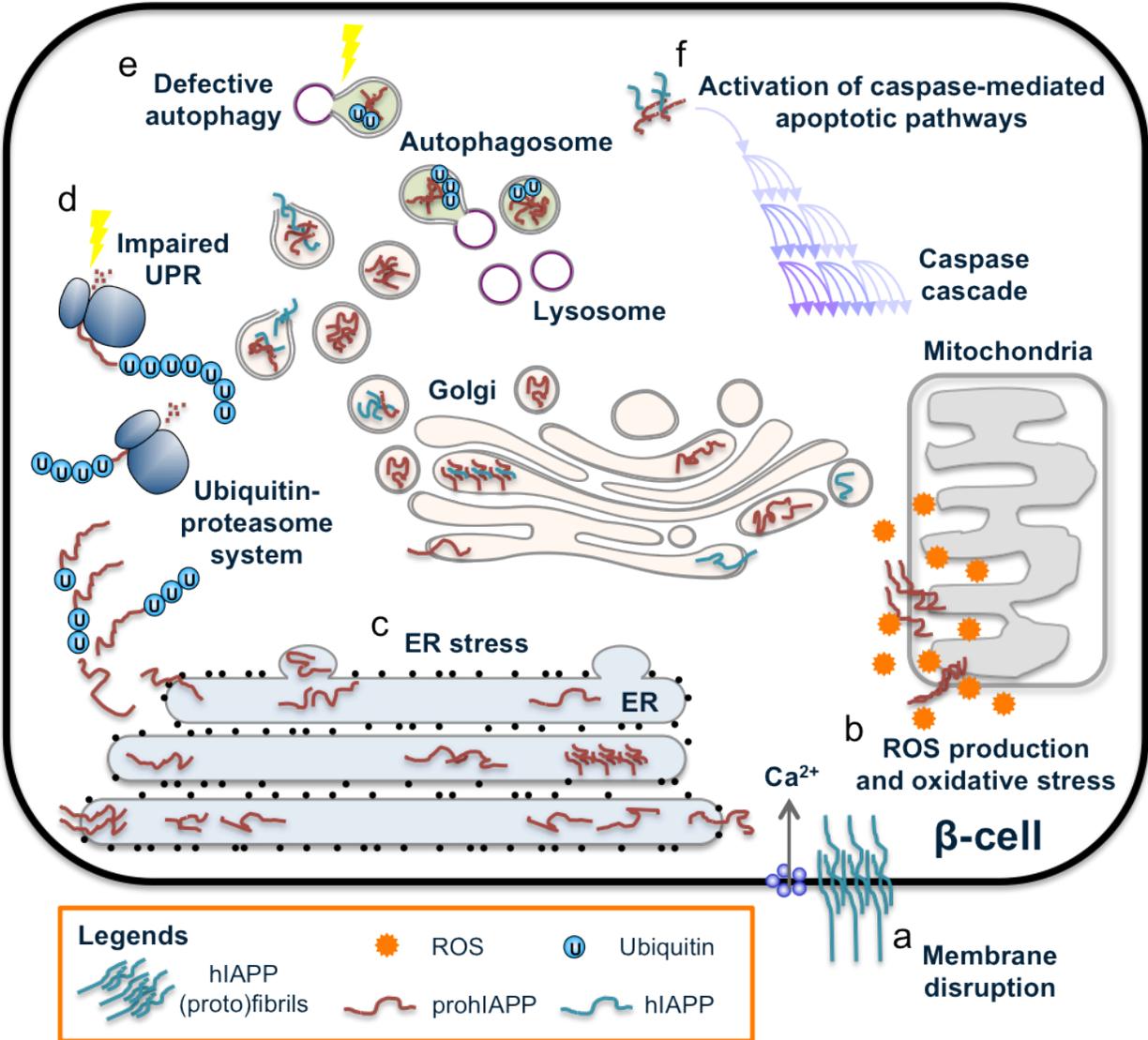


Figure 1-4. The proposed mechanisms of hIAPP-induced β -cell toxicity.

Human IAPP aggregation induces β -cell death through several mechanisms including: (a) membrane disruption and pore formation leading to destabilization of intracellular ion homeostasis; (b) production of ROS which triggers oxidative stress response; (c) activation of ER stress and (d) disruption of UPR due to impaired proIAPP processing and accumulation of misfolded hIAPP aggregates in the ER; (e) defective autophagy by blocking autophagosome formation and lysosomal degradation; and (f) activation of caspase-mediated apoptotic pathways.

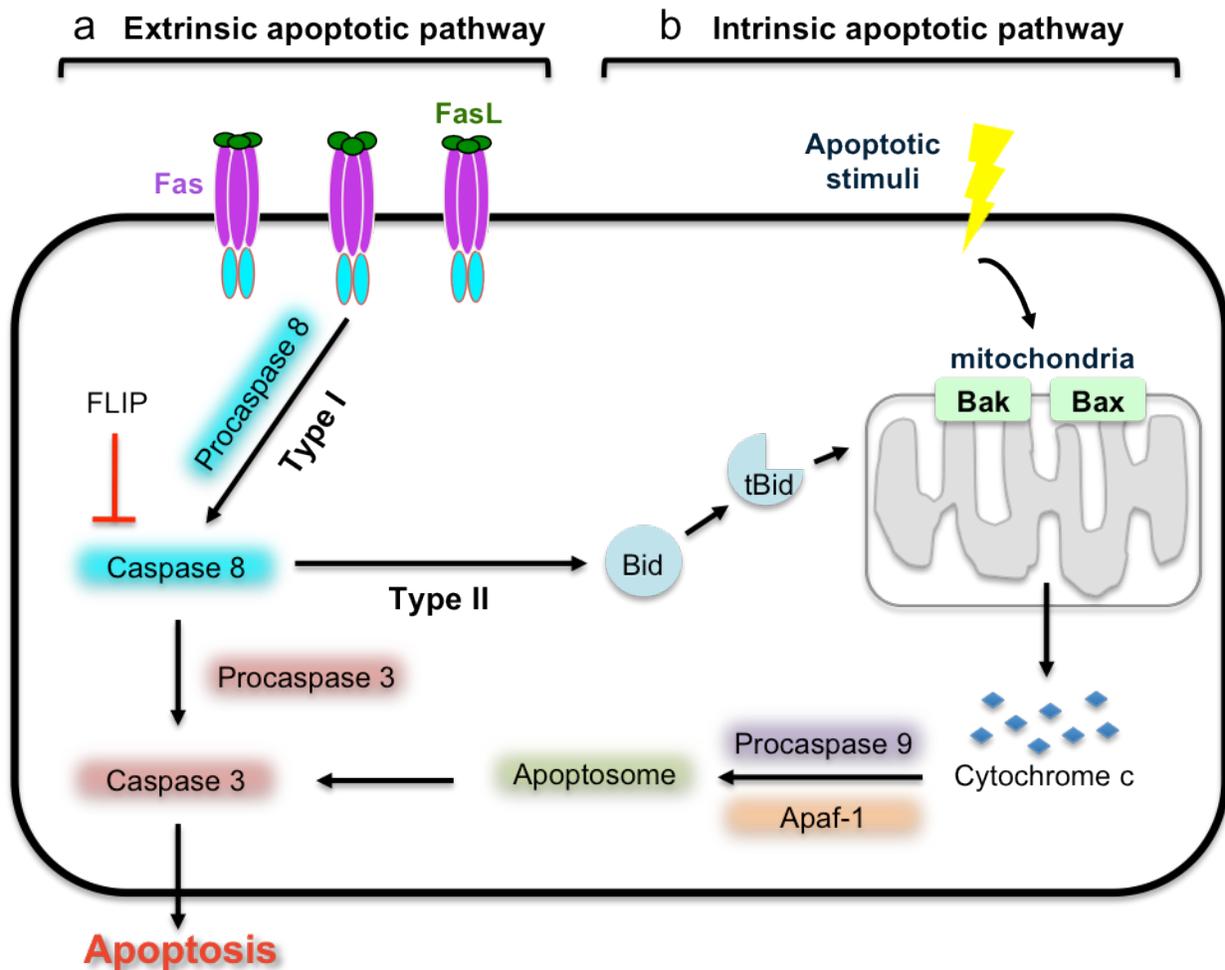


Figure 1-5. The two major apoptotic pathways mediating cell death.

(a) The Fas cell death receptor-mediated (extrinsic) apoptotic pathway is initiated by the Fas and FasL interaction leading to the activation of caspase-8, which in turn activates caspase-3 and apoptosis (Type I). Alternatively, caspase-8 can cleave Bid, a cytosolic pro-apoptotic protein, to its truncated form (tBid), indirectly activating the intrinsic apoptotic pathway (Type II). **(b)** In mitochondrial-mediated (intrinsic) apoptotic pathway, cellular stress can activate pro-apoptotic proteins such as Bax and Bak, which in turn triggers the release of cytochrome c from the mitochondria. Then, the apoptotic protease activation factor-1 (Apaf-1) oligomerises and forms a complex called apoptosome with cytochrome c and procaspase-9, resulting in caspase-9 activation. Active caspase-9 can then cleave procaspase-3 to its active form, resulting in cell apoptosis.

Chapter 2: Materials and methods

2.1 Materials

Thioflavin S, dithizone, bovine serum albumin (BSA), HEPES buffer, Hoechst-33342, 2-mercaptoethanol, sodium bicarbonate, poly-L-lysine, Triton X-100, trypan blue, dimethyl sulfoxide (DMSO), 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), Avertin, collagenase (Type XI), phenylmethylsulfonyl fluoride (PMSF), and aprotinin were obtained from Sigma-Aldrich (Oakville, ON, Canada). RPMI-1640, fetal bovine serum (FBS), penicillin, streptomycin, gentamycin, cell dissociation buffer enzyme free Hank's-based, Ham's-F10, trypsin-EDTA, and glutamax were from Invitrogen Canada Inc. (Burlington, ON, Canada). CMRL culture medium was from Mediatech Inc. (Herndon, VA, USA). All electrophoresis chemicals were from Bio-Rad Laboratories (Mississauga, ON, Canada).

2.2 INS-1 cell culture

INS-1 (832/13) cells, a transformed rat insulinoma β -cell line, were provided by Dr. C Newgard (Duke University Medical Center, NC, USA). Cells were grown in RPMI-1640 containing 11.1 mmol/l glucose supplemented with 10 % (vol/vol) FBS, 50 U/ml penicillin, 50 μ g/ml streptomycin, 50 μ mol/l 2-mercaptoethanol, 2 mmol/l glutamax, and 1 mmol/l sodium pyruvate.

2.3 Animals

The list of mouse strains used for studies and their sources as shown in Table 2-1. All animals were fed a mouse chow containing 9% (wt/wt) fat (Purina 5021; LabDiet, Richmond, IN, USA). Animals were cared in accordance with the guidelines and principles of the laboratory animal care and the standard procedures established by the Canadian Council on Animal Care and the University of British Columbia's Animal Policy and Welfare Committee.

2.3.1 hIAPP-expressing transgenic mice

Hemizygous C57BL/6 transgenic mice with β -cell specific hIAPP expression (hIAPP^{+/-}) were kindly provided by Dr. S Kahn (University of Washington, Seattle, WA, USA) (351) and maintained by cross-breeding with DBA/2J mice (Jackson Laboratory, Bar Harbour, ME, USA). Male hIAPP-expressing transgenic mice form islet amyloid *in vivo* and develop diabetes in about one year in the presence of a predisposing factor such as high fat diet (351) whereas isolated islets cultured *in vitro* form amyloid within days under elevated glucose conditions (365,390). The primers used for genotyping of the hIAPP gene are shown in Table 2-2. The genotyping PCR reaction mixture also included primers for the β -2-microglobulin (*β 2M*) gene, which was used as an indication of successful hIAPP transgene amplification.

2.3.2 Generation of hIAPP-expressing mice with β -cell Fas deletion

Mice with β -cell specific Fas deletion (RIPcre⁺Fas^{fl/fl}) were generated using the Cre/loxP recombinase system as previously described (457-459). RIPcre⁺Fas^{fl/fl} mice (background: C57BL/6) were maintained by breeding RIPcre⁺Fas^{fl/+} mice kindly provided by Dr. M Woo (University of Toronto, ON, Canada). Islets from RIPcre⁺Fas^{fl/fl} have normal islet mass and enhanced insulin secretion (459). To generate mice with β -cell specific hIAPP expression and Fas deletion, hIAPP^{+/-} mice were cross-bred with RIPcre⁺Fas^{fl/fl} mice to produce hIAPP^{+/-}/RIPcre⁺Fas^{fl/+} mice which then were bred together to generate hIAPP⁺/RIPcre⁺Fas^{fl/fl} and hIAPP⁺/RIPcre⁺Fas^{+/+} mice (Figure 2-1).

2.3.3 Generation of hIAPP-expressing mice with β -cell caspase-8 deletion

Similarly, mice with β -cell specific caspase-8 deletion (RIPcre⁺Casp8^{fl/fl}) were generated from Casp8^{fl/fl} mice using the Cre/loxP recombinase system and maintained by inter-breeding RIPcre⁺Casp8^{fl/+} mice (provided by Dr. M Woo; background: C57BL/6) (460,461). Homozygous RIPcre⁺Casp8^{fl/fl} mice have age-dependent (8-12 months) defects in β -cell mass but with enhanced glucose-stimulated insulin secretion *in vitro* (461). To generate mice with β -cell specific hIAPP expression and caspase-8 deletion, hIAPP^{+/-} and RIPcre⁺Casp8^{fl/fl} mice were cross-bred to produce hIAPP^{+/-}/RIPcre⁺Casp8^{fl/+} mice, which were then inter-bred to generate RIPcre⁺Casp8^{fl/fl}/hIAPP⁺ and hIAPP⁺/RIPcre⁺Casp8^{+/+} mice (Figure 2-2).

For hIAPP-expressing mice lacking Fas or caspase-8, the first generation of the offspring was used for the studies. Islets from wild-type (hIAPP^{-/-}) mice lacking Fas or caspase-8 expression were used as controls to detect any hIAPP-independent effects of Fas or caspase-8 deletion on amyloid-induced β -cell death. Moreover, to reduce the effects of genetic background variation, wild-type littermates were used for each study. The presence of the hIAPP transgene (462), insulin-Cre transgene (463) and disrupted Fas (458) or caspase-8 gene (460) was determined by PCR using the primers shown in Table 2-2.

2.4 Islet isolation and culture

2.4.1 Human islets

Human islets isolated from cadaveric pancreatic donors were provided by the Ike Barber Human Islet Transplant Laboratory (Vancouver, BC, Canada) in accordance with approved procedures and guidelines of the Clinical Research Ethics Board of the University of British Columbia. Human islets were then hand-picked to achieve >90% purity as assessed by dithizone staining prior to culture studies. Human islets were cultured in CMRL supplemented with 5.5 or 11.1 mmol/l glucose, 10% (vol./vol.) FBS, 50 U/ml penicillin, 50 μ g/ml streptomycin, and 50 μ g/ml gentamicin in humidified 5% CO₂/95% air incubator at 37°C for up to 7 days.

2.4.2 Mouse islets

Animals were anesthetised with Avertin (0.25 mg/g body weight, i.p.) and sacrificed by cervical dislocation. Ice-cold collagenase (1000 U/ml) in 2 ml calcium-free Hank's buffer was injected via the common bile duct. Harvested pancreas was incubated with collagenase (1000 U/ml) in Hank's buffer in a shaker water bath (14 min, 37°C, 120 rpm), followed by gentle shaking for 2 min. Digestion was stopped by addition of ice-cold Hank's buffer containing 1 mmol/l CaCl₂. Digested pancreatic tissues were rinsed, centrifuged (250 g, 30 s, 4°C), and resuspended in the same solution, then filtered through a 70 μ m mesh cell strainer (BD Biosciences, Oakville, ON, Canada) into Ham's-F10. To allow recovery, hand-picked islets (purity >95% as assessed by dithizone staining) were cultured overnight in Ham's-F10 supplemented with 16.7 mmol/l glucose, 0.5% (wt/vol.) BSA, 50 U/ml penicillin, 50 μ g/ml

streptomycin, and 50 µg/ml gentamicin. For culture studies, islets were kept in a humidified 5% CO₂/95% air incubator (37°C) for up to 7 days.

2.5 Islet dissociation

Isolated human or mouse islets were cultured overnight to allow recovery and dissociated as previously described (364). Briefly, ~300 islets were dispersed in 150 µl of dissociation buffer by gently pipetting up and down for 1 min followed by 1 min rest repeated for a total of 5 min. After 2 min, 2 µl trypsin-EDTA (0.25%) and 1 µl DNase I (0.4 mg/ml) were added. Dissociation was stopped by adding 150 µl medium (CMRL: human; Ham's-F10: mouse). Cells were centrifuged (2000 rpm, 5 min, 4°C) and cell pellets were resuspended in 100 µl of culture medium. Cell viability was assessed by trypan blue. Then, cells were cultured in poly-L-lysine coated (100 µg/ml, 4 h) 8-well chamber slides (BD Biosciences) in CMRL (human) or Ham's-F10 (mouse) containing 5.5 and 10 mmol/l glucose, respectively. Chamber slides containing cells were kept in a humid chamber at 37°C prior to the treatment studies.

2.6 Treatment with peptides and inhibitors

The list of peptides and inhibitors for treatment studies are shown in Table 2-3. hIAPP and rIAPP aliquots were prepared from synthetic peptides (1-37 aa; Bachem, Torrance, CA, USA) by dissolving in HFIP, then lyophilised and frozen at -20°C as before (364). Immediately prior to each experiment, peptides were dissolved in appropriate culture medium and added to cells at a final concentration of 10 µmol/l. Either 25 µmol/l amyloid inhibitor Congo red (dissolved in DMSO), 10 mmol/l Fas antagonist (Kp7-6; EMD Chemicals, Gibbstown, NJ, USA), or 100 µmol/l caspase-8 inhibitor (z-LETD-FMK; Bachem) were prepared in culture medium and added to cells 1 h before hIAPP treatment.

2.7 Adenoviral transduction of human and mouse islets

Human or hIAPP-expressing transgenic mouse islets were transduced overnight with an adenovirus that delivers a human proIAPP specific siRNA (Ad-prohIAPP-siRNA; multiplicity of infection: 20) to suppress hIAPP expression and thereby prevent amyloid formation (366). Islets

transduced with Ad-cont-siRNA that delivers a non-specific siRNA (MOI: 20) were used as a control to detect any potential β -cell adverse effects of adenoviral transduction. Adenoviral-siRNA transduced human or hIAPP-expressing transgenic mouse islets were then rinsed and cultured for 6 days in CMRL (11.1 mmol/l glucose) or 7 days in Ham's-F10 (16.7 mmol/l glucose), respectively.

2.8 Treatment with the GLP-1R agonist exenatide

Freshly isolated human islets were cultured free-floating in non-adherent 24-well culture plates (50 islets per well, duplicate) in normal (5.5 mmol/l) or elevated (11.1 mmol/l) glucose for up to 7 days. Exenatide (Byetta; Amylin Pharmaceuticals, San Diego, CA, USA) was added to the islet culture medium in a final concentration of 10 nmol/l. To determine the optimal concentration of exenatide for these studies, human islets were cultured with exenatide at different concentrations (1-10 nmol/l) and β -cell apoptosis was quantified. The concentration of exenatide (10 nmol/l) at which islets had lowest β -cell apoptosis was used for following studies.

2.9 Treatment with IL-1R antagonist anakinra

Freshly isolated human or mouse islets were cultured free-floating in non-adherent 24-well culture plates (50 islets per well, duplicate) in normal or elevated glucose as indicated in each figure legend for 7 days. Anakinra (Kineret; Sobia Pharmaceuticals, Denton, MD, USA) was added to the islet culture medium at a final concentration of 10 μ g/ml. The optimal concentration of anakinra (10 μ g/ml) used in these studies to block the binding of IL-1 β to its receptor on islet β -cells was determined by culturing human islets in the presence of recombinant IL-1 β (2 ng/ml; R&D Systems, Minneapolis, MN, USA) with or without different concentrations of anakinra for 3 days. Paraffin-embedded islet sections were double immunostained for insulin and IL-1 β or insulin and TUNEL to assess the level of IL-1 β bound to IL-1R on β -cells and inhibition of IL-1 β -induced β -cell death, respectively. The lowest concentration of anakinra (10 μ g/ml) at which islets showed significant reduction in IL-1 β -induced β -cell apoptosis was used for following studies.

2.10 Islet (pro)insulin and hIAPP content and release

Human or mouse islets (25 per condition) were pre-incubated (1 h, 37°C) in KRB buffer containing 10 mmol/l HEPES (pH 7.4), 0.25% BSA (wt/vol.) and 1.67 mmol/l glucose, followed by 1 h incubation in KRB containing 1.67 mmol/l glucose (basal insulin release) and another 1 h in KRB containing 16.7 mmol/l glucose (stimulated insulin release). Islets were lysed in 100 µl of 1 mol/l acetic acid/0.1% BSA (10 min, 100°C). Incubation media and islet lysates were centrifuged and supernatants were frozen (−20°C) until assayed. Insulin and proinsulin levels were measured using human or mouse specific insulin and proinsulin ELISA kits (ALPCO Diagnostics, Salem, NH, USA). Islet hIAPP content and release were assessed by a human amylin (total) ELISA kit (EZHAT-51K; EMD Millipore, Billerica, MA, USA). All values were normalised to islet protein levels measured by bicinchoninic acid (BCA) protein assay (Pierce/Thermo Scientific, Rockford, IL, USA).

2.11 Immunolabelling of cells and islet sections

Islet cells were fixed in 4% paraformaldehyde (wt/vol.) and permeabilised with Triton X-100. Paraffin-embedded human or mouse islet sections (5 µm) were dewaxed and rehydrated, followed by antigen retrieval using citrate buffer in a steamer for 10 min. Fixed cells or islet sections were blocked in 2% normal goat and/or donkey serum (Vector Laboratories, Burlingame, CA, USA) for 1 h and incubated overnight with guinea pig anti-insulin alone or with each of primary antibodies listed in Table 2-4. Islet sections (or cells) were then incubated with Texas red-conjugated anti-guinea pig (Jackson Laboratories, West Grove, PA, USA) and Alexa 488-conjugated anti-rabbit (Molecular Probes, Eugene, OR, USA) for 1 h. For insulin and A11 staining, Alexa 488-conjugated anti-guinea pig (Molecular Probes) and Texas red-conjugated anti-rabbit (Jackson) were used as secondary antibodies, respectively. For triple immunolabelling, islet cells were first incubated overnight with anti-insulin and anti-Fas primary antibodies. Next, cells were incubated with AMCA-conjugated anti-guinea pig (1:200; Jackson) and Texas red-conjugated anti-rabbit (1:100; Jackson) secondary antibodies for 1 h followed by overnight incubation with cleaved caspase-3 antibody directly conjugated to Alexa 488. For detection of apoptotic β-cells, following immunolabelling for insulin, cells or islet sections were incubated with the TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling)

reagent from Roche Diagnostics (Laval, QC, Canada) for 30 min at 37°C. To detect amyloid formation, islet sections were stained with 0.5% (wt/vol.) thioflavin S solution for 5 min at room temperature. Finally, islet sections (or cells) were counterstained with the nuclear dye DAPI (Vector Laboratories) or Hoechst-33342 for quantification studies or with 7-aminoactinomycin D (Molecular Probes). Images were taken using an Olympus IX81 inverted fluorescence microscope and Image-Pro analyzer software (version 6.3; Media Cybernetics Inc., MD, USA).

2.12 Immunoprecipitation, electrophoresis and immunoblotting

About 100 islets were lysed in 30 µl NP-40 lysis buffer containing 50 mmol/l Tris-HCl (pH 8.0), 150 mmol/l NaCl, 0.02% sodium azide, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mmol/l PMSF and 10 µg/ml aprotinin, for 25 min on ice, and vortexed every 5 min. Samples were centrifuged (15,000 × g, 10 min, 4°C) and the supernatants were frozen at -70°C until analysis. Aliquots of islet protein (10 or 15 µg) were electrophoresed on a polyacrylamide gel (using Tris-tricine buffer for IAPP) then transferred to polyvinyl difluoride membrane (15 V, 20-23 min) using a Trans-Blot semidry transfer cell (Bio-Rad Laboratories). Then, the membranes were blocked with 5% skim milk overnight, washed, and incubated for 1 h at room temperature with each of the antibodies or antisera listed in Table 2-5. For detection of phosphorylated JNK, membranes were incubated with phospho-JNK antibody (Cell Signaling Technology) overnight at 4°C. Membranes were then washed and incubated (1 h) with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (1:5000; Amersham, Baie d'Urfe, QC, Canada) at room temperature. Immunodetection was performed using an enhanced chemiluminescence detection kit (Amersham). Protein bands on films (X-OMAT; Kodak, Rochester, NY, USA) were analyzed by densitometry using the Image Lab software (Bio-Rad).

2.13 Statistical analysis

Data are expressed as mean ± SEM. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Newman-Keuls test or by Student's *t*-test as appropriate. A *p* value of <0.05 was taken as significant. All experiments were performed in duplicate or triplicate as detailed in each figure legend and repeated at least three times unless stated otherwise.

Table 2-1. Mouse strains.

Strain name	Source
hIAPP ^{+/-}	Provided by Dr. S Kahn (University of Washington)
DBA-2J	Jackson Laboratory (Bar Harbour, ME, USA)
PC2 ^{-/-}	Provided by Dr. D Steiner (University of Chicago)
RIPcre ⁺ Fas ^{fl/fl}	Provided by Dr. M Woo (University of Toronto)
RIPcre ⁺ Fas ^{+/+}	Generated as detailed in the breeding scheme (Figure 2-1)
hIAPP ⁺ /RIPcre ⁺ Fas ^{fl/fl}	
hIAPP ⁺ /RIPcre ⁺ Fas ^{+/+}	
RIPcre ⁺ Casp8 ^{fl/fl}	Provided by Dr. M Woo (University of Toronto)
RIPcre ⁺ Casp8 ^{+/+}	Generated as detailed in the breeding scheme (Figure 2-2)
hIAPP ⁺ /RIPcre ⁺ Casp8 ^{fl/fl}	
hIAPP ⁺ /RIPcre ⁺ Casp8 ^{+/+}	

Table 2-2. PCR primers for mice genotyping.

Gene name	Primer sequence	
<i>IAPP</i>	sense	5'-CTG AAG CTG CAA GTA TTT CTC A-3'
	antisense	5'-AGA TGA GAG AAT GGC ACC AAA-3'
<i>β2M</i>	sense	5'-CAC CGG AGA ATG GGA AGC CGA A-3'
	antisense	5'-TCC ACA CAG ATG GAG CGT CCA G-3'
<i>PC2</i>	common	5'-CGC TGC AAC AAG AAG GAT T-3'
	wild-type	5'-TAG AGA AAC TTA CCA GGT ACC-3'
	mutant	5'-CCA CTT GTG TAG CGC CAA GT-3'
<i>Cre</i>	sense	5'-GGC AGT AAA AAC TAT CCA GCA A-3'
	antisense	5'-GTT ATA AGC AAT CCC CAG AAA TG-3'
<i>Fas</i>	sense	5'-TGC AGT TGC TGA GAT GAA CCA TTT TCT CTG TCT-3'
	antisense	5'-GGC TTT GGA AAG GAA TTT CCT CCT AAG AGG-3'
<i>Casp8</i>	sense	5'-CCA GGA AAA GAT TTG TGT CTA GC-3'
	antisense	5'-GGC CTT CCT GAG TAC TGT CAC CTG T-3'

Table 2-3. List of peptides and inhibitors for treatment studies.

Name	Abbreviation	Concentration	Source
hIAPP	hIAPP	10 μmol/l	Bachem
rIAPP	rIAPP	10 μmol/l	Bachem
Congo red	CR	25 μmol/l	Sigma
Fas antagonist	Kp7-6	10 mmol/l	EMD Chemicals
Caspase-8 inhibitor (z-LETD-FMK)	Casp8 inh	100 μmol/l	Bachem
Recombinant IL-1β	rIL-1β	2 ng/ml	R&D Systems

Table 2-4. List of primary antibodies for immunolabelling.

Target	Host	Concentration	Source	Catalog #
Insulin	Guinea pig	Cell: (1:400); Islet: (1:1000)	Dako	A0564
Glucagon	Rabbit	Islet: (1:1000)	Dako	A0565
Fas	Rabbit	Cell: (1:100); Islet: (1:50)	Santa Cruz Biotechnology	SC-7886
IL-1 β	Rabbit	Islet: (1:100)	Santa Cruz Biotechnology	SC-7884
Mouse cleaved caspase-8 (Asp387)	Rabbit	Cell: (1:100); Islet: (1:100)	Cell Signaling Technology	8592
Human cleaved caspase-8 (Asp391)	Rabbit	Cell: (1:100); Islet: (1:100)	Cell Signaling Technology	9406
Mouse caspase-8	Rabbit	Islet: (1:50)	Santa Cruz Biotechnology	5263
Cleaved caspase-3 (Asp175)	Rabbit	Cell: (1:400); Islet: (1:100)	Cell Signaling Technology	9661
Cleaved caspase-3 (Asp175) conjugated with AF488	Rabbit	Cell: (1:10)	Cell Signaling Technology	9669
A11	Rabbit	Islet: (1:400)	Invitrogen	AHB0052
PCNA	Mouse	Islet: (1:400)	Cell Signaling Technology	2586
Phospho-PKB (Ser473)	Rabbit	Islet: (1:100)	Cell Signaling Technology	4060
Phospho-JNK (Thr183/Tyr185)	Rabbit	Islet: (1:100)	Cell Signaling Technology	4668

Table 2-5. List of primary antibodies/antisera for immunoblotting.

Target	Host	Concentration	Source	Catalog #
Amylin (IAPP)	Rabbit	(1:1000)	Peninsula Laboratories	T-4145
FLIP _L	Rabbit	(1:500)	Santa Cruz Biotechnology	SC-8347
Phospho-JNK	Rabbit	(1:750)	Cell Signaling Technology	4668
GAPDH	Mouse	(1:1000)	Advanced ImmunoChemical	RGM2
Human proIAPP N- terminal fragment	Rabbit	(1:100)	Provided by Dr. B Verchere (University of British Columbia)	8546
Human proIAPP C- terminal fragment	Rabbit	(1:100)	Provided by Dr. B Verchere	8548
PC1/3	Rabbit	(1:2000)	Provided by Dr. I Lindberg (University of Maryland)	-
PC2	Rabbit	(1:1000)	Provided by Dr. C Rhodes (University of Chicago)	-

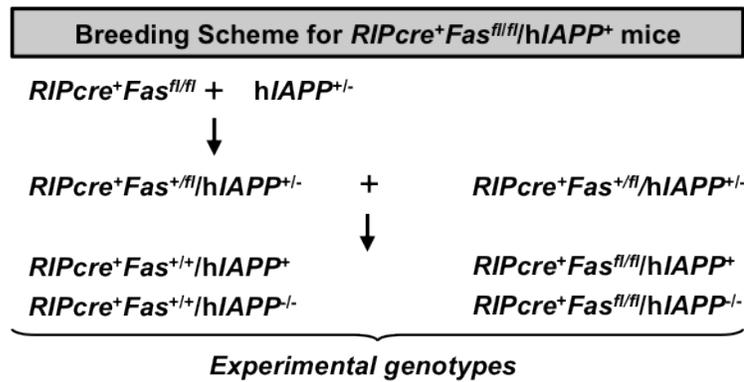


Figure 2-1. Breeding scheme for generation of $hIAPP^+/RIPcre^+Fas^{fl/fl}$ mice.

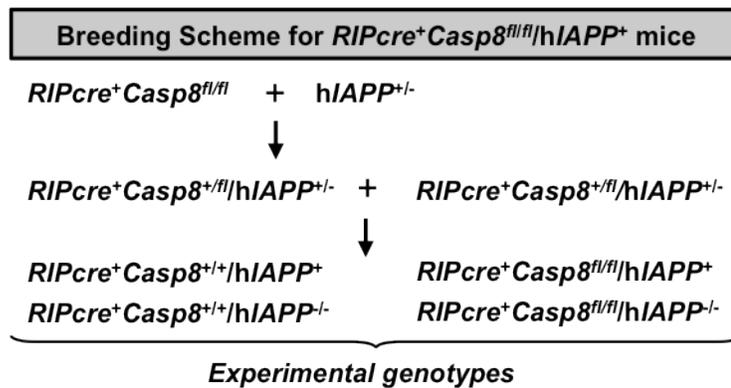


Figure 2-2. Breeding scheme for generation of $hIAPP^+/RIPcre^+Casp8^{fl/fl}$ mice.

Chapter 3: Amyloid formation induces β -cell Fas upregulation

3.1 Background

Despite considerable studies during the past decade, it is still not clear why normally soluble hIAPP molecules form toxic aggregates in type 2 diabetes. The presence of an amyloidogenic sequence in the hIAPP molecule (193,319), elevated hIAPP production and secretion from β -cells associated with an increased demand for insulin (346,464,465), and defects in trafficking/processing of proIAPP associated with β -cell dysfunction (346,365,464,466), have been proposed to contribute to aggregation of hIAPP molecules in type 2 diabetes. Importantly, widespread amyloid deposition has also been reported in human islets transplanted into patients with type 1 diabetes (367,384) and animal models of type 1 diabetes (389,467).

Aggregates of hIAPP, including small oligomeric species, are toxic to β -cells and have been shown to induce both β -cell dysfunction and apoptosis in isolated human islets (366,468). Previous *in vitro* studies have suggested different mechanisms for β -cell apoptosis mediated by hIAPP aggregates as described in detail in Chapter 1.2.4.3 and Figure 1-4. Briefly, disruption of membrane integrity and formation of non-selective ion-channel-like structures (322,403,406,468-470), activation of the caspase pathways (364,439,442,471), and interaction of hIAPP aggregates with components of β -cell membranes such as heparan sulphate proteoglycans (348,472) or touch receptors (473) have been shown to mediate hIAPP-induced β -cell apoptosis. Moreover, recent studies suggest that oxidative stress (200,414,415,474), ER stress (424,425,475), and disruption in autophagy/lysosomal pathway (432), due to formation of intracellular hIAPP aggregates, also contribute to the cytotoxic effects of hIAPP. It therefore appears that, at least *in vitro*, hIAPP-induced β -cell apoptosis involves several mechanisms, some of which likely share the same apoptotic signalling pathways.

Fas (CD95/APO-1) is a transmembrane receptor protein belonging to the tumour necrosis factor superfamily of receptors (369,476,477). Fas is comprised of three domains which include an extracellular domain responsible for direct binding with its ligand (FasL), a transmembrane domain, and a cytoplasmic domain that contains the death domain required for relaying the

apoptotic signalling through activation of downstream caspases (478). Islet β -cells do not normally express Fas at detectable levels whereas they constitutively express FasL (479,480). However, conditions associated with β -cell stress such as exposure to elevated glucose, leptin, or cytokines can induce upregulation of Fas and β -cell apoptosis in isolated human islets (217,480,481). These findings led us to the idea that hIAPP aggregates, similar to elevated glucose, may induce Fas upregulation in islet β -cells. Thus, hIAPP aggregates may destroy islet β -cells by common mechanisms in all conditions associated with islet amyloid formation such as type 2 diabetes, islet culture and transplantation.

In this chapter, we performed mechanistic studies to test whether hIAPP aggregates derived from exogenously applied or endogenously produced hIAPP can induce upregulation of Fas in islet β -cells. To investigate the role of Fas in mediating hIAPP-induced β -cell toxicity, we first used transformed INS-1 and primary (human and mouse) islet cells treated with synthetic hIAPP peptides to examine if hIAPP can induce Fas upregulation in β -cells. Conversely, we examined if inhibition of hIAPP fibril formation and its interaction with β -cells can prevent upregulation of Fas in transformed INS-1 and mouse islet β -cells. Next, we tested if endogenous formation of hIAPP aggregates can induce β -cell Fas upregulation and apoptosis in cultured human and hIAPP-expressing transgenic mouse islets and whether prevention of amyloid formation can reduce Fas expression thereby enhance β -cell viability. Finally, we generated a new mouse model with β -cell specific hIAPP expression and Fas deletion to examine if deletion of Fas can protect islet β -cells from hIAPP aggregates derived from endogenously secreted hIAPP.

3.2 Results

3.2.1 Optimization of synthetic hIAPP concentration for *in vitro* studies

Transformed INS-1 cells and dispersed mouse islet cells (BALB/c, 8-10 weeks) were treated with freshly prepared synthetic hIAPP peptide at different concentrations (10 - 40 $\mu\text{mol/l}$; 24 h), to optimise the concentration of synthetic hIAPP peptide for studies presented in this thesis. As expected, exposure to hIAPP aggregates derived from synthetic hIAPP induced cell death in both transformed INS-1 and dispersed mouse islet β -cells in a concentration-dependent

manner (Figure 3-1). The morphological changes observed following hIAPP treatment indicated that cells were undergoing apoptosis at all concentrations of hIAPP (Figure 3-1a). The lowest concentration of synthetic hIAPP (10 $\mu\text{mol/l}$) at which β -cell apoptosis was significantly increased compared to the untreated β -cells was chosen for studies throughout this thesis (Figure 3-1b, c).

3.2.2 Exogenously applied hIAPP induces Fas upregulation, caspase-3 activation, and β -cell apoptosis in both transformed INS-1 and primary islet β -cells

INS-1 cells and dispersed mouse (C57BL/6) or human islet cells were treated with synthetic hIAPP (10 $\mu\text{mol/l}$) for 8 and 12 h, to investigate the effects of hIAPP on β -cell Fas expression. Fas was not detectable in non-treated INS-1 cells by immunolabelling but treatment with hIAPP induced Fas expression in a time-dependent manner with low levels of Fas present after 8 h and higher levels detectable after 12 h treatment with hIAPP (Figure 3-2a). Treatment with non-fibrillogenic rIAPP did not have any noticeable effect on β -cell Fas expression and apoptosis (Figures 3-2a and 3-4c). Similarly, exposure to exogenously applied hIAPP induced upregulation of Fas in dispersed mouse islet cells, mainly in β -cells, which was detectable after 8 and 12 h treatment with hIAPP (Figure 3-2b). Upregulation of Fas was associated with increased number of active caspase-3 and TUNEL-positive (apoptotic) β -cells (Figure 3-2b, c), resulting in lower islet β -cell to total islet cell ratio in hIAPP-treated islet cells compared to non-treated islet cells (Figure 3-2d). The time point of Fas expression preceded that of caspase-3 activation (16 h) and apoptosis (24 h). Furthermore, immunolabelling studies showed a marked increase in co-localization of Fas and active caspase-3 in hIAPP-treated human and mouse islet β -cells compared to non-treated islet β -cells (Figure 3-3). Moreover, unlike INS-1 cells, low levels of Fas-positive β - and non- β islet cells were also present in non-treated dispersed mouse and human islets (Figures 3-2b and 3-3a).

3.2.3 Preventing the interaction between hIAPP aggregates and β -cells markedly reduces β -cell apoptosis in hIAPP-treated INS-1 and mouse islet cells

We examined whether the interaction between hIAPP aggregates and islet β -cells is required for inducing Fas expression. INS-1 and dispersed mouse islet (C57BL/6) β -cells were

cultured without or with synthetic hIAPP (10 $\mu\text{mol/l}$) in the presence or absence of the amyloid inhibitor Congo red (25 $\mu\text{mol/l}$) for 12 h. We previously showed that Congo red at this concentration does not have any adverse effects on β -cell survival (364). Treatment with Congo red markedly reduced Fas expression, caspase-3 activation, and apoptosis in hIAPP-treated INS-1 β -cells (Figure 3-4a, b, c). Similarly, the number of apoptotic β -cells as detected by double insulin and TUNEL immunolabelling was lower in hIAPP-treated mouse islet cells cultured in presence of Congo red (Figure 3-4b).

3.2.4 Blocking or deletion of Fas reduces β -cell apoptosis induced by exogenously applied hIAPP

INS-1 cells or dispersed mouse islet cells (C57BL/6) were cultured with or without synthetic hIAPP (10 $\mu\text{mol/l}$) in the absence or presence of a Fas antagonist (Kp7-6; 10 mmol/l) for 12 h, to test if blocking Fas can protect β -cells from hIAPP aggregates. Both INS-1 and mouse islet cells treated with hIAPP and Fas antagonist had significantly lower β -cell apoptosis compared to those treated with hIAPP only (Figure 3-4b, c, d).

Dispersed wild-type and mouse islet cells with β -cell specific deletion of Fas (RIPcre⁺Fas^{fl/fl}) were treated with synthetic hIAPP peptides to assess the effects of Fas deletion on hIAPP-induced β -cell apoptosis. There was no significant difference between basal β -cell apoptosis in wild-type and RIPcre⁺Fas^{fl/fl} mouse islet cells (Figure 3-5a, b). Interestingly, hIAPP-treated RIPcre⁺Fas^{fl/fl} islets had markedly lower β -cell death compared to hIAPP-treated wild-type islet cells (Figure 3-5b), indicating that deletion of Fas reduces β -cell death induced by aggregation of exogenously applied hIAPP.

3.2.5 Endogenously formed hIAPP aggregates induce upregulation of Fas mRNA and protein expression, which are associated with increased β -cell apoptosis in cultured human islets

Human islets were cultured in CMRL containing elevated glucose (11.1 mmol/l glucose; to potentiate amyloid formation) at different time points for up to 7 days. Islet amyloid formation was not detectable in freshly isolated human islets but was easily detectable after 7 days culture

(Figure 3-6a). Amyloid formation in cultured human islets was associated with increased Fas mRNA (Figure 3-6b) and protein levels (Figure 3-6c, d) and β -cell apoptosis (Figure 3-6a).

3.2.6 Inhibition of amyloid formation reduces Fas expression, IL-1 β levels, and β -cell apoptosis in human and hIAPP-expressing transgenic mouse islets

Human islets or hIAPP-expressing mouse islets were transduced with Ad-prohIAPP-siRNA to suppress hIAPP expression and thereby prevent amyloid formation (366). Non-transduced and transduced human and mouse islets were then cultured in 11.1 and 16.7 mmol/l glucose, respectively, to potentiate amyloid formation. Islets transduced with Ad-cont-siRNA that delivers a non-specific siRNA were used as a control to detect any potential β -cell adverse effects of adenoviral transduction. As expected (365,390), hIAPP-expressing mouse islets formed amyloid during culture and had higher rate of β -cell apoptosis as compared to wild-type islets (Figure 3-7). Aggregation of endogenously produced hIAPP in cultured hIAPP-expressing mouse islets was associated with Fas upregulation in β -cells (Figure 3-7a) and increased islet IL-1 β levels (Figure 3-7b). Adenoviral siRNA-mediated suppression of hIAPP expression markedly reduced amyloid formation, islet IL-1 β levels, β -cell Fas upregulation, and apoptosis (Figure 3-7). Similarly, formation of hIAPP aggregates in human islets during culture was associated with increased islet IL-1 β levels and upregulation of Fas in β -cells (Figure 3-8), both of which were reduced by adenoviral-siRNA-mediated suppression of amyloid formation. Transduction with Ad-cont-siRNA did not have any noticeable effect on amyloid formation or β -cell survival in human or hIAPP-expressing mouse islets (Figures 3-7 and 3-8).

3.2.7 β -cell specific deletion of Fas in hIAPP-expressing mouse islets prevents apoptosis induced by aggregation of endogenously produced hIAPP during culture

We generated a mouse model with β -cell specific expression of hIAPP and deletion of Fas (hIAPP⁺/RIPcre⁺Fas^{fl/fl}) by cross-breeding hIAPP-expressing mice with RIPcre⁺Fas^{fl/fl} mice, to further examine the role of Fas in β -cell apoptosis induced by endogenously formed hIAPP aggregates. The presence of the hIAPP transgene and the disruption of the Fas gene in hIAPP⁺/RIPcre⁺Fas^{fl/fl} mice were confirmed through PCR genotyping (Figure 3-9). Islets from

hIAPP^{-/-}/RIPcre⁺Fas^{+/+} and hIAPP^{-/-}/RIPcre⁺Fas^{fl/fl} mice were used as controls to detect any hIAPP-independent effects of Fas deletion on β -cell apoptosis.

Cultured islets from hIAPP⁺/RIPcre⁺Fas^{fl/fl} and hIAPP⁺/RIPcre⁺Fas^{+/+} mice had comparable levels of amyloid formation (Figure 3-10a, b). Basal β -cell death in hIAPP^{-/-}/RIPcre⁺Fas^{fl/fl} mice was somewhat lower than wild-type (hIAPP^{-/-}/RIPcre⁺Fas^{+/+}) mice but this difference was not statistically significant (Figure 3-10c). Despite similar levels of amyloid formation, islets from hIAPP⁺/RIPcre⁺Fas^{fl/fl} mice had a markedly lower number of active caspase-3 and TUNEL-positive β -cells (Figure 3-10a, c) but comparable number of α -cells, resulting in increased β - to α -cell ratio in these islets compared to hIAPP⁺/RIPcre⁺Fas^{+/+} mice (Figure 3-10d). Islet IL-1 β immunoreactivity was markedly higher in hIAPP-expressing mouse islets with amyloid formation than in wild-type islets (Figure 3-10a).

3.2.8 Reduced amyloid-induced β -cell death in hIAPP-expressing mouse islets with Fas deletion is associated with enhanced β -cell function

Amyloid formation in hIAPP-expressing mouse islets during culture was associated with lower islet insulin content, glucose-stimulated insulin release, and higher proinsulin release compared to wild-type islets (Figure 3-11). This resulted in a higher ratio of proinsulin to insulin secreted from hIAPP-expressing islets as compared to wild-type islets. Glucose-stimulated insulin release was increased by 28% (Figure 3-11a) and proinsulin release was reduced by ~18% (Figure 3-11c) in hIAPP-expressing mouse islets with β -cell Fas deletion compared to those expressing Fas, indicating that Fas deletion in hIAPP-expressing mouse islets enhances insulin secretion and improves the proinsulin to insulin ratio released from those islets. Deletion of Fas in hIAPP-expressing islets did not have any significant effect on islet insulin or proinsulin content during 7 days culture (Figure 3-11b, d). Finally, glucose-stimulated insulin release was somewhat higher in wild-type islets with Fas deletion than those expressing Fas but this difference was not statistically significant (Figure 3-11a).

3.3 Discussion

Growing evidence suggests that formation of hIAPP aggregates in pancreatic islets of patients with type 2 diabetes and human islet grafts in patients with type 1 diabetes is associated with progressive β -cell dysfunction and death (193,367,467,482). Moreover, previous studies have shown that amyloid formation in cultured human (366) and hIAPP-expressing mouse (365) islets induces β -cell apoptosis. Conversely, prevention of amyloid formation can enhance survival and function of human islets during culture (366). The molecular mechanisms by which aggregation of endogenously produced hIAPP induces β -cell apoptosis are still not well understood. It is likely that the β -cell toxic effects of hIAPP aggregates are mediated through similar mechanisms in all conditions associated with islet amyloid formation.

Fas cell death receptor has been implicated in both autoimmune β -cell death in type 1 diabetes (483) and non-immune β -cell death in type 2 diabetes (481). Interestingly, a recent study suggested that Fas may be involved in hIAPP-induced β -cell death. In this study, exposure to hIAPP aggregates derived from supraphysiological concentrations of synthetic hIAPP induced Fas expression in transformed and mouse islet β -cells (471). In another study, Zhang *et al.* (439) showed that fibrillogenic hIAPP aggregates at supraphysiological concentrations can induce activation of the caspase cascade in transformed β -cells and that inhibition of caspases using a pan caspase inhibitor results in a substantial reduction of hIAPP-induced β -cell apoptosis. Similarly, our previous study demonstrated that blocking the activation of caspase-3, a key downstream effector caspase in the Fas apoptotic pathway, protects islet β -cells from apoptosis induced by fibrillogenesis of hIAPP produced and secreted from islet β -cells (364). Additionally, other studies have reported that multiple apoptotic signalling pathways are activated in the presence of hIAPP aggregates including MAPK (JNK/p38) pathways that can activate the caspase cascade in the Fas-mediated extrinsic apoptotic pathway (439,441). Moreover, there is evidence that A β , the amyloidogenic peptide in Alzheimer's disease, induced neuronal cell death is associated with caspase-8 activation and increased expression of Fas and FasL (484,485). Finally, ultrastructural analysis of β -cells showed that in the presence of hIAPP aggregates, clathrin-coated pits are formed on the membrane surface of β -cells, which is indicative of receptor clustering at the plasma membrane (411). Similarly, Fas-FasL interaction occurs in

trimers or multiple of trimers, which leads to clustering of receptor-ligand complexes at the plasma membrane and initiation of the Fas-mediated apoptotic pathway (486). Based on these findings, we hypothesised that Fas may be an important mediator of amyloid-induced β -cell death.

Studies in this chapter, using two *ex vivo* models of islet amyloid formation, human islets and hIAPP-expressing mouse islets with β -cell specific Fas deletion, demonstrated that Fas has a key role in β -cell apoptosis induced by aggregation of biosynthetic hIAPP produced by islet β -cells. Moreover, our data showed that prevention of hIAPP-induced Fas upregulation or deletion of Fas can protect islet β -cells from hIAPP aggregates formed during *in situ* islet culture. These findings suggest that endogenously produced hIAPP aggregates induce β -cell apoptosis, at least partially, through upregulation of Fas and likely activation of the Fas associated death-receptor signalling pathway.

Under normal conditions, INS-1 cells did not express Fas at detectable levels with immunolabelling techniques that we used. Consistent with a previous *in vitro* study performed on transformed and isolated mouse islet β -cells (471), we found that treatment with micromolar concentrations of synthetic hIAPP aggregates can induce upregulation of Fas in INS-1 cells as well as in human and mouse islet β -cells in a time-dependent manner. Low levels of Fas were present as early as 8 h and higher levels detectable following 12 h exposure to hIAPP, which correlates with our previous finding that maximal activation of caspase-3 is detected after 16 h treatment with exogenous hIAPP (364). In hIAPP-treated dispersed human and mouse islet cells, the majority of Fas-positive islet cells were insulin-positive (β -cells), indicating that hIAPP induces upregulation of Fas mainly in β -cells. Moreover, synthetic hIAPP aggregates induced upregulation of Fas in transformed and primary islet β -cells under normal glucose conditions, suggesting that hIAPP aggregates can induce Fas expression independently from glucose-induced Fas upregulation. Taken together, these findings suggest that in both transformed and primary islet β -cells, exposure to hIAPP aggregates derived from exogenously applied hIAPP induces upregulation of Fas and that hIAPP-induced Fas expression is associated with caspase-3 activation and subsequent β -cell apoptosis.

Previous studies have demonstrated that the cell-to-cell communication or contact between islet cells is of importance in maintaining β -cell survival and function. In support, physical separation of islet cells can induce β -cell death, which is partially reversible upon re-establishment of cell contacts (487-489). When cells are in close proximity, contact between β -cells or other islet cells allows for the interaction of surface receptors expressed on one cell with the surface ligands carried by an adjacent cell. Thus, it has been proposed that once Fas is expressed on β -cell membrane, it may interact with FasL, which is constitutively expressed in β -cells (438), on neighboring β -cells to trigger the Fas-mediated apoptotic pathway and subsequent β -cell death. In support, *in vitro* culture in the presence of a Fas antagonist (Kp7-6) significantly reduced hIAPP-induced apoptosis in INS-1 and dispersed islet β -cells. Kp7-6 is a small exocyclic cysteine-knot peptide (originally derived from Fas) that blocks the Fas-FasL interaction by binding to either Fas or FasL (471). This is consistent with the previous finding that blocking the Fas and FasL interaction using anti-Fas/FasL antibodies can prevent hIAPP-induced β -cell death (471). Interestingly, Zhang *et al.* (471) also suggested that Kp7-6 may play a dual role in inhibition of hIAPP-induced β -cell death either by prevention of Fas/FasL assembly or interfering with hIAPP aggregation as demonstrated by structural similarity between the Kp7-6 cysteine-knot structure and the hIAPP NH₂-terminal ring structure.

Moreover, treatment of INS-1 cells with the amyloid binding dye Congo red, which prevents amyloid formation and its interaction with β -cell membranes, markedly reduced hIAPP-induced Fas upregulation and the proportion of active caspase-3 and apoptotic β -cells. This finding supports the notion that interaction of extracellular hIAPP aggregates with β -cell membranes might be important for Fas expression and subsequent β -cell apoptosis and that the inhibitors of islet amyloid formation may provide a potential approach to prevent amyloid-induced Fas expression.

Unlike INS-1 cells, however, low levels of β - and non- β Fas-positive islet cells were present in dispersed human and mouse islets. One possible explanation is that cytokines or other Fas-inducing factors that are released from islet cells and other cells in the digested pancreatic tissue during islet isolation and culture contribute to low levels of Fas detected in non-treated islet cells. In support of our findings, IL-1 β and elevated glucose have been reported to induce

Fas upregulation in cultured human islets (480,481). Therefore, islet amyloid and elevated glucose, both of which contribute to β -cell death in type 2 diabetes, may induce Fas expression in an additive manner.

Next, we used human and hIAPP-expressing transgenic mice with β -cell hIAPP expression (365,390) to examine whether hIAPP aggregates formed by endogenous hIAPP produced and secreted from islets β -cells can induce Fas expression. hIAPP-expressing mouse islets, but not wild-type mouse islets, formed hIAPP aggregates during culture that was associated with an increase in Fas expression. In cultured human islets, islet amyloid formation was associated with a time-dependent increase in Fas mRNA and protein expression. Moreover, our immunolabelling studies showed that Fas-positive islet areas co-localised with thioflavin S (amyloid)-positive islet areas, indicating the correlation between amyloid formation and Fas expression in cultured human and hIAPP-expressing mouse islets. In support, a recent study showed that amyloid formation in cultured hIAPP-expressing mouse islets induces expression of Fas and FADD in a JNK-dependent manner and that inhibition of JNK pathway protects islet β -cells from islet amyloid-induced apoptosis (490). Conversely, prevention of amyloid formation in both human and hIAPP-expressing mouse islets by adenoviral-mediated suppression of hIAPP expression markedly reduced Fas expression and subsequent β -cell apoptosis.

We generated a new mouse model expressing hIAPP with β -cell specific Fas deletion to further investigate the role of Fas in mediating the β -cell toxic effects of endogenously produced hIAPP aggregates in islets. Islets from hIAPP-expressing mice with or without Fas expression had comparable levels of amyloid formation confirming that the absence of Fas *per se* does not have any significant effect on amyloid formation in hIAPP-expressing mouse islets. Interestingly, hIAPP-expressing mouse islets lacking Fas had a significantly lower number of active caspase-3 and apoptotic β -cells, higher islet insulin response to glucose, and lower proinsulin release compared to islets expressing hIAPP and Fas, suggesting that deletion of Fas protects islet β -cells from endogenously produced hIAPP aggregates and improves both their survival and function. Despite significant increase in the proportion of active caspase-3 and apoptotic β -cells, the number of α -cells remained unchanged in cultured hIAPP-transgenic

mouse islets expressing Fas resulting in a lower β - to α -cell ratio in hIAPP-expressing islets with Fas expression compared to those lacking Fas.

Enhanced glucose stimulated insulin secretion in hIAPP-expressing mouse islets with β -cell Fas deletion is likely a result of both reduced amyloid toxicity mediated by Fas upregulation or effects of Fas deletion on β -cell secretory mechanism (459). However, under the culture conditions used in our study, glucose-stimulated insulin secretion was not significantly different between non-transgenic (hIAPP^{-/-}) islets with Fas deletion and those expressing Fas, indicating that enhanced insulin secretion in hIAPP-expressing mouse islets without Fas expression is due to the effects of Fas deletion on preventing hIAPP-induced β -cell apoptosis.

The formation of hIAPP-aggregates and Fas upregulation in both human and hIAPP-expressing mouse islets was associated with increased islet IL-1 β immunoreactivity, which was reduced by adenoviral-siRNA-mediated prevention of amyloid formation, suggesting that IL-1 β may contribute to hIAPP-induced Fas upregulation. The source of IL-1 β detected by immunolabelling in thioflavin S (amyloid)-positive islets might be islet β -cells, non- β -cells, or non-islet cells. β -cells have been shown to produce IL-1 β in conditions such as exposure to elevated glucose, resulting in Fas upregulation (481), so it is likely that amyloid formation similarly induces β -cell production of IL-1 β . In support of this notion, our immunolabelling studies showed co-localization of insulin and IL-1 β staining in cultured human and mouse islets. Furthermore, two recent studies have demonstrated increase in IL-1 β release from non-islet cells such as macrophages activated by hIAPP aggregates (491,492). Taken together, these findings suggest a role for IL-1 β in hIAPP-induced Fas upregulation, although further studies are required to identify the cell type(s) that release IL-1 β in conditions associated with islet amyloid formation.

In summary, data presented in this chapter show that hIAPP-induced β -cell death is mediated, at least partially, via upregulation of Fas and that deletion of Fas protects islet β -cells from the cytotoxic effects of endogenously secreted and exogenously applied hIAPP aggregates. Blocking Fas upregulation may therefore provide a new approach to prevent amyloid toxicity

thereby enhance survival and function of human islets in conditions associated with islet amyloid formation such as type 2 diabetes, islet culture, and transplantation.

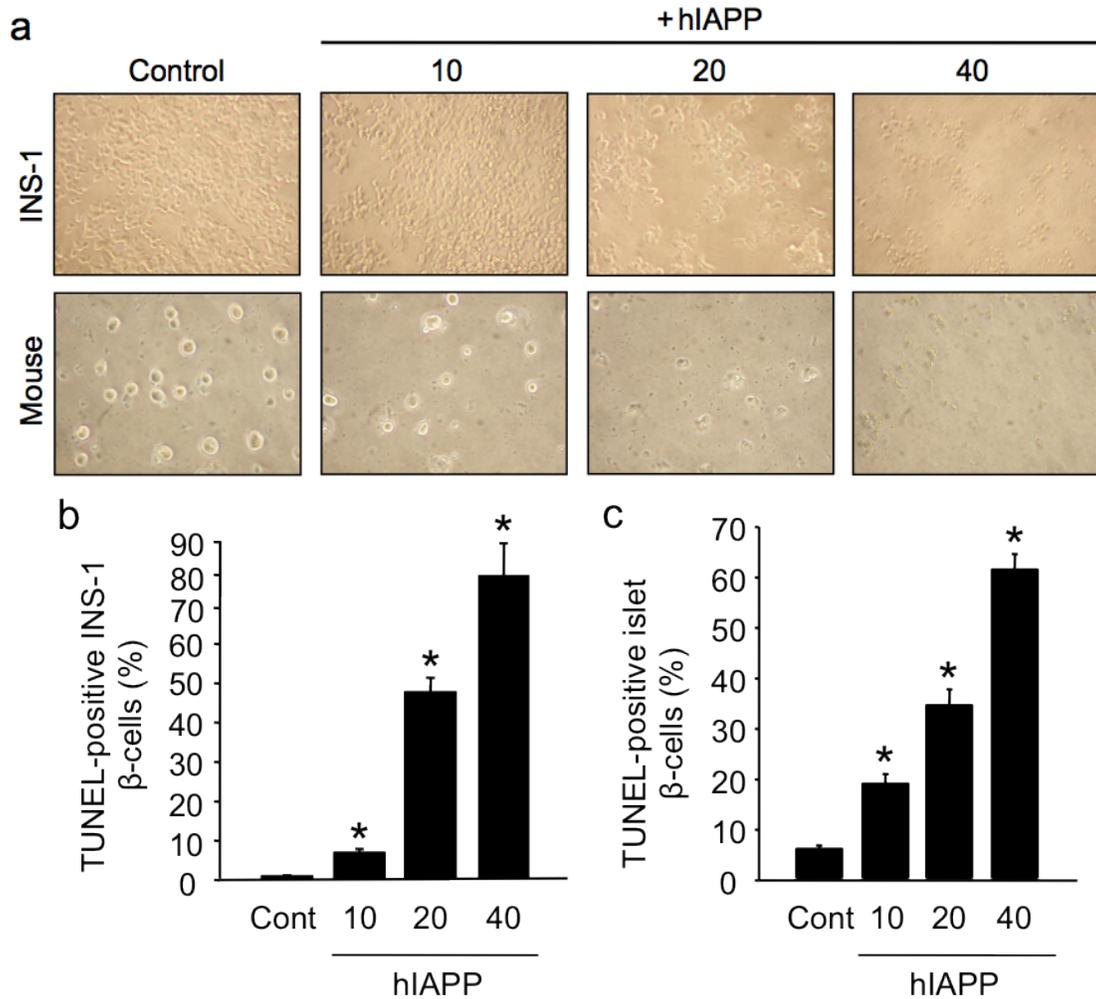


Figure 3-1. Synthetic hIAPP aggregates induce β-cell apoptosis in a concentration dependent manner.

INS-1 or dispersed mouse islet (BALB/c) β-cells were cultured in the absence or presence of synthetic hIAPP (10-40 μmol/l) for 24 h. **(a)** Pictures were taken using an inverted phase contrast microscope. **(b)** INS-1 or **(c)** dispersed mouse islet cells were immunolabelled for insulin/TUNEL and the number of TUNEL-positive (apoptotic) β-cells was quantified. The results are mean of three independent studies performed in triplicate. *vs. non-treated control ($p < 0.01$, Student's *t*-test)

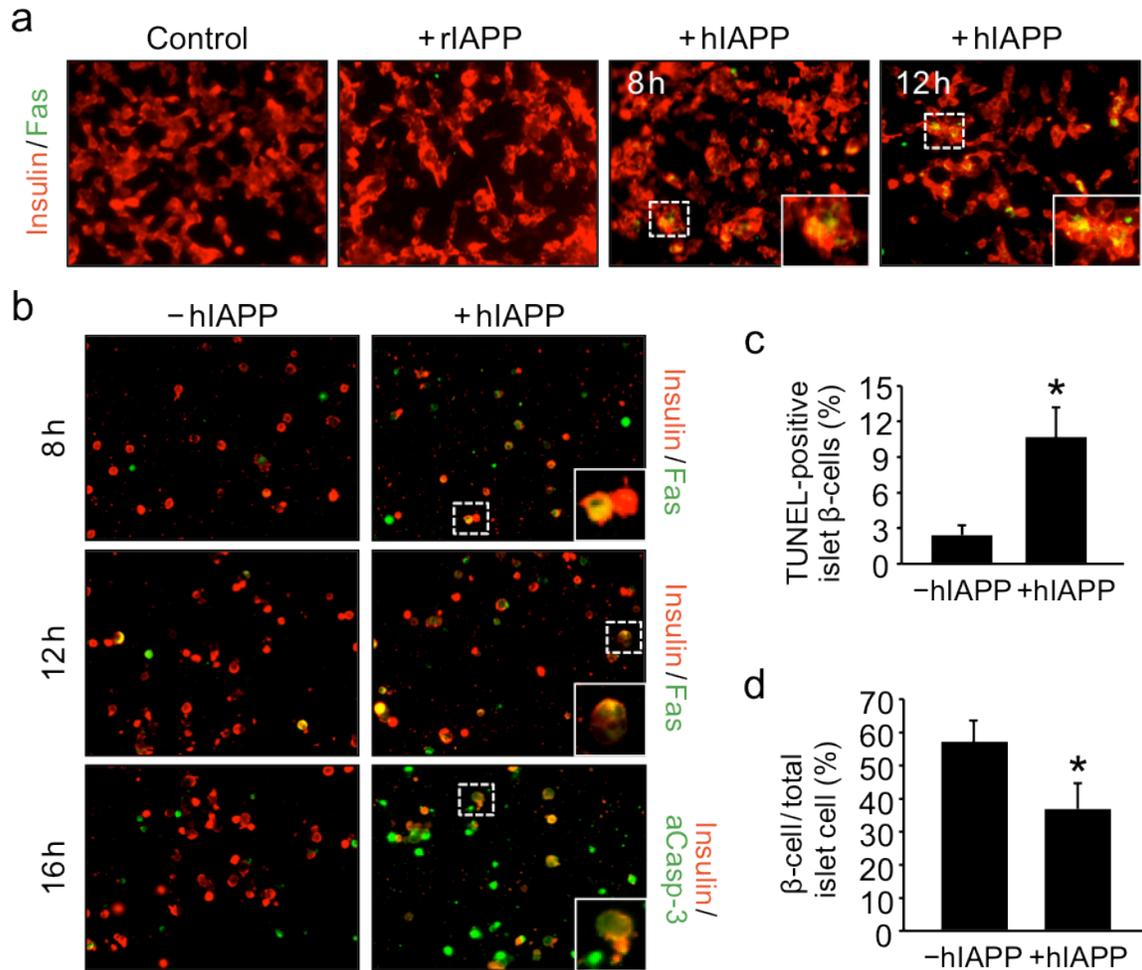


Figure 3-2. Exposure to synthetic hIAPP induces Fas upregulation associated with caspase-3 activation and apoptosis in INS-1 and mouse islet β -cells.

INS-1 cells and dispersed mouse islet cells (C57BL/6) were treated with 10 $\mu\text{mol/l}$ synthetic hIAPP or non-fibrillogenic rIAPP at different time points. **(a)** Immunolabelling of INS-1 cells for insulin and Fas after 8 and 12 h exposure to hIAPP or rIAPP. **(b)** Immunolabelling of dispersed mouse islet cells for insulin and Fas or cleaved (active) caspase-3 following exposure to hIAPP for 8 and 12 h or 16 h, respectively. The white squares denote regions enlarged in each image. The proportion of **(c)** TUNEL-positive islet β -cells and **(d)** β -cell to total islet cell ratio in dispersed mouse islets following culture with or without hIAPP for 24 h. The proportion of apoptotic β -cells was quantified by manual counting of double insulin and TUNEL-positive islet cells in a minimum of ten microscopic fields each containing 100-150 dispersed islet cells. β -cell/total islet cell ratio represents the mean of insulin-positive islet cells divided by total islet cells in each microscopic field. Results are expressed as means \pm SEM of three independent studies performed in triplicate. *vs non-treated group ($p < 0.05$, Student's *t*-test)

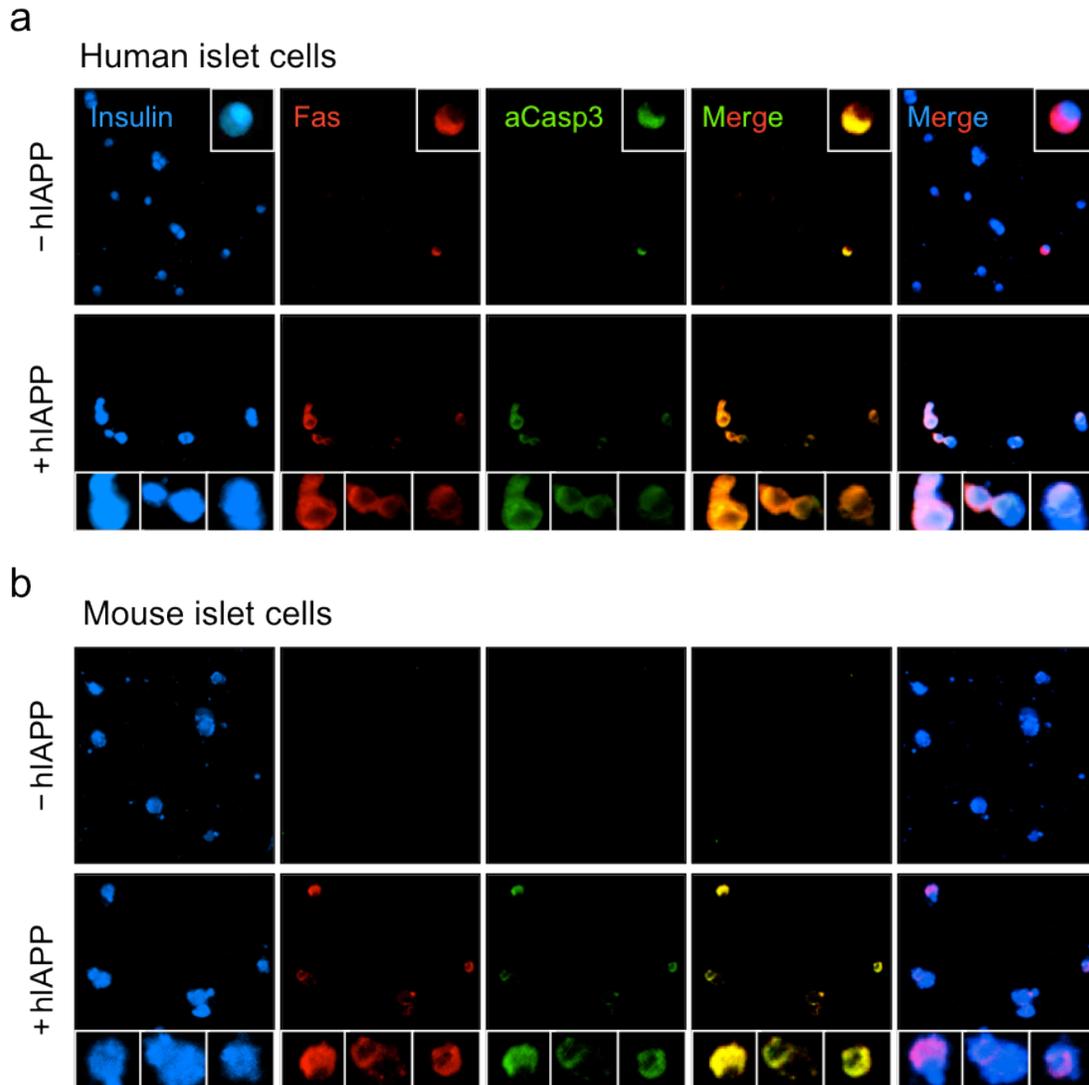


Figure 3-3. Co-localization of Fas and active caspase-3 in human and mouse islet β -cells treated with synthetic hIAPP.

(a) Dispersed human or (b) mouse islet (C57BL/6) cells cultured without or with synthetic hIAPP (10 μ mol/l, 12 h) were immunolabelled for insulin, Fas, and cleaved (active) caspase-3. The white squares denote regions enlarged in each image. Images represent three independent human or mouse studies performed in duplicate.

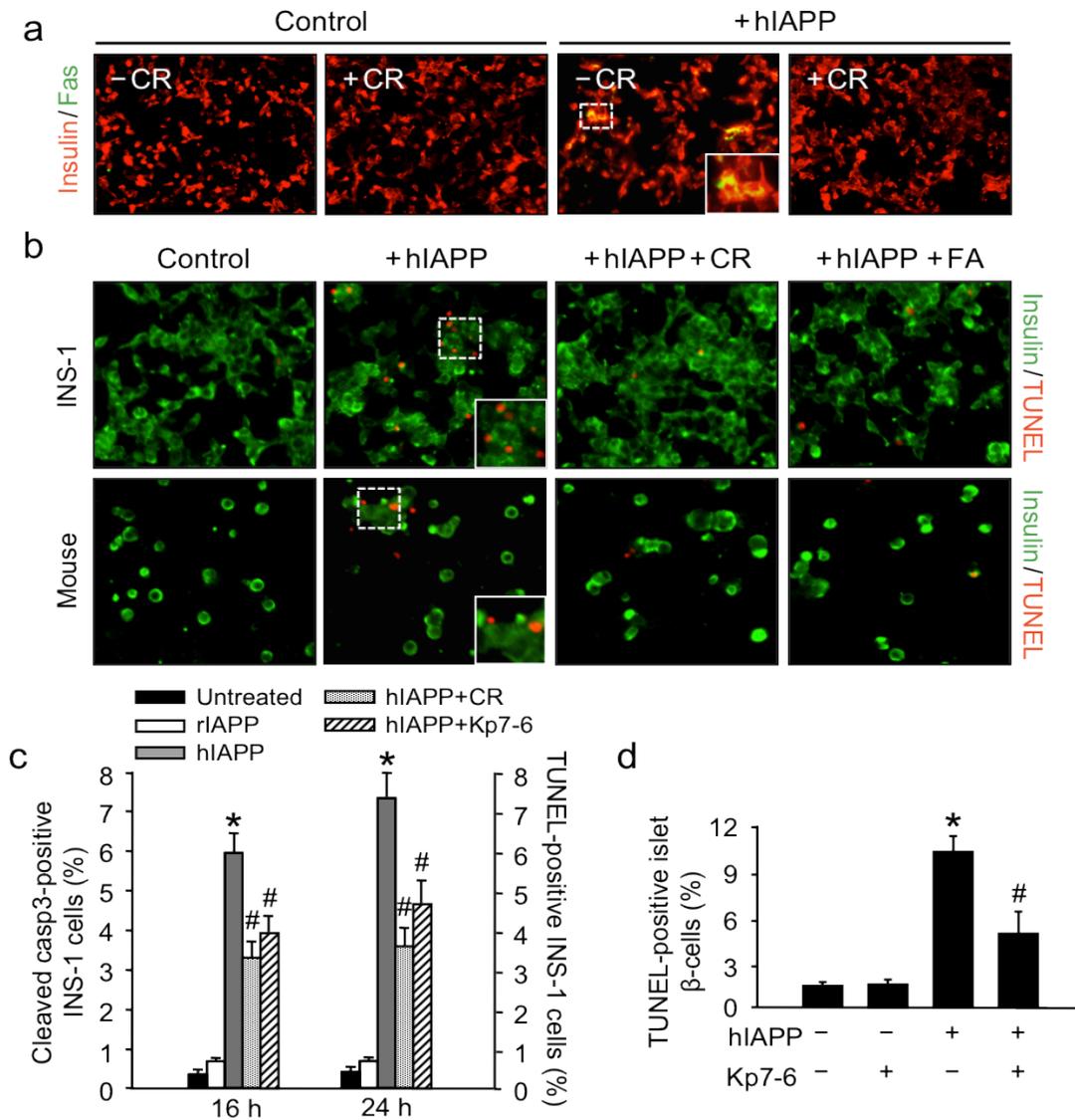


Figure 3-4. Culture with islet amyloid inhibitor or Fas antagonist markedly reduces apoptosis induced by exogenously applied hIAPP.

(a) INS-1 cells treated with hIAPP (10 $\mu\text{mol/l}$, 12 h) without or with Congo red (CR; 25 $\mu\text{mol/l}$) were immunolabelled for insulin and Fas. (b) INS-1 and mouse islet cells (C57BL/6) were treated with hIAPP alone (24 h) or with CR or Kp7-6 (10 mmol/l) and immunolabelled for insulin and TUNEL. The white squares denote regions enlarged in each image. (c) The proportion of cleaved caspase-3 and TUNEL-positive INS-1 cells treated with hIAPP (or rIAPP) in the absence and presence of CR or Kp7-6. (d) The proportion of apoptotic mouse islet cells treated with hIAPP alone or with Kp7-6 for 24 h. The proportion of apoptotic β -cells was quantified by manual counting of insulin and cleaved caspase-3 or TUNEL-positive INS-1 or islet cells in a minimum of ten microscopic fields each containing 200-300 INS-1 cells or 100-150 dispersed islet cells. Results are expressed as means \pm SEM of three independent studies performed in triplicate. *vs non-treated group; #vs hIAPP-treated group ($p < 0.05$, one-way ANOVA)

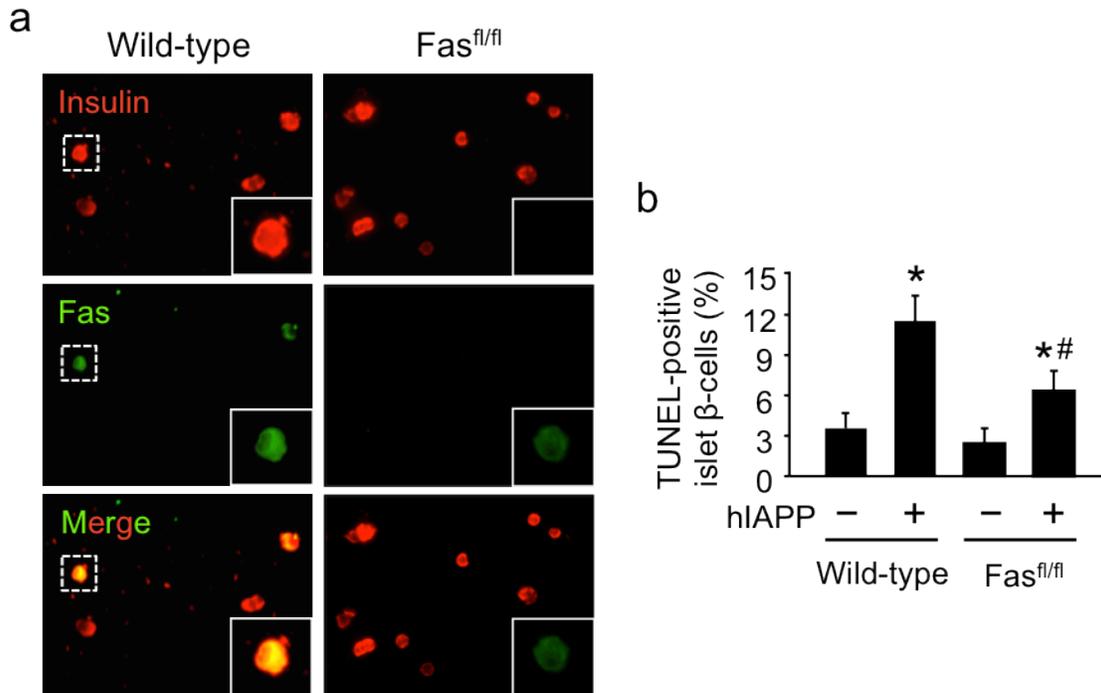


Figure 3-5. β -cell specific deletion of Fas markedly reduces hIAPP-induced β -cell apoptosis in dispersed islet cells.

Dispersed RIPcre⁺Fas^{fl/fl} and wild-type (RIPcre⁺Fas^{+/+}) mouse islet cells were treated with hIAPP (10 μ mol/l, 24 h) and **(a)** immunolabelled for insulin and Fas. The white squares denote regions enlarged in each image. **(b)** The proportion of apoptotic β -cells in mouse islet cells with and without Fas deletion after treatment with hIAPP. The proportion of apoptotic β -cells was quantified by manual counting of insulin and TUNEL-positive islet cells in a minimum of ten microscopic fields each containing 100-150 dispersed islet cells. Results are expressed as means \pm SEM of three independent studies performed in triplicate. *vs corresponding non-treated group; #vs corresponding Fas expressing group ($p < 0.05$, one-way ANOVA)

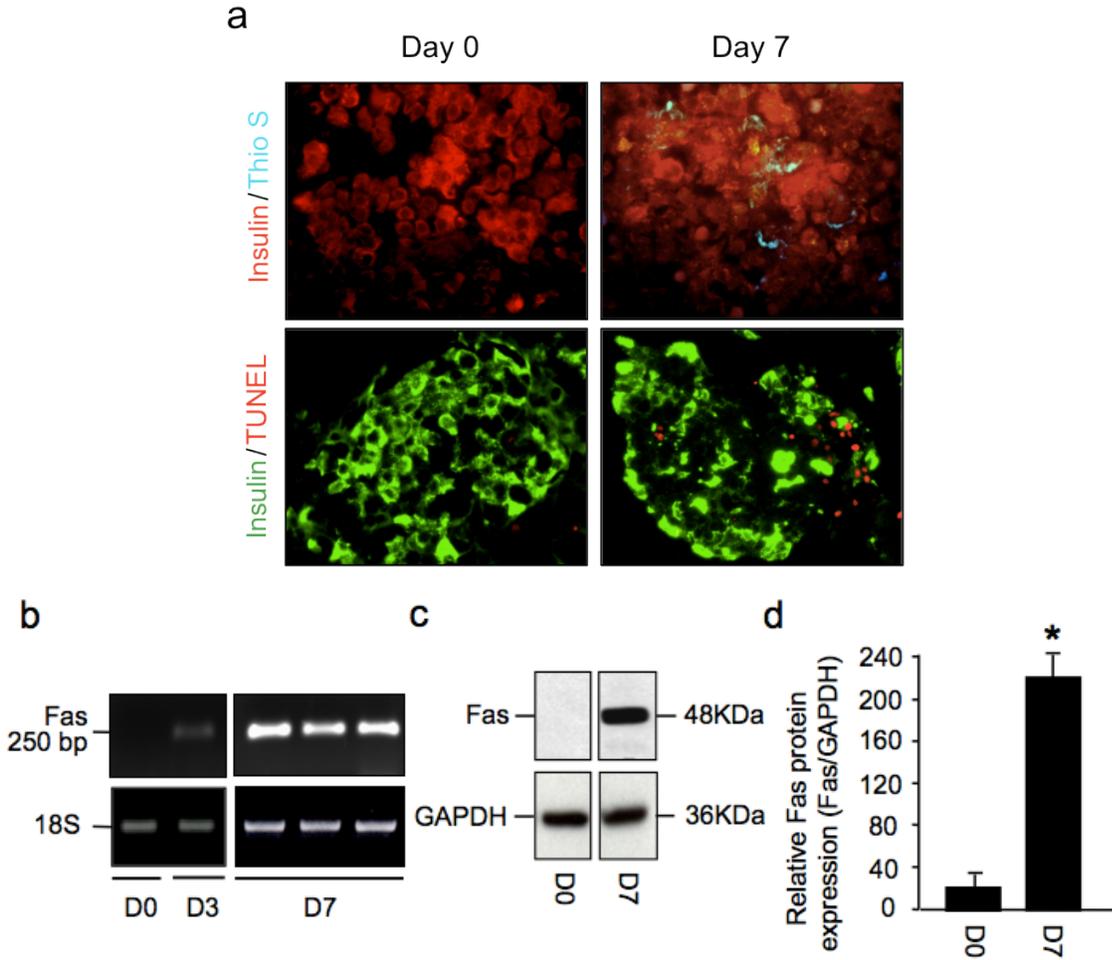


Figure 3-6. Increased Fas mRNA and protein expression in cultured human islets is associated with progressive amyloid formation and β -cell apoptosis.

Freshly isolated human islets were cultured in CMRL containing 11.1 mmol/l glucose for up to 7 days. **(a)** Paraffin-embedded islet sections were double immunolabelled for insulin/thioflavin S and insulin/TUNEL to detect amyloid formation and β -cell apoptosis, respectively. **(b)** Fas mRNA and **(c)** protein expression in human islets before and after culture. **(d)** Densitometric analysis of the immunoblots. Results are expressed as means \pm SEM of three independent studies. *vs day 0 group ($p < 0.05$, Student's t -test)

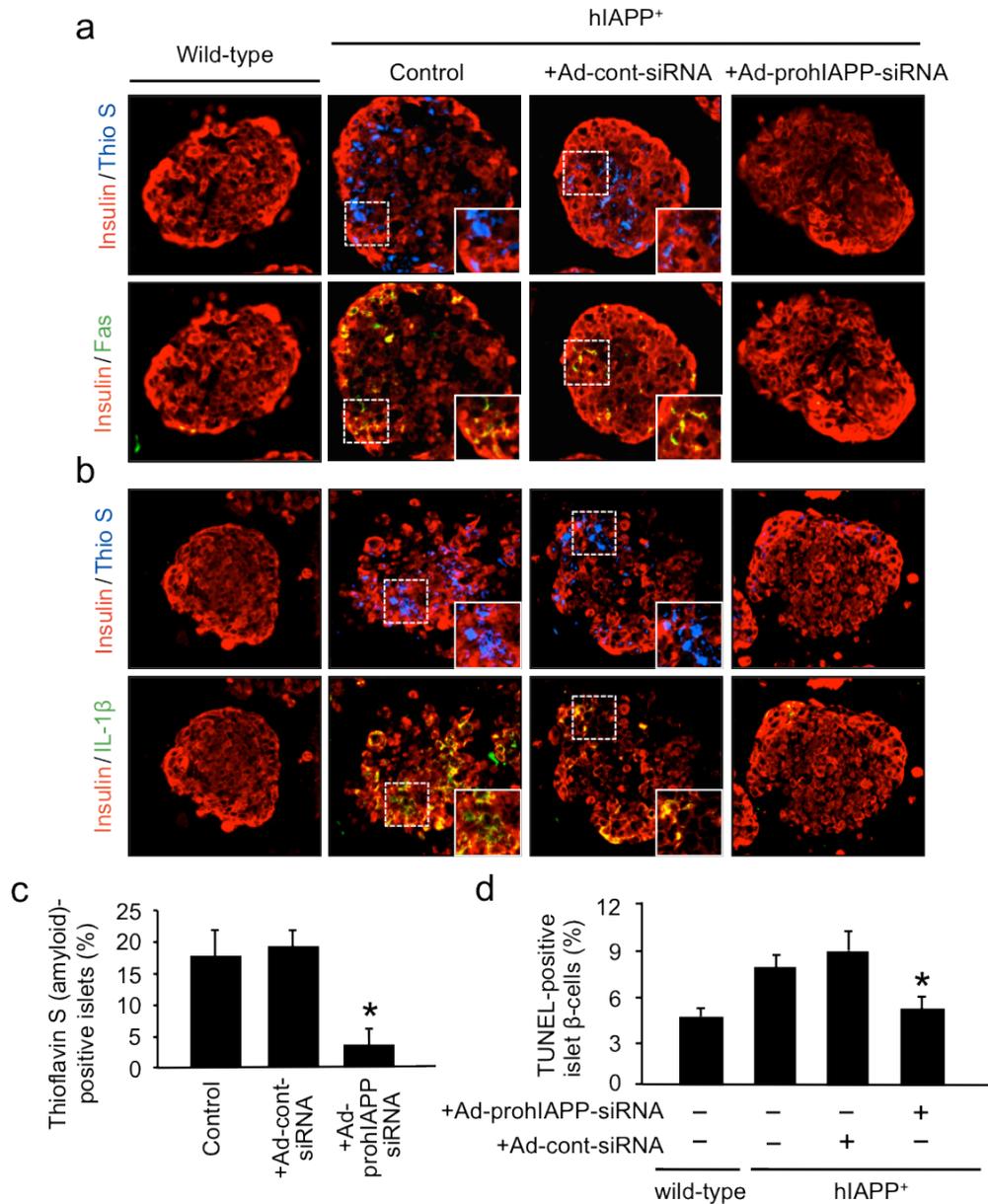


Figure 3-7. Suppression of islet amyloid formation in cultured hiAPP-expressing mouse islets reduces IL-1β levels, Fas expression, and β-cell apoptosis.

Islets isolated from hiAPP mice (8-12 weeks) were transduced overnight with Ad-prohIAPP-siRNA or Ad-cont-siRNA (as control). Non-transduced and transduced islets were cultured in Ham's-F10 (16.7 mmol/l glucose) for 6 days to allow amyloid formation. Paraffin embedded islet sections were immunolabelled for **(a)** insulin/thioflavin S or insulin/Fas; and **(b)** insulin/thioflavin S or insulin/IL-1β. **(c)** The percentage of thioflavin S (amyloid)-positive islets; and **(d)** the proportion of apoptotic β-cells in non-transduced and transduced islets following culture. The number of double insulin and TUNEL-positive islet cells was counted in each islet in a total of 15-20 islets from each condition. Results are expressed as means ± SEM of three independent studies. *vs corresponding non-transduced group ($p < 0.05$, one-way ANOVA)

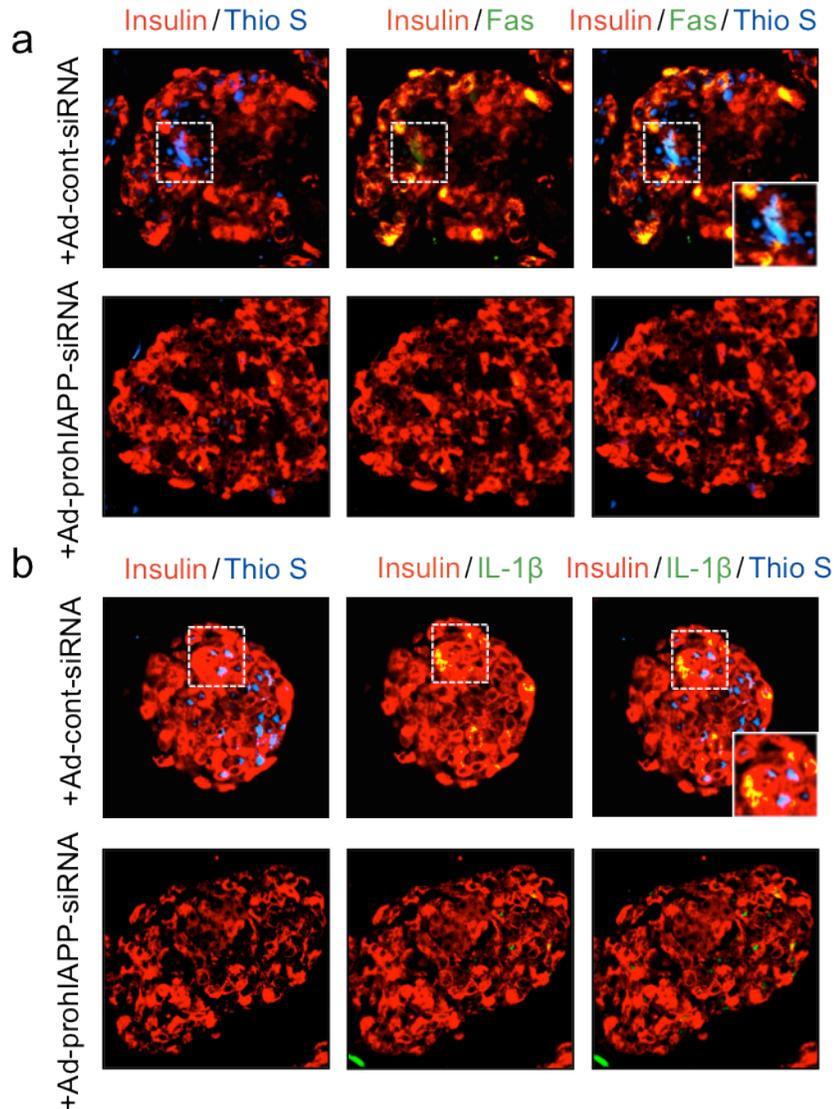


Figure 3-8. Adenoviral-siRNA-mediated suppression of islet amyloid formation in cultured human islets reduces IL-1 β levels and Fas expression and enhances β -cell survival.

Isolated human islets were transduced overnight with Ad-prohIAPP-siRNA or Ad-cont-siRNA (as control). Transduced islets were cultured for 7 days in CMRL containing 11.1 mmol/l glucose to allow amyloid formation. Paraffin embedded islet sections were immunolabelled for **(a)** insulin, Fas, and thioflavin S; and **(b)** insulin, IL-1 β , and thioflavin S. The white squares denote regions enlarged in each image. Images are representative of studies performed on three human islet preparations.

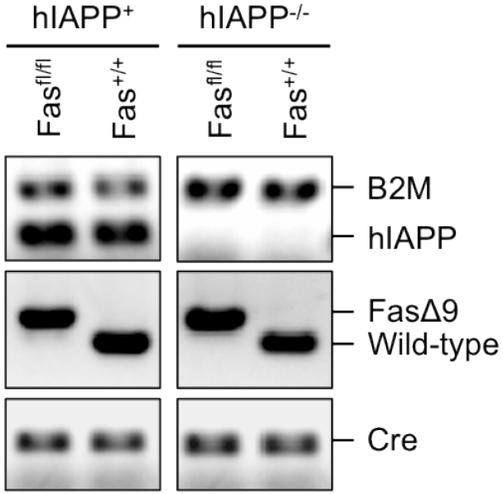


Figure 3-9. Verification of RIP-Cre mediated Fas deletion in hIAPP-expressing transgenic mice.

hIAPP transgene, Fas gene, and insulin-Cre transgene were detected by PCR performed on tail sample DNA from 3-week-old mice with each genotype as indicated in figure labels.

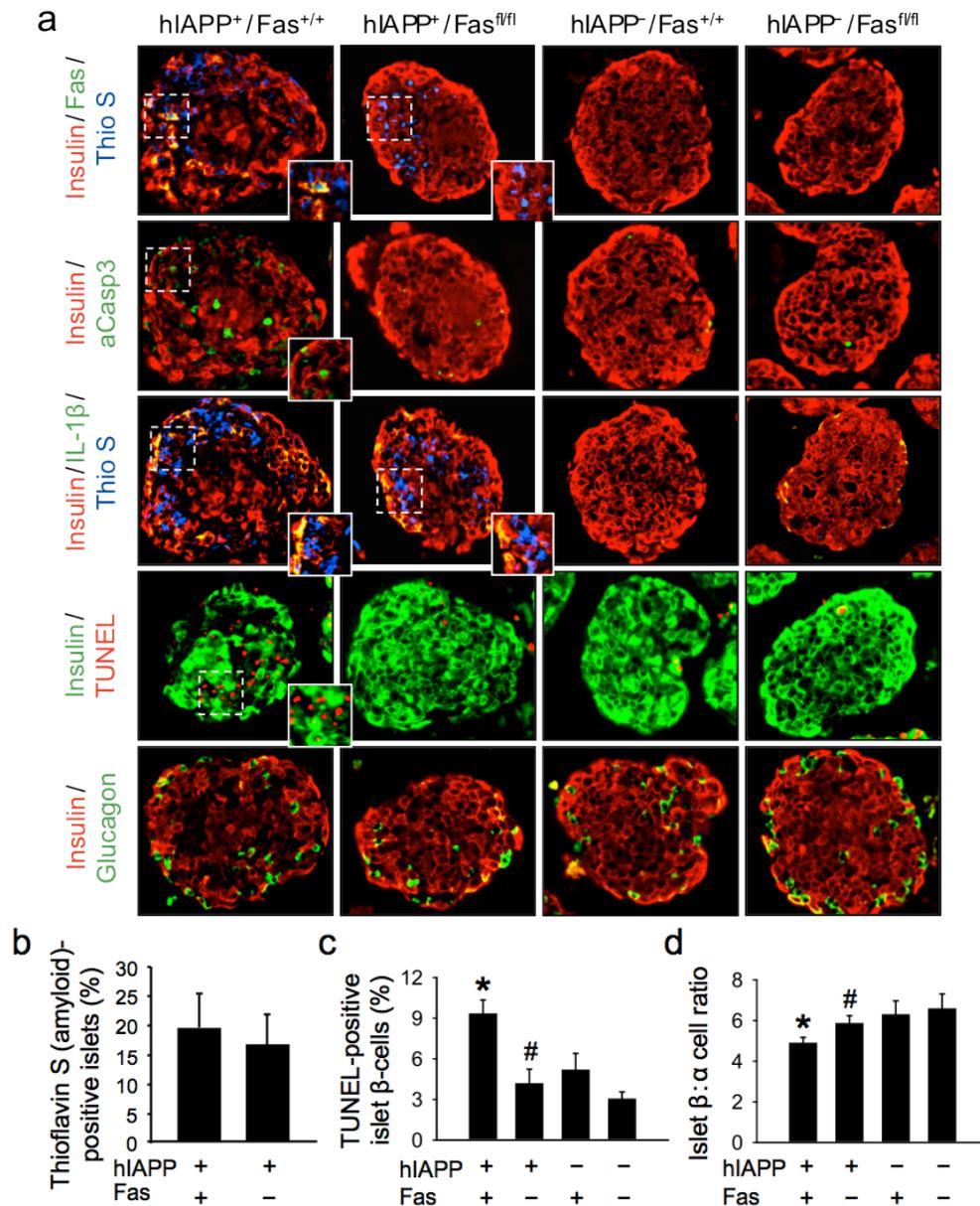


Figure 3-10. β -cell specific deletion of Fas in hIAPP-expressing mouse islets markedly reduces apoptosis induced by aggregation of endogenously secreted hIAPP during culture. Freshly isolated mouse islets from wild-type (hIAPP^{-/-}/Fas^{+/+}) and hIAPP-expressing mice (8-18 weeks) with or without Fas deletion were cultured in Ham's-F10 (16.7 mmol/l glucose) for 7 days. **(a)** Paraffin-embedded sections of cultured islets were immunolabelled as shown in figure labels. **(b)** The percentage of thioflavin S (amyloid)-positive hIAPP-expressing islets with or without Fas deletion after 7 days culture. The proportion of **(c)** TUNEL-positive β -cells and **(d)** islet β to α cell ratio were quantified. The number of double insulin and TUNEL-positive islet cells was counted in each islet in a total of 15-25 islets per animal from each genotype (6-8 animals per group). Islet β to α cell ratio represents the mean of insulin-positive cells divided by glucagon-positive cells in each islet. Results are presented as the means \pm SEM. *vs wild-type group; #vs corresponding Fas expressing group ($p < 0.05$, one-way ANOVA)

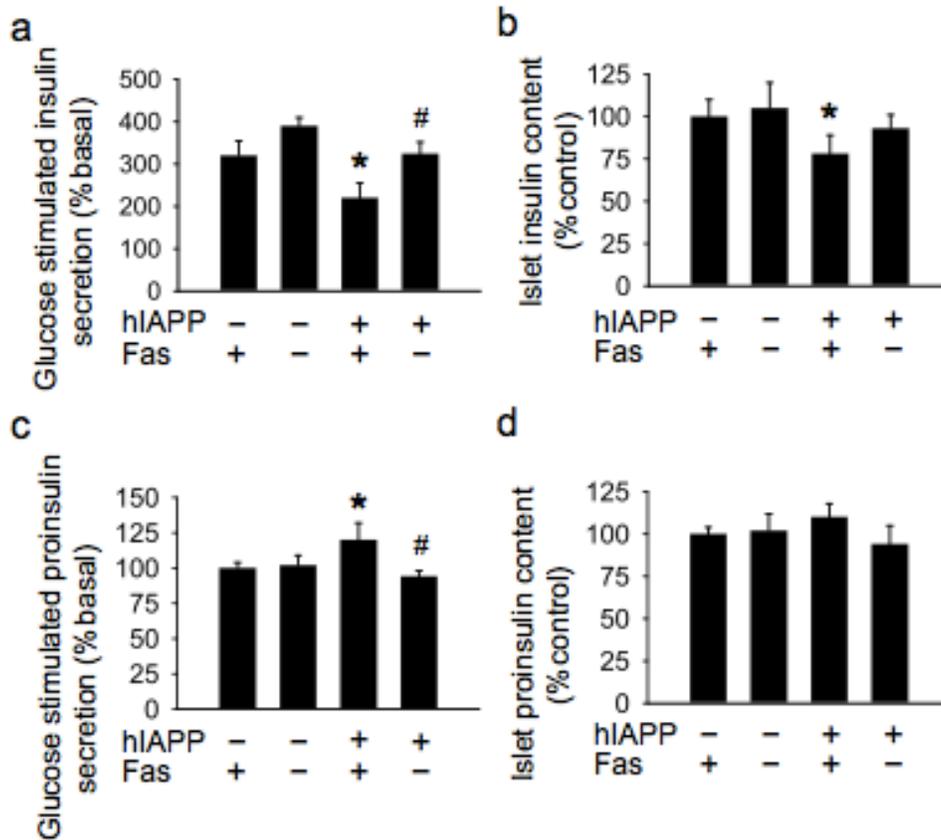


Figure 3-11. Reduced amyloid-induced β -cell death in hIAPP-expressing mouse islets with Fas deletion is associated with enhanced β -cell function.

Wild-type (hIAPP^{-/-}/Fas^{+/+}) and hIAPP-expressing mouse islets with or without Fas deletion were cultured for 7 days in Ham's-F10 (16.7 mmol/l glucose). (a) Insulin response to glucose stimulation (16.7 mmol/l), (b) insulin content, (c) glucose stimulated proinsulin release, and (d) proinsulin content in cultured islets from each genotypes were assessed by performing glucose-stimulated insulin secretion test. Islet insulin and proinsulin content are reported as percentage of insulin or proinsulin content in cultured wild-type islets taken as 100% and glucose stimulated insulin or proinsulin release as percent increase over basal release (1.67 mmol/l glucose). Results are expressed as means \pm SEM of 4-6 animals per genotype. *vs wild-type group; #vs corresponding Fas expressing group ($p < 0.05$, one-way ANOVA)

Chapter 4: Amyloid-induced Fas upregulation promotes Fas and FasL interaction and caspase-8 activation

4.1 Background

Growing evidence suggests that the Fas cell death receptor signalling pathway plays an important role in mediating β -cell death in type 2 diabetes (438,480,493). While Fas expression is negligible in pancreatic sections from non-diabetic individuals, localization of Fas in β -cells has been shown in the pancreatic sections from type 2 diabetic patients using immunohistochemistry and mRNA *in situ* hybridization methods (480). The factor(s) that induce Fas upregulation in islet β -cells in patients with type 2 diabetes are still not well understood. In isolated human islets, exposure to elevated glucose induces Fas upregulation and activation of downstream caspases, leading to β -cell apoptosis (480). However, Fas upregulation and caspase activation also occur when islets are treated with glucose at very high concentrations (e.g. 33.3 mmol/l) that is not typically found in patients with type 2 diabetes, indicating that other factors likely contribute to Fas upregulation and Fas-mediated β -cell apoptosis in type 2 diabetes (480). Studies in Chapter 3 demonstrated that exposure to aggregates derived from both synthetic and biosynthetic hIAPP in human and hIAPP-expressing transgenic mouse islets induces Fas upregulation in β -cells and that blocking or deletion of Fas can protect islets from amyloid-induced β -cell death. Our results further showed that inhibition of amyloid formation prevents hIAPP-induced β -cell Fas upregulation in human and hIAPP-expressing transgenic mouse islets. These studies indicate that islet amyloid formation may be an important factor that contributes to Fas upregulation in islet β -cells in type 2 diabetes.

As shown in Figure 1-5, caspase-8 is the initiator caspase in the Fas-mediated apoptotic pathway, which is responsible for propagating the apoptotic signal once FasL binds to Fas expressed on the cell membrane (494). Following the Fas-FasL ligation, formation of the death-inducing signalling complex (DISC) occurs instantly by recruiting procaspase-8 (495,496). At the DISC, procaspase-8 dimerises and becomes activated through autoproteolytic cleavage of the molecule (497). The active (cleaved) caspase-8 is then released from the DISC to trigger the apoptotic cascade of downstream effector caspases such as caspase-3, -6 and -7, which leads to

cleavage and breakdown of essential cellular components eventually resulting in cell death (Type I) (497,498). Moreover, caspase-8 can mediate a cross-talk between the Fas-mediated apoptotic pathway and the mitochondrial-mediated (intrinsic) apoptotic pathway (Type II) (494,499). Cellular FLIP (cFLIP), a structural caspase-8 homologue, is the major endogenous regulator of the Fas-mediated apoptotic pathway, which can prevent the cleavage of procaspase-8 thereby blocking the subsequent apoptotic signalling cascade (498).

In this study, we hypothesised that amyloid-induced β -cell Fas upregulation may promote the interaction between Fas and FasL on islet and/or non-islet cells in close proximity, leading to the activation of caspase-8, the key upstream enzyme in the Fas signalling pathway, resulting in β -cell apoptosis. To test this hypothesis, we first used dispersed islet cells to examine if Fas upregulation induced by exogenously applied hIAPP aggregates can promote activation of caspase-8 and β -cell death. Conversely, we tested if amyloid inhibitor Congo red can prevent caspase-8 activation and apoptosis in hIAPP-treated β -cell cells. Moreover, we tested if blocking the interaction between Fas and FasL using a Fas blocker or selective inhibition of caspase-8 using a caspase-8 specific inhibitor, can prevent hIAPP-induced caspase-8 activation thereby reduce β -cell apoptosis. Next, we used cultured islets from human and hIAPP-expressing transgenic mice, to examine if β -cell Fas upregulation induced by endogenously formed hIAPP aggregates can promote caspase-8 activation leading to increased β -cell apoptosis. Finally, using a cross-breeding approach, we generated a new mouse model expressing hIAPP but lacking β -cell specific caspase-8, to investigate the effects of caspase-8 deletion on amyloid-induced β -cell toxicity.

4.2 Results

4.2.1 Exposure to exogenous hIAPP induces caspase-8 activation in human islet β -cells, which is associated with Fas upregulation and precedes caspase-3 activation and apoptosis

First, we examined if exposure to synthetic hIAPP aggregates can induce activation of caspase-8 in islet β -cells. Human islet cells were cultured in normal glucose with or without synthetic hIAPP (10 μ mol/l) or non-fibrillogenic rIAPP (10 μ mol/l) at different time points (8-24 h). Non-treated and rIAPP-treated islet cells had very low numbers of β -cells positive for active

caspase-8 (Figure 4-1a). Treatment with hIAPP induced caspase-8 activation in islet β -cells in a time-dependent manner. The maximum number of β -cells positive for active caspase-8 was detectable by immunolabelling between 8 and 12 h after exposure to synthetic hIAPP (Figure 4-1a). The time-point of caspase-8 activation (8-12 h; Figure 4-1a) correlated with that of Fas upregulation (8-12 h; Figures 3-2a and 4-1b), and preceded caspase-3 activation (16 h; Figures 3-2b and 4-1b, c) and β -cell apoptosis (24 h; Figures 3-2c and 4-1b, d) in hIAPP-treated islet β -cells. Increased caspase-8 activation in human islet cells treated with synthetic hIAPP was associated with higher number of TUNEL-positive (apoptotic) β -cells as compared to non-treated islets cells (Figure 4-1d).

4.2.2 Blocking hIAPP-mediated caspase-8 activation by amyloid, Fas or caspase-8 inhibitors markedly reduces β -cell apoptosis induced by aggregation of exogenously applied hIAPP

To identify the mechanisms underlying amyloid-induced caspase-8 activation, we tested the effects of the amyloid inhibitor Congo red, Fas antagonist and caspase-8 inhibitor on hIAPP-treated human islet β -cells. The amyloid-binding dye Congo red significantly reduced the number of active caspase-8 and TUNEL-positive β -cells in hIAPP-treated islet cells (Figure 4-2a, b, c). Similarly, blockade of Fas (induced by hIAPP aggregates) with a Fas antagonist markedly lowered the number of active caspase-8-positive and apoptotic β -cells in hIAPP-treated islet cells (Figure 4-2a, b, c). Inhibition of caspase-8 by treatment with a cell permeable caspase-8 specific inhibitor effectively reduced hIAPP-induced caspase-8 activation (Figure 4-2a, b), and increased islet cell viability in hIAPP-treated cells (Figure 4-2c). Moreover, deletion of caspase-8 protected islet cells from synthetic hIAPP-induced β -cell death as shown by a lower number of TUNEL-positive β -cells in hIAPP-treated islet cells from RIPcre⁺Casp8^{fl/fl} mice than wild-type mice that express caspase-8 (Figure 4-3). As expected, active caspase-8 positive β -cells were absent in hIAPP-treated islet cells from mice lacking caspase-8 (Figure 4-3a).

4.2.3 Amyloid formation in human islets during culture induces caspase-8 activation and β -cell death, which is associated with Fas upregulation

Next, we examined whether formation of endogenous hIAPP aggregates in human islets during culture can induce caspase-8 activation in β -cells. Isolated human islets from six donors were cultured for 7 days in elevated glucose to potentiate amyloid formation. Thioflavin S (amyloid)-positive islets were not detectable in freshly isolated islets from five of six donors, but small amyloid-positive areas were detectable in about 1% of islets from one donor (Figure 4-4a, b, c). Islet culture resulted in progressive amyloid formation in all human islet preparations (Figure 4-4a, b, c). Similarly, hIAPP oligomers were detectable in cultured human islets from all donors by A11 immunolabelling (Figure 4-4a, d). Importantly, thioflavin S- or A11 (oligomer)-positive areas in islets closely correlated with active caspase-8-positive β -cell areas, both of which were associated with increased β -cell apoptosis (Figure 4-4a, e) and reduced islet β -cell area (Figure 4-4a, f).

4.2.4 Generation of hIAPP-expressing mice with β -cell specific caspase-8 deletion

We generated a new mouse model with β -cell specific hIAPP expression and caspase-8 deletion by a cross-breeding approach as detailed in Chapter 2 (Figure 2-2). The presence of the hIAPP transgene and the caspase-8 gene with a deletion mutation were confirmed in hIAPP-expressing mice lacking caspase-8 (hIAPP⁺/RIPcre⁺Casp8^{fl/fl}) by PCR genotyping (Figure 4-5a). Deletion of caspase-8 in hIAPP-expressing mice was further confirmed by the absence of caspase-8 positive islet β -cells in double insulin and (full-length) caspase-8 immunolabelled pancreatic sections of hIAPP⁺/RIPcre⁺Casp8^{fl/fl} mice (Figure 4-5b). A few caspase-8 positive but insulin-negative islet cells were detectable in the islet periphery, indicating that the disruption of caspase-8 was specific to β -cells and did not affect caspase-8 expression in non- β -cells such as α -cells in hIAPP⁺/RIPcre⁺Casp8^{fl/fl} mice (Figure 4-5b). Moreover, deletion of caspase-8 had no effect on amyloid formation as shown by double insulin and thioflavin S immunolabelling of cultured islets from hIAPP-expressing mice with or without caspase-8 deletion (Figure 4-5c).

4.2.5 Deletion of caspase-8 in hIAPP-expressing mouse islet β -cells markedly reduces apoptosis induced by the aggregation of endogenously produced hIAPP during culture

Using hIAPP-expressing mice with β -cell specific caspase-8 deletion, we tested whether blockade of caspase-8 activation can protect β -cells from amyloid toxicity in this *ex vivo* model of islet amyloid formation. During 7 days of culture, hIAPP-expressing transgenic mouse islets formed amyloid, which was detectable by insulin/thioflavin S and insulin/A11 immunolabelling (Figure 4-6a). Cultured islets from hIAPP-expressing transgenic mice with or without caspase-8 deletion had comparable levels of amyloid formation, hIAPP release and content (Figure 4-6). Similar to human islets shown in Figure 4-4a, the aggregation of endogenously produced hIAPP in transgenic mouse islets during culture induced activation of caspase-8 (Figure 4-6a, b). A few active caspase-8-positive β -cells were present in cultured islets from wild-type mice expressing caspase-8, but such cells were not detectable in wild-type or hIAPP-expressing transgenic mouse islets with β -cell caspase-8 deletion (Figure 4-6a). Also, a small number of active caspase-8-positive non- β islet cells were present in all genotypes.

Amyloid formation in cultured hIAPP-expressing mouse islets with and without caspase-8 deletion was associated with β -cell Fas upregulation and elevated islet IL-1 β immunoreactivity (Figure 4-7a, b). As expected, amyloid formation was undetectable in cultured islets from hIAPP wild-type (hIAPP^{-/-}) mice, which correlated with very low levels of islet Fas and IL-1 β expression that is consistent with our findings in Figures 3-7a, b and 3-10a. Despite similar islet IL-1 β and β -cell Fas levels, hIAPP-expressing mouse islets lacking caspase-8 had significantly lower rates of β -cell apoptosis during culture compared to islets expressing hIAPP and caspase-8 (Figure 4-8a, b), resulting in higher β - to α -cell ratios and islet β -cell areas in those islets (Figure 4-8d, e). The number of apoptotic β -cells, β - to α -cell ratio, and islet β -cell areas were comparable in cultured hIAPP wild-type islets with or without caspase-8 deletion (Figure 4-8a, b, d). Furthermore, the β -cell proliferation rate was not significantly different in the four genotypes (Figure 4-8c). Finally, there was no detectable difference between FLIP levels in islet lysates from wild-type and hIAPP-expressing transgenic mice following 7 days culture (Figure 4-7c).

4.2.6 Cultured hIAPP-expressing mouse islets with caspase-8 deletion have enhanced β -cell function compared with those expressing caspase-8

Increased β -cell apoptosis in cultured islets from hIAPP-expressing mice was associated with reduced insulin response to elevated glucose (stimulation index) and lower insulin content as compared with wild-type littermates (Figure 4-9). The stimulation index was 32% higher in cultured islets from hIAPP-expressing mice lacking caspase-8 than in islets expressing hIAPP and caspase-8, a finding that correlated with the higher (36%) insulin content in those islets. Cultured islets from mice with caspase-8 deletion and without hIAPP expression had a greater (15%) insulin response to elevated glucose and higher (20%) insulin content than their wild-type littermates. However, this increase was not as profound as the difference observed between hIAPP-expressing mice lacking or expressing caspase-8, suggesting that deletion of caspase-8 improves β -cell dysfunction caused by amyloid formation in hIAPP-expressing mouse islets during culture.

4.3 Discussion

Our findings in Chapter 3 demonstrated that both exogenously applied and endogenously produced hIAPP aggregates induce β -cell Fas upregulation, which is associated with caspase-3 activation and apoptosis. Based on these findings, we proposed that amyloid-induced Fas upregulation may trigger the activation of Fas-mediated signalling pathway by promoting Fas and FasL interaction, leading to the activation of caspase-8, the key mediator of this pathway, and subsequent β -cell death.

In human islet cells, we showed that aggregates of exogenously applied hIAPP induced β -cell Fas upregulation and activation of caspase-8 and -3, resulting in apoptosis. The time point of caspase-8 activation preceded caspase-3 activation and apoptosis, and followed Fas upregulation. We observed very low levels of caspase-8 activation in non- β islet cells following treatment with synthetic hIAPP peptides, indicating that these cells are minimally affected by toxic hIAPP aggregates. Importantly, treatment with a Fas antagonist, which blocks Fas-FasL assembly, prevented hIAPP-induced caspase-8 activation and β -cell apoptosis in dispersed human islet cells, suggesting that hIAPP-induced β -cell Fas upregulation leads to Fas and FasL

interaction resulting in caspase-8 activation and apoptosis. The selective inhibition of caspase-8 in human islet cells treated with synthetic hIAPP aggregates protected islet β -cells from hIAPP-induced apoptosis. Moreover, prevention of hIAPP aggregation by the amyloid inhibitor Congo red markedly reduced β -cell caspase-8 activation and death. Basal death in non-treated islet β -cells was comparable with that in cells treated with Congo red alone, but was slightly lower in those treated with the Fas antagonist or caspase-8 inhibitor alone, likely due to blocking the effects of Fas-inducing factors released during islet isolation, dispersion and culture.

Consistent with our findings in human islet β -cells, *in vitro* studies performed on transformed and mouse islet β -cells showed that treatment with supraphysiological concentrations of synthetic hIAPP results in increased caspase-8 and -3 activation in β -cells, both of which were prevented by selective caspase inhibitors (438,439). Additionally, Zhang *et al.* (438) showed that treatment with synthetic hIAPP has no effect on mRNA expression of caspase precursors (procaspase-8 and -3) in transformed β -cells, indicating that hIAPP aggregates may promote the activation of caspase zymogens rather than affecting the transcriptional expression of these caspases.

Taken together, these findings suggest that Fas upregulation induced by hIAPP aggregates results in the interaction between Fas on β -cells and FasL on neighbouring cells such as β -cells, other islet or non-islet cells, leading to the activation of caspase-8 and subsequent β -cell apoptosis. Similarly, aggregates of amyloidogenic A β peptide were shown to induce caspase-8 activation and apoptosis in neural cells, likely via the Fas-mediated apoptotic pathway (446). In support, active caspase-8 and Fas-positive cells have been reported in the brain of patients with Alzheimer's disease (500,501). Thus, it seems likely that hIAPP aggregates in type 2 diabetes and A β aggregates in Alzheimer's disease share the same apoptotic signalling pathway(s). Furthermore, caspase inhibitors are being considered as potential therapeutic strategies for treatment of Alzheimer's disease as well as other amyloid-associated neurodegenerative disorders (502).

Unlike hIAPP, treatment with synthetic rIAPP peptides did not elicit Fas upregulation and activation of caspase-8 or -3 in β -cells. Zhang *et al.* (439) has proposed that the sequence

differences between fibrillogenic hIAPP and non-fibrillogenic rIAPP in the amyloidogenic region of IAPP (IAPP₂₀₋₂₉) may be important in triggering caspase activation. Accordingly, treatment with modified hIAPP peptide, [^{25,28,29}tripoly]hIAPP, which does not form characteristic β -sheet structures due to substitution of prolyl residues in the hIAPP₂₀₋₂₉ region, did not induce caspase activation as compared to normal hIAPP peptides (439). These findings support the idea that formation of hIAPP β -sheet structures may be required for inducing β -cell Fas upregulation and activation of downstream caspases. In contrast, hIAPP peptides lacking Cys²-Cys⁷ intramolecular disulphide bond and N-terminal amino acid sequences evoked similar responses as normal hIAPP peptides, indicating that the intramolecular disulphide bond or N-terminal amino acid sequences are likely not essential for mediating the toxic effects of hIAPP in β -cells (439).

Interestingly, disruption of the Fas apoptotic pathway using a cell permeable inhibitor of caspase-8 or a Fas antagonist were more effective in reducing hIAPP-induced caspase-8 activation than the amyloid inhibitor Congo red. However, Congo red-treated islet cells had a lower number of apoptotic β -cells than Fas or caspase-8 inhibitor-treated islet cells, suggesting that hIAPP aggregates, in addition to the Fas-mediated apoptotic pathway, may also simultaneously induce other apoptotic mechanism(s) in β -cells. Previous studies have shown that activated caspase-8 may interact with other pathways such as stress-activated protein kinase (JNK and p38 MAPK) pathways in controlling hIAPP-induced β -cell apoptosis (439,441). *In vitro* studies performed on transformed rat and human β -cells have reported that hIAPP-induced caspase-8 activation correlates with the activation of JNK and p38 MAPK (439,441). Similarly, in human neural cells, A β aggregates have been shown to activate caspase-8 and JNK, leading to apoptosis (446). However, whether caspase-8 acts upstream or downstream of JNK and p38 MAPK signalling pathways is still under investigation.

Next, we used human islets isolated from cadaveric pancreatic donors, to assess if aggregation of endogenously produced hIAPP during culture can activate caspase-8 in β -cells. Amyloid deposits were undetectable in the majority of freshly isolated islets, except in a small portion of islets (~1%) from one donor. Islet culture potentiated amyloid formation in human islets, which was associated with β -cell Fas upregulation, caspase-8 activation and apoptosis,

resulting in reduced islet β -cell area. These findings indicate that similar to the effects of synthetic hIAPP aggregates, formation of endogenous hIAPP aggregates in human islets during culture promotes β -cell Fas upregulation, leading to activation of Fas apoptotic pathway mediated by caspase-8.

Further, we generated a new mouse model with β -cell specific hIAPP expression and caspase-8 deletion, to directly examine the potential role of caspase-8 in mediating the cytotoxic effects of endogenously secreted hIAPP aggregates in islets. Consistent with our findings in cultured human islets, formation of endogenous hIAPP aggregates in hIAPP-expressing mouse islets during culture was associated with β -cell Fas upregulation and caspase-8 activation, leading to increased β -cell apoptosis and reduced β -cell mass. As expected, both hIAPP oligomers (small aggregates) and hIAPP fibrils (larger aggregates) were undetectable in freshly isolated islets, but were present in cultured islets. Islets from hIAPP-expressing mice with or without caspase-8 had comparable levels of amyloid formation following culture, indicating that deletion of caspase-8 *per se* does not have any detectable effects on amyloid formation in hIAPP-expressing mouse islets during culture. Despite comparable levels of amyloid, IL-1 β and Fas in cultured hIAPP-expressing mouse islets with or without caspase-8 expression, islets lacking caspase-8 had markedly lower β -cell apoptosis, higher β - to α -cell ratio and greater islet β -cell area than islets expressing caspase-8. Moreover, deletion of caspase-8 in hIAPP-expressing mouse islets enhanced β -cell function manifested as higher insulin content and insulin response to elevated glucose in these islets compared to islets expressing hIAPP and caspase-8. Taken together, our data suggest that deletion of caspase-8 can reduce β -cell death and dysfunction caused by endogenously produced hIAPP aggregates.

Interestingly, the majority of islets with amyloid formation were positive for IL-1 β , Fas and caspase-8 immunoreactivity, whereas islets with no detectable amyloid formation had very low levels of all three, suggesting that amyloid may induce Fas upregulation and caspase-8 activation by promoting IL-1 β release in islets. In support, studies in Chapter 3 showed that inhibition of amyloid formation reduces islet IL-1 β levels, which was associated with decreased β -cell Fas expression and β -cell apoptosis. In light of these findings, we further investigated the role of IL-1 β in mediating hIAPP-induced β -cell Fas upregulation and death in Chapter 5.

A previous study has demonstrated that FLIP has a dual role in the Fas apoptotic pathway through which cellular level of FLIP can switch Fas signalling from mediating β -cell apoptosis to cell proliferation (503). It has been reported that in patients with type 2 diabetes, FLIP expression in islet β -cells is reduced compared to healthy individuals, which may contribute to increased β -cell loss in those patients (503). This raises the possibility that hIAPP aggregates, like elevated glucose in type 2 diabetes, may reduce β -cell FLIP levels, thereby switching Fas signalling towards apoptosis. Although we did not detect any significant difference between FLIP protein levels in lysates from 7-day cultured wild-type and hIAPP-expressing transgenic mouse islets, it is possible that the potential effects of hIAPP aggregates on β -cell FLIP levels were masked in our experimental model for the following reasons: 1) elevated glucose (used to potentiate amyloid formation) can increase FLIP levels in wild-type and hIAPP-expressing transgenic mouse islets; 2) FLIP protein levels were measured in lysates from islet cultures that contained amyloid-positive and -negative islets; and 3) islet lysates contain a mixture of cell types including non- β islet cells.

In summary, our studies in this chapter showed that β -cell Fas upregulation induced by exogenously applied and endogenously formed hIAPP aggregates promotes the Fas and FasL interaction thereby initiating Fas-mediated apoptosis, leading to caspase-8 activation and β -cell death. We further demonstrated that inhibition or deletion of caspase-8 protects islet β -cells from amyloid toxicity, suggesting that caspase-8 plays an important role in amyloid-induced β -cell death.

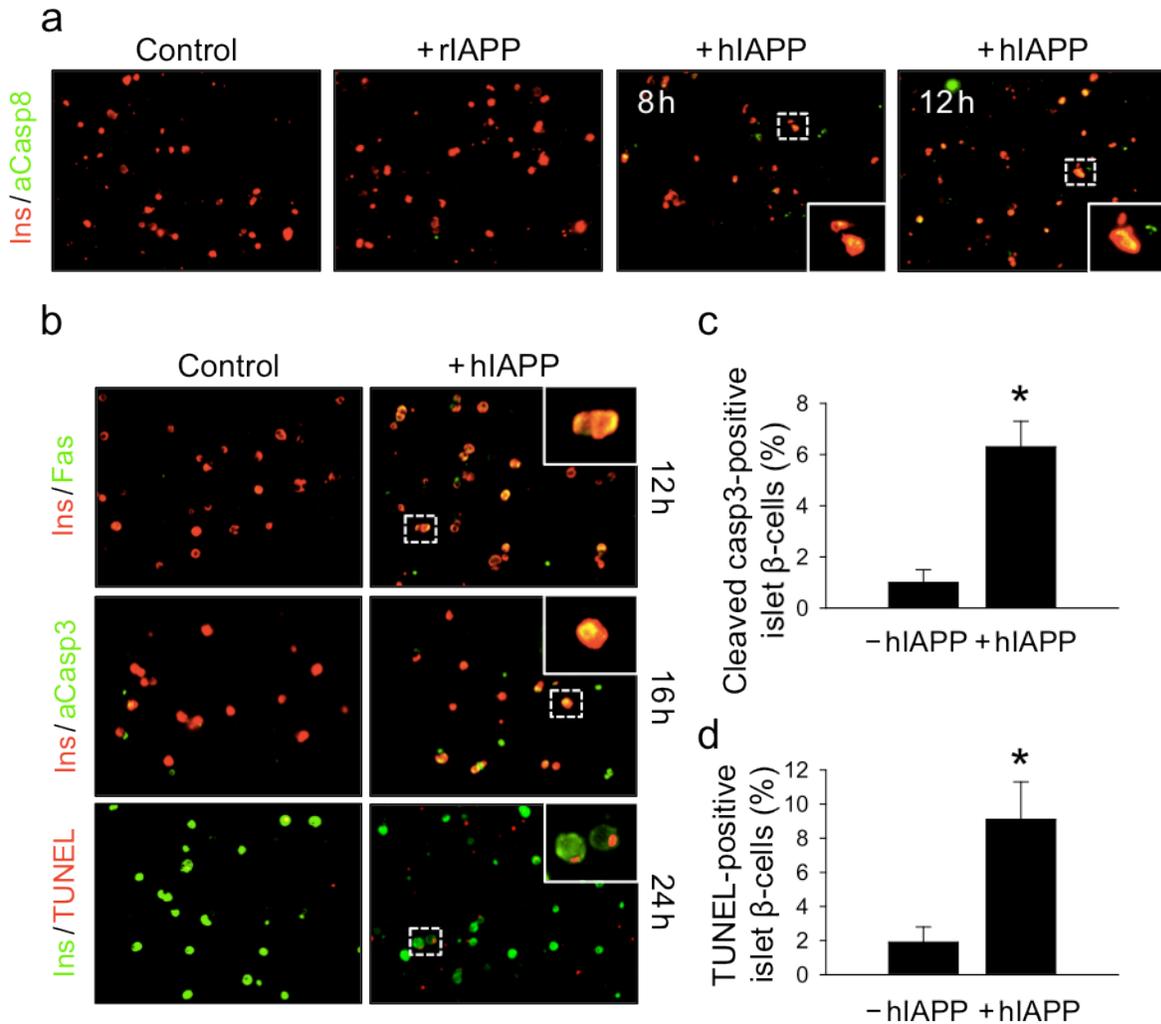


Figure 4-1. Exposure to exogenous hIAPP induces caspase-8 activation and apoptosis in human islet β - cells, which is associated with Fas upregulation.

Dispersed human islet cells were treated with 10 μ mol/l synthetic hIAPP or non-fibrillogenic rIAPP at different time points. **(a)** Dispersed human islet cells were immunolabelled for insulin and active caspase-8 (aCasp8) following 8 or 12 h of treatment with hIAPP or rIAPP (control). **(b)** Immunolabelling for insulin and Fas, insulin and active caspase-3 (aCasp3), or insulin and TUNEL as indicated after 12, 16 or 24 h of hIAPP treatment, respectively. The white squares denote regions enlarged in each image. The proportion of β -cells positive for **(c)** active caspase-3 or **(d)** TUNEL in hIAPP-treated and non-treated cells. Quantifications represent a minimum of ten microscopic fields each containing 100 to 150 cells. Results are expressed as means \pm SEM of four independent studies performed in triplicate. *vs non-treated group ($p < 0.05$ by Student's t -test)

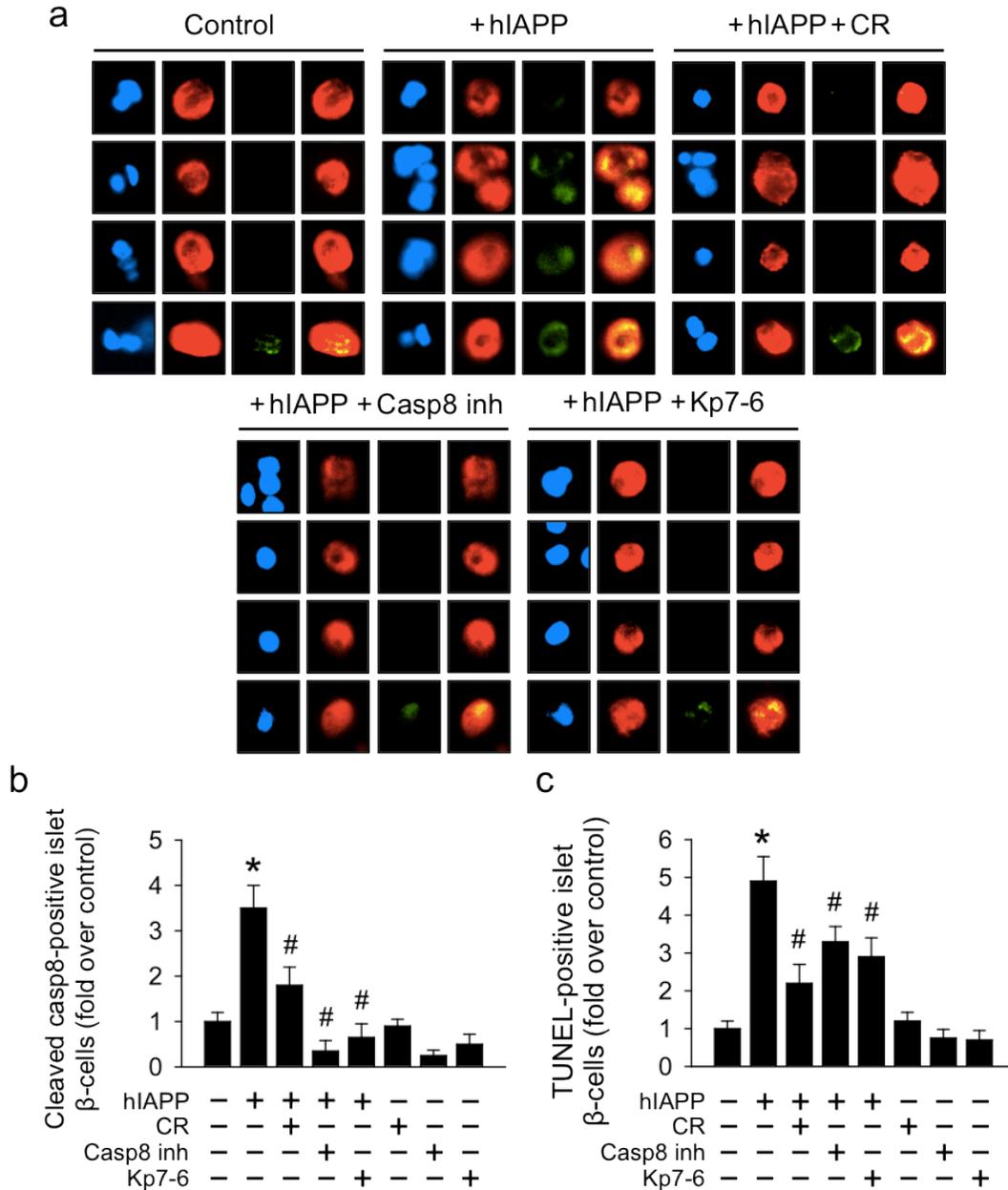


Figure 4-2. Blockade of hIAPP-mediated caspase-8 activation markedly reduces β -cell apoptosis induced by aggregation of exogenously applied hIAPP.

Dispersed human islet cells were treated with hIAPP alone or with each of the following: amyloid inhibitor Congo red (CR; 8 h), caspase-8 inhibitor (Casp8 inh; 12 h), or Fas antagonist (Kp7-6; 12 h). **(a)** Immunolabelling for DAPI (blue), insulin (red), active caspase-8 (green) and insulin/active caspase-8 (merged). The proportion of β -cells positive for **(b)** active caspase-8 (12 h) or **(c)** TUNEL (24 h). Quantifications represent a minimum of ten microscopic fields each containing 100 to 150 islet cells. Results are expressed as means \pm SEM of three independent studies performed in triplicate. *vs non-treated control group, #vs hIAPP-treated group ($p < 0.05$ by one-way ANOVA)

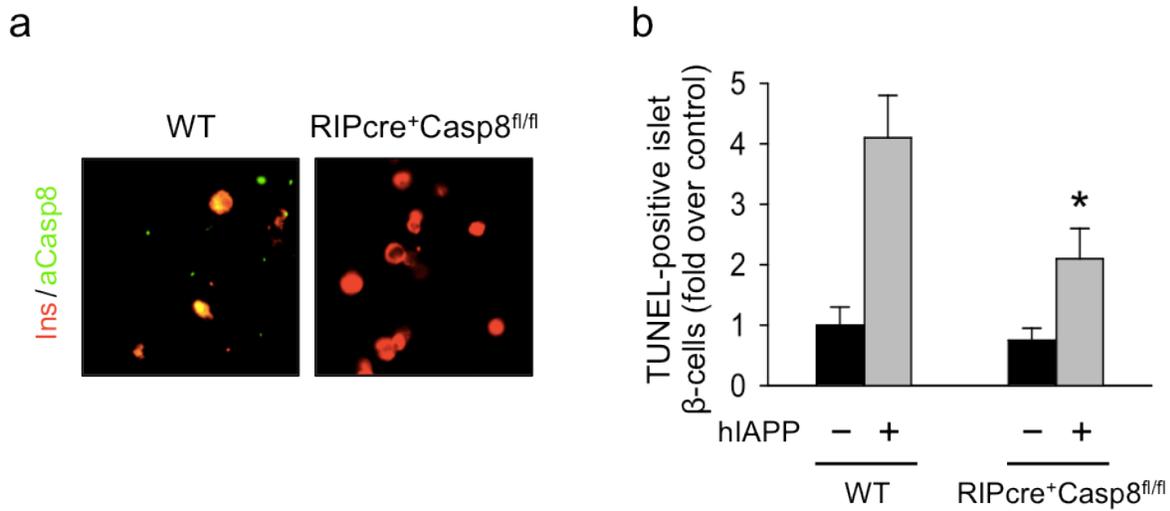


Figure 4-3. Deletion of caspase-8 reduces β -cell apoptosis induced by synthetic hIAPP aggregates.

Dispersed RIPcre⁺Casp8^{fl/fl} and wild-type (WT: RIPcre⁺Casp8^{+/+}) mouse islet cells were treated with hIAPP for 8 or 24 h. **(a)** Immunolabelling for insulin and active caspase-8 (aCasp8), and **(b)** the proportion of TUNEL-positive β -cells in hIAPP-treated mouse islets with or without caspase-8 deletion. Quantifications represent a minimum of ten microscopic fields each containing 100 to 150 islet cells. Non-treated WT islet cells used as a control. Results are expressed as means \pm SEM of three independent studies performed in triplicate. *vs WT hIAPP-treated group ($p < 0.05$ by one-way ANOVA)

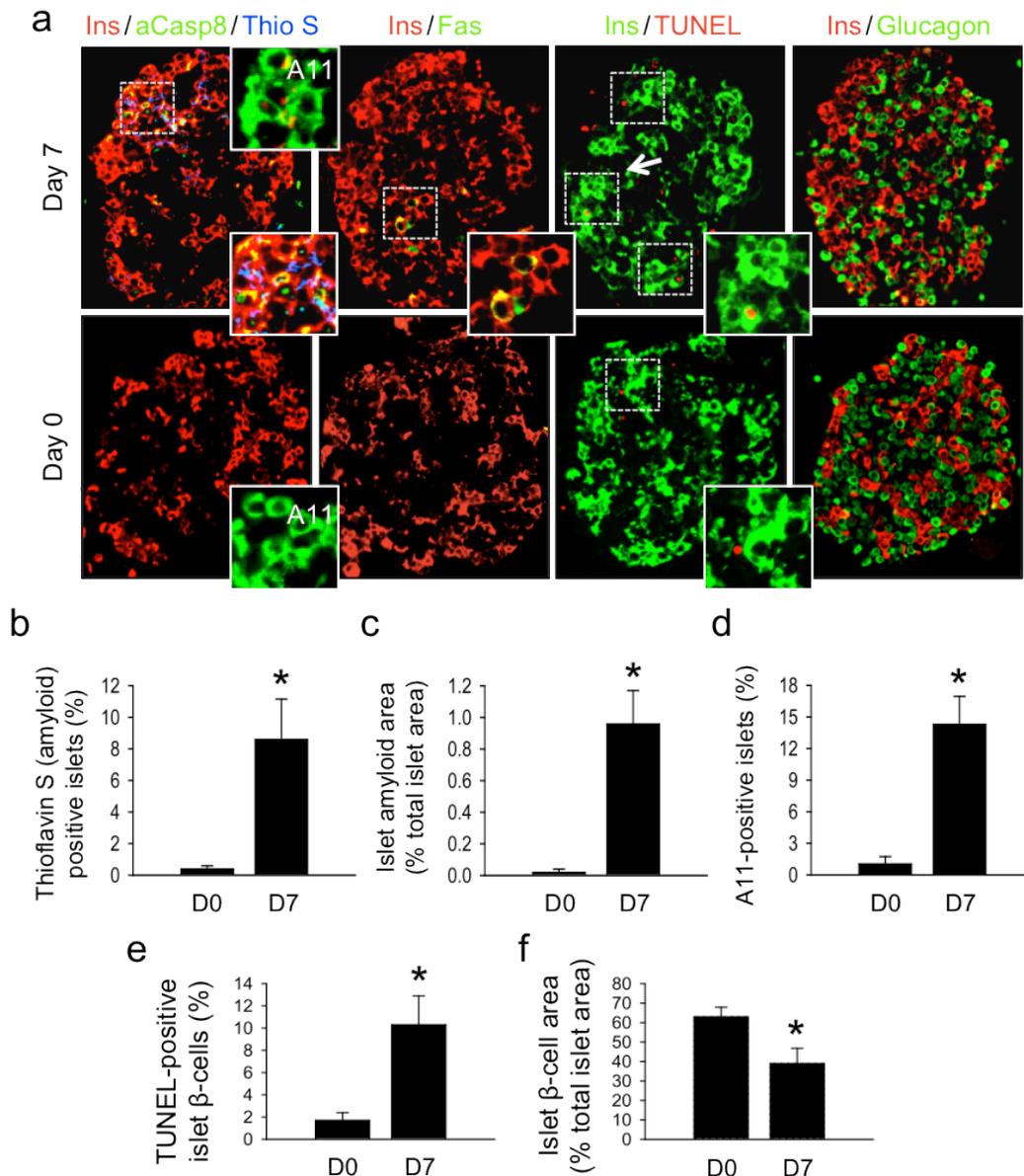


Figure 4-4. The formation of endogenous hIAPP aggregates in human islets during culture induces caspase-8 activation and β -cell apoptosis, which is associated with Fas upregulation.

Isolated human islets were cultured for 7 days in CMRL containing 11.1 mmol/l glucose to allow amyloid formation. (a) Pre-culture and 7-day cultured human islets were immunolabelled as indicated in figure labels. Inserts show immunolabelling for insulin (green) and A11 (red). The white squares denote regions enlarged in each image. The proportion of (b) thioflavin S-positive islets to the total number of islets, (c) amyloid area to total islet area, (d) A11-positive islets to the total number of islets, (e) TUNEL-positive β -cells, and (f) β -cell area to total islet area. Results are expressed as means \pm SEM of six independent studies (25-30 islets per condition in each study). *vs day 0 group ($p < 0.05$ by Student's t -test)

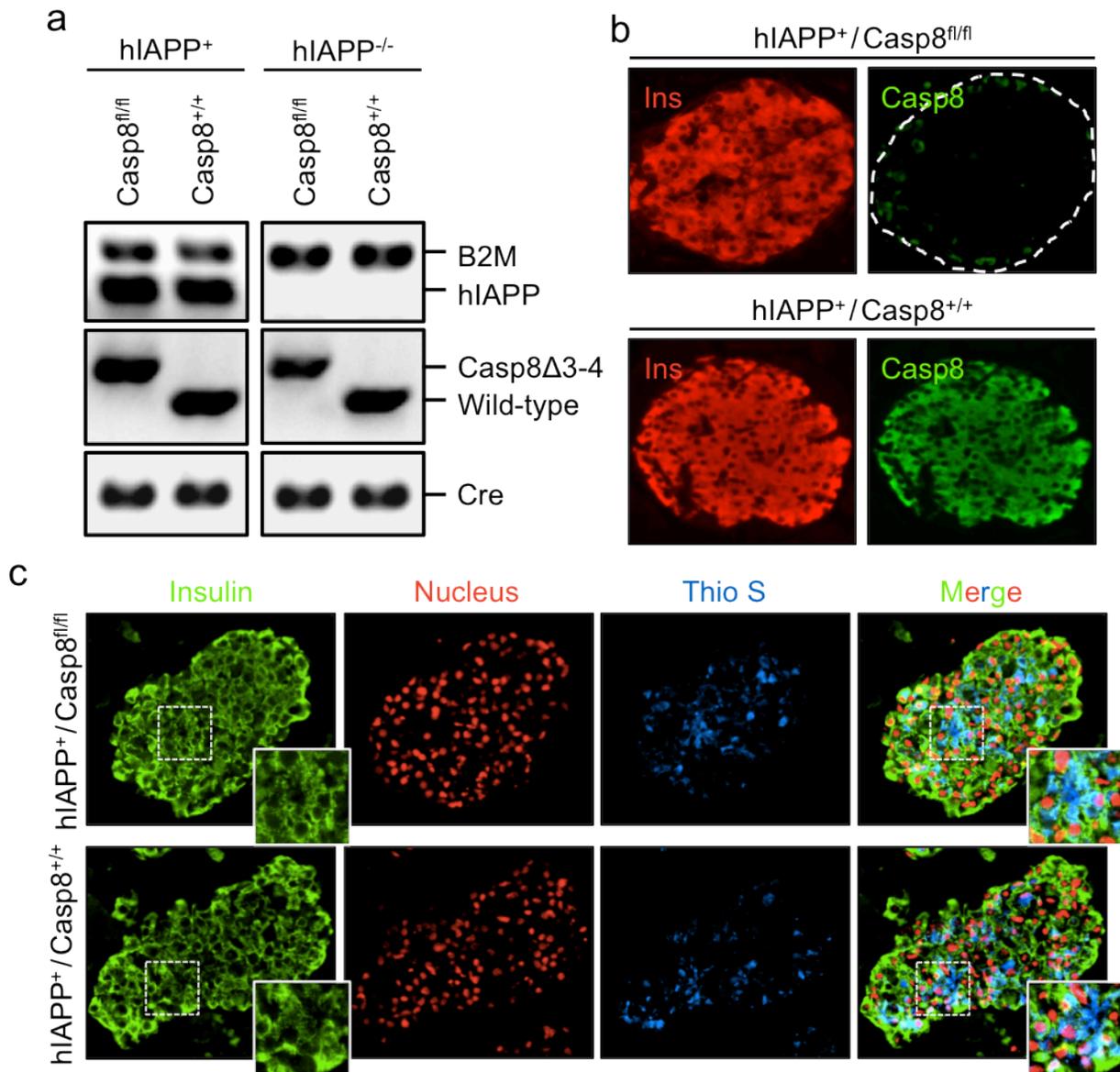


Figure 4-5. RIP-Cre mediated deletion of caspase-8 in hIAPP-expressing transgenic mice. (a) hIAPP transgene, disrupted caspase-8 and insulin-Cre transgene were detected by PCR performed on tail sample DNA from 3-week-old mice. Wild-type littermates with or without caspase-8 deletion which do not express hIAPP are shown for comparison. (b) Pancreatic sections from 8-week-old hIAPP⁺/RIPcre⁺Casp8^{fl/fl} and hIAPP⁺/RIPcre⁺Casp8^{+/+} mice immunolabelled for insulin and full-length caspase-8 (Casp8). (c) Islet sections from each genotype immunolabelled for insulin, thioflavin S (Thio S) and nucleus staining. The white squares denote regions enlarged in each image.

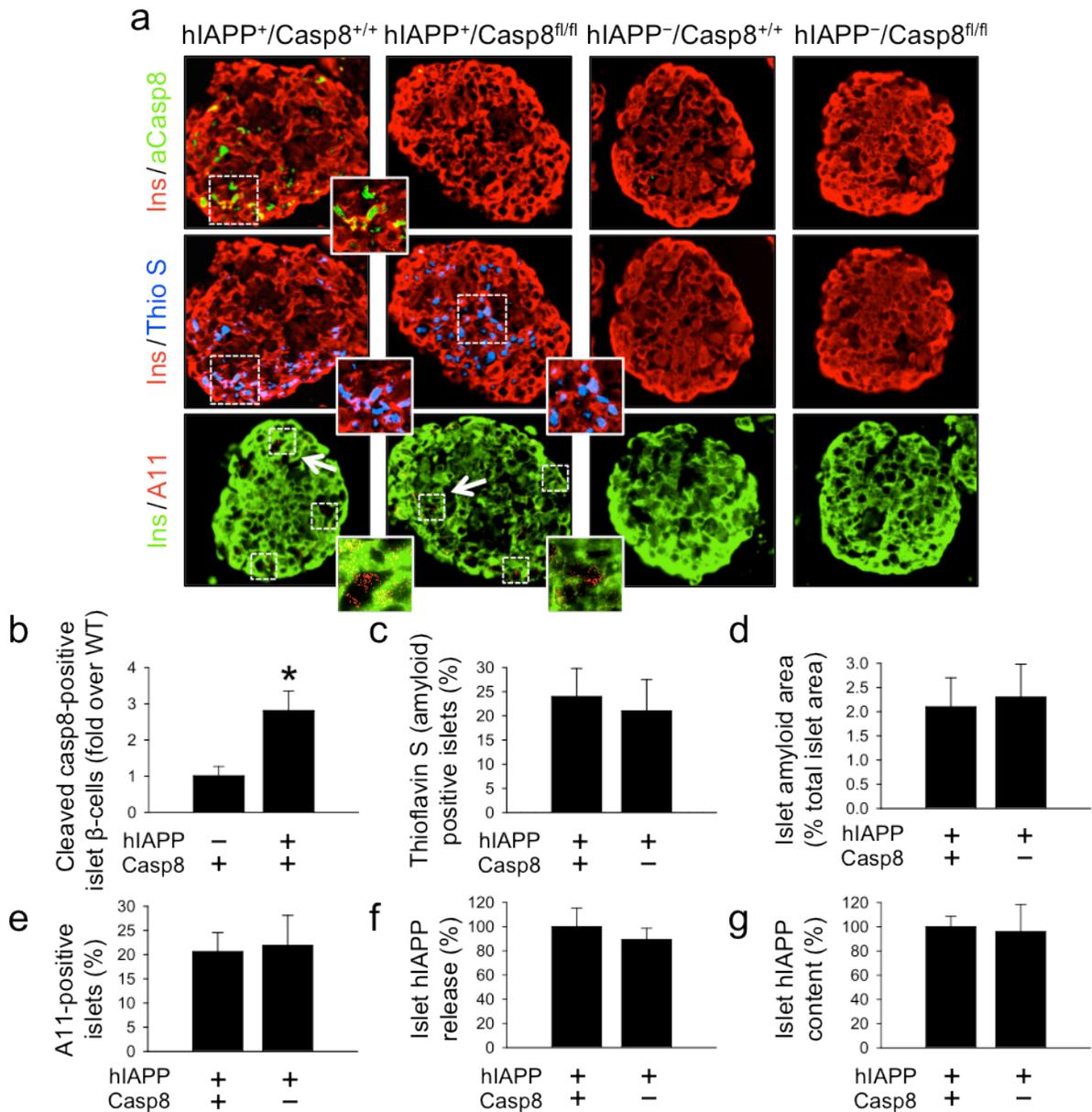


Figure 4-6. Cultured hiAPP-expressing mouse islets with or without β -cell-specific caspase-8 deletion have comparable levels of amyloid formation, hiAPP release and content.

(a) Islets from wild-type (WT: hiAPP^{-/-}/Casp8^{+/+}) and hiAPP-expressing mice with or without β -cell caspase-8 deletion were cultured for 7 days in Ham's-F10 (16.7 mmol/l glucose) and then immunolabelled as indicated for insulin and active caspase-8 (aCasp8); insulin and thioflavin S (Thio S); and insulin and A11. The white squares denote regions enlarged in each image. The proportion of (b) active caspase-8-positive β -cells, (c) thioflavin S-positive islets to the total number of islets, (d) amyloid area to total islet area, and (e) A11-positive islets to the total number of islets. (f) Islet hiAPP release and (g) content were assessed in cultured islets from hiAPP-expressing mice with or without caspase-8 deletion (hiAPP⁺/Casp8^{+/+} taken as 100%). Quantifications represent 17-25 islets per animal from each genotype (6-9 mice per group). Results are expressed as means \pm SEM. *vs WT group ($p < 0.05$ by Student's *t*-test)

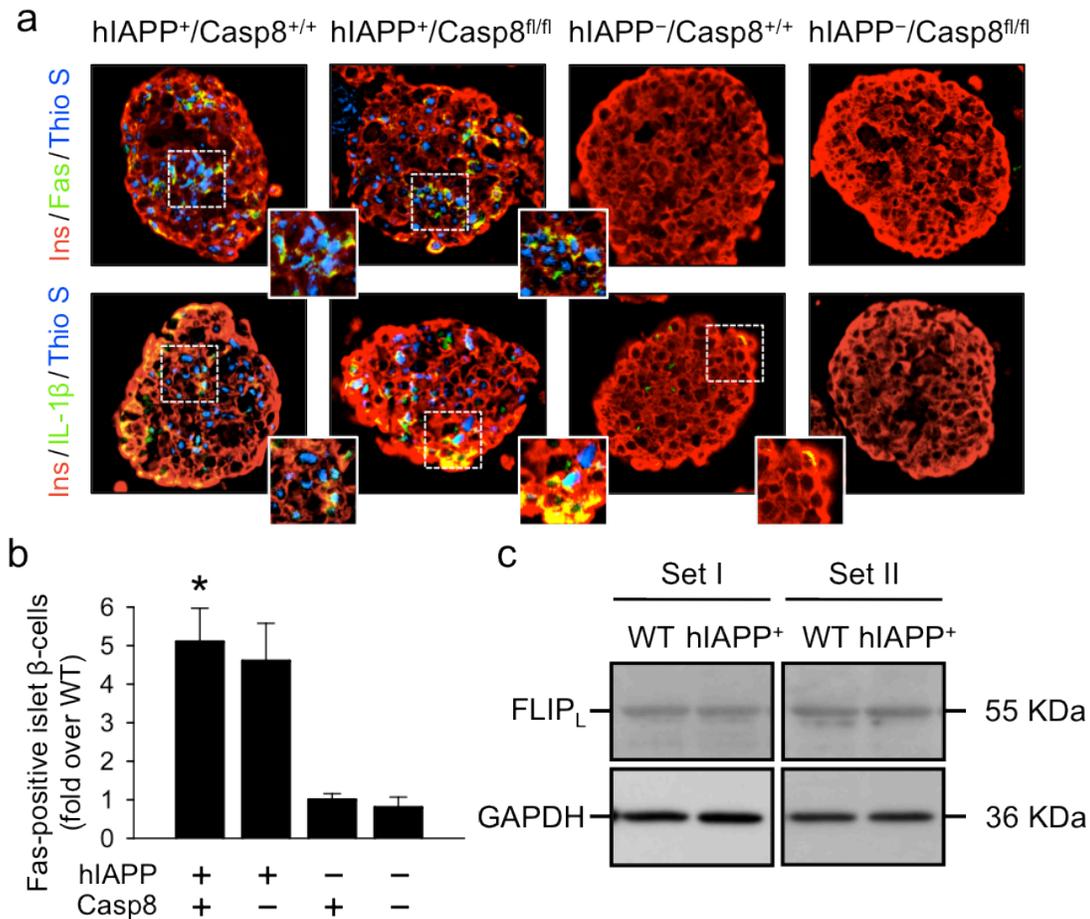


Figure 4-7. Amyloid formation in cultured hiAPP-expressing mouse islets with and without caspase-8 deletion is associated with β-cell Fas upregulation and elevated islet IL-1β immunoreactivity with no changes to islet FLIP protein levels.

Freshly isolated islets from wild-type (WT: hiAPP^{-/-}/Casp8^{+/+}) and hiAPP-expressing mice with or without β-cell caspase-8 deletion were cultured for 7 days in Ham's-F10 containing 16.7 mmol/l glucose. **(a)** Paraffin-embedded islet sections were immunolabelled as indicated for insulin, Fas and thioflavin S (Thio S), or insulin, IL-1β and Thio S. The white squares denote regions enlarged in each image. **(b)** The proportion of Fas-positive islet β-cells. **(c)** FLIP protein levels in the lysates from 7-day cultured WT and hiAPP-expressing transgenic mouse islets (12-14 weeks). Quantifications represent 20-25 islets per animal from each genotype (5-6 mice per group). Results are expressed as means ± SEM. *vs WT group ($p < 0.05$ by one-way ANOVA)

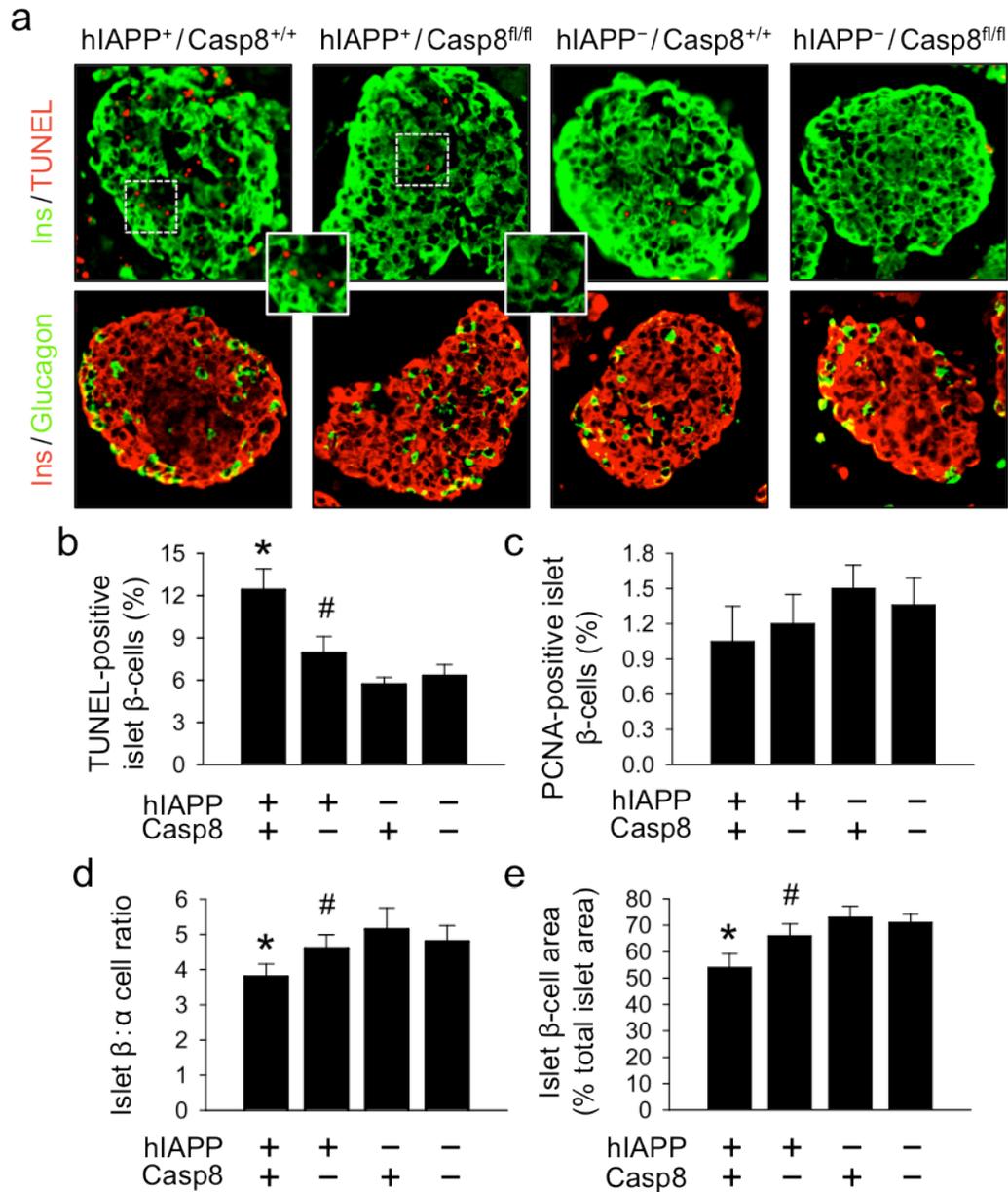


Figure 4-8. β-cell specific deletion of caspase-8 in hiAPP-expressing mouse islets markedly reduces apoptosis induced by aggregation of endogenous hiAPP during culture. Islets from wild-type (WT: hiAPP⁻/Casp8^{+/+}) and hiAPP-expressing mice with or without caspase-8 deletion were cultured for 7 days in Ham's-F10 (16.7 mmol/l glucose). **(a)** Paraffin-embedded islet sections were immunolabelled as indicated for insulin and TUNEL, or insulin and glucagon. The white squares denote regions enlarged in each image. The proportion of **(b)** apoptotic or **(c)** proliferating islet β-cells, **(d)** islet β- to α-cell ratio, and **(e)** β-cell area per total islet area in each genotype. Quantifications represent 17-25 islets per animal from each genotype (6-9 mice per group). Results are expressed as means ± SEM. *vs WT group, #vs corresponding caspase-8 expressing group ($p < 0.05$ by one-way ANOVA)

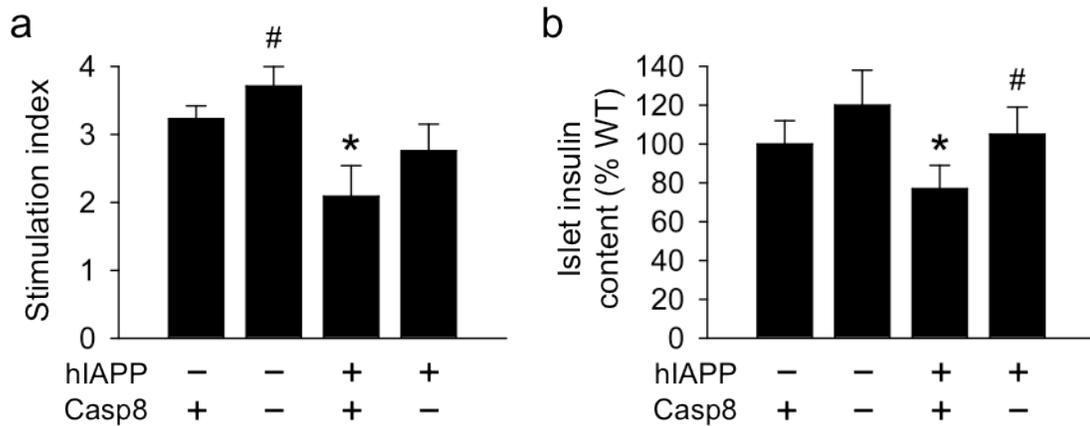


Figure 4-9. Cultured hIAPP-expressing mouse islets with caspase-8 deletion have enhanced β -cell function compared with those expressing caspase-8.

Wild-type (WT: hIAPP^{-/-}/Casp8^{+/+}) and hIAPP-expressing mouse islets with or without caspase-8 deletion were cultured for 7 days in Ham's-F10 (16.7 mmol/l glucose). **(a)** Insulin response to glucose stimulation and **(b)** insulin content in islets following culture. Glucose-stimulated insulin release (stimulation index) indicates the amount of insulin secreted during 1 h of incubation at 1.67 (basal) and 16.7 mmol/l (stimulated) glucose. Insulin content is reported as percent of the insulin content in WT islets, which was set at 100%. Results are expressed as means \pm SEM of 5-6 animals per genotype. *vs WT group, #vs corresponding caspase-8 expressing group ($p < 0.05$ by one-way ANOVA)

Chapter 5: Dual role of IL-1 β in islet amyloid formation and its β -cell toxicity

5.1 Background

Islet inflammation mediated primarily by pro-inflammatory cytokines of the innate immune system contributes to pathogenesis of not only autoimmune type 1 diabetes (104,504,505) but also non-immune-mediated type 2 diabetes (506-508). In particular, IL-1 β has been shown to be an important mediator of islet inflammation in type 2 diabetes (507). Analysis of β -cells from patients with type 2 diabetes using *in situ* hybridization method displayed increased IL-1 β expression (481); similarly, real-time PCR of nearly-pure β -cells from pancreatic sections of type 2 diabetic patients obtained by the laser capture micro-dissection method showed elevated IL-1 β mRNA levels compared to those from non-diabetic individuals (509). Moreover, high-fat diet fed diabetes-prone *Psammomys obesus* exhibited elevated β -cell IL-1 β expression whereas low-fat diet fed controls did not (481). Importantly, IL-1 β can stimulate its own production as well as other pro-inflammatory cytokines and chemokines (eg. IL-8, CXCL1, MCP-1), which in turn may attract immune cells to the inflamed islets (509-512). Increased macrophage infiltration has been reported in pancreatic sections from type 2 diabetic patients (97,214) and in animal models of type 2 diabetes (215,513). These recruited immune cells can further aggravate islet inflammatory response by releasing IL-1 β and other cytokines/chemokines (214,507,514,515).

The role of IL-1 β in mediating islet β -cell dysfunction and death has been well documented (515-520). Sustained exposure to high concentrations (2-5 ng/ml) of IL-1 β can promote β -cell apoptosis and reduce β -cell insulin secretion in cultured human islets (521), indicating that long-term exposure to pathologically elevated islet IL-1 β levels associated with islet inflammation likely results in reduced β -cell mass and function under diabetic conditions. Similarly, in clinical islet transplantation, IL-1 β released during the early stages of islet transplantation process (eg. islet isolation) may trigger islet inflammatory response, leading to reduced β -cell mass and function and eventually islet graft failure (104,522-526). IL-1 β -induced β -cell dysfunction and death involve activation of multiple signalling pathways including the intracellular MAPK (extracellular signal-regulated kinase, p38, JNK) pathways, nuclear factor

kappa B (NF- κ B) signalling, and the Fas-mediated apoptotic pathway (481,509,527-529). Blocking IL-1 β with neutralizing anti-IL-1 β antibodies has been shown to protect islets from the cytotoxic effects of IL-1 β (530,531).

Growing evidence suggests that exposure to type 2 diabetes associated metabolic stress such as high glucose (509,529), free fatty acids (532), and leptin (217) can increase IL-1 β expression and β -cell death in cultured human islets. Interestingly, our data in Chapter 3 have demonstrated that formation of islet amyloid during culture is associated with increased islet IL-1 β immunoreactivity in human and mouse islets, which can be prevented by suppressing amyloid formation. In addition, we have shown in Chapters 3 and 4 that hIAPP aggregates induce upregulation of Fas in β -cells, which correlates with increased islet IL-1 β levels. Together with a previous finding, which showed that IL-1 β can induce Fas upregulation in human islet β -cells (481), we proposed that hIAPP-induced β -cell Fas upregulation is likely mediated by IL-1 β . Therefore, in this chapter, we investigated the role of IL-1 β in hIAPP-induced Fas upregulation in β -cells.

First, we examined if treatment with synthetic hIAPP aggregates can promote islet IL-1 β production and Fas expression in dispersed mouse islet β -cells and tested if blocking IL-1 β signalling can prevent hIAPP-induced Fas upregulation. Next, we examined if endogenous amyloid formation in human islets during culture can induce islet IL-1 β expression leading to β -cell Fas upregulation and activation of the Fas-mediated apoptotic pathway. We then tested if blocking IL-1 β signalling can prevent amyloid-induced Fas upregulation and thereby reduce amyloid-induced β -cell apoptosis. In a parallel study, we examined if inhibition of amyloid formation using the amyloid inhibitor Congo red or by adenoviral-mediated hIAPP suppression can reduce amyloid-induced islet IL-1 β expression, resulting in reduced Fas upregulation and activation of the Fas apoptotic pathway in islet β -cells.

In addition to its role in mediating β -cell apoptosis, IL-1 β has been shown to induce β -cell dysfunction manifested as reduced glucose-stimulated insulin secretion and increased proinsulin to insulin ratio (481,512,533). Since proinsulin and proIAPP are both processed by the same enzymes in the β -cell granules, it led us to the idea that IL-1 β may also cause impaired

prohIAPP processing thereby potentiate amyloid formation. Thus, we examined the potential effect of IL-1 β induced β -cell dysfunction on prohIAPP processing and amyloid formation. We assessed (pro)hIAPP immunoreactive forms in cultured human islets with or without exposure to exogenous IL-1 β . Finally, we tested if blocking IL-1 β signalling by treatment with IL-1 receptor (IL-1R) antagonist anakinra can restore impaired prohIAPP processing and reduce amyloid formation in cultured human islets.

5.2 Results

5.2.1 Blocking IL-1 signalling by treatment with IL-1R antagonist markedly reduces β -cell Fas upregulation and apoptosis in hIAPP-treated mouse islet cells

To examine the role of IL-1 β in mediating hIAPP-induced Fas upregulation in β -cells, dispersed C57BL/6 mouse islets were pre-treated with IL-1R antagonist anakinra (5 μ g/ml) for 1 h followed by treatment with synthetic hIAPP peptides (10 μ mol/l) for 12 or 24 h. As expected, non-treated or non-fibrillogenic rIAPP treated (10 μ mol/l) human islet cells had low numbers of IL-1 β -, Fas-, and TUNEL-positive (apoptotic) β -cells (Figure 5-1). Increased IL-1 β and Fas immunoreactivity were detected in β -cells following 12 h exposure to synthetic hIAPP aggregates (Figure 5-1a, b), which correlated with elevated β -cell apoptosis (24 h; Figure 5-1c). Pre-treatment with anakinra effectively reduced the number of IL-1 β -positive islet cells following hIAPP treatment (Figure 5-1a), which was associated with lower number of Fas- and TUNEL-positive β -cells compared to those treated with hIAPP alone (Figure 5-1).

5.2.2 Anakinra-treated human islets have markedly lower IL-1 β levels during culture, which correlates with decreased β -cell Fas expression, caspase-8 activation, and apoptosis

To investigate whether β -cell Fas upregulation and activation of Fas-mediated apoptotic pathway induced by aggregation of endogenous hIAPP is mediated by IL-1 β in primary islet β -cells, human islets were cultured in CMRL containing normal (5.5 mmol/l) or elevated glucose (11.1 mmol/l glucose) for 7 days in the presence or absence of IL-1R antagonist anakinra (10 μ g/ml). Similar to our results in Chapters 3 and 4, amyloid formation in cultured human islets increased islet IL-1 β immunoreactivity, which was associated with β -cell Fas upregulation and

caspase-8 activation (Figure 5-2). Importantly, thioflavin S (amyloid)- or A11 (oligomer)-positive areas in islets closely correlated with IL-1 β , Fas, and cleaved (active) caspase-8-positive β -cell areas (Figure 5-2a). Blocking IL-1 β signalling markedly reduced the number of Fas-positive and active caspase-8-positive β -cells in anakinra-treated islets as compared to non-treated cultured islets (Figure 5-2). Moreover, treatment with anakinra markedly reduced the proportion of TUNEL-positive (apoptotic) β -cells in 7-day cultured human islets in both normal and elevated glucose conditions compared to non-treated cultured islets (Figure 5-3a, b). This reduced β -cell apoptosis in anakinra-treated islets was associated with increased islet β - to α -cell ratio and β -cell area to total islet area, which were more profound in islets cultured under elevated glucose (Figure 5-3c, d).

5.2.3 Prevention of amyloid formation blocks amyloid-induced IL-1 β production and reduces β -cell Fas upregulation, caspase-8 activation, and apoptosis in cultured human islets

We further examined if prevention of amyloid formation can reduce islet IL-1 β production using two different approaches. Isolated human islets were transduced with an adenovirus that delivers a proIAPP specific siRNA to suppress hIAPP expression. As an independent approach, human islets were cultured with the amyloid inhibitor Congo red to prevent amyloid formation and its interaction with β -cells. Non-treated human islets cultured in the same glucose conditions (11.1 mmol/l) were used as a comparison to assess any potential effects of elevated glucose on mediating islet IL-1 β production. As expected, amyloid deposits formed in non-treated human islets during 7-day culture but to a much lower extent in islets transduced with Ad-proIAPP siRNA or treated with Congo red (Figure 5-4). Moreover, inhibition of amyloid formation markedly reduced islet IL-1 β expression, which was associated with a lower number of Fas and cleaved (active) caspase-8 positive islet β -cells in these islets than non-treated cultured islets (Figure 5-4).

5.2.4 Treatment with anakinra improves β -cell function manifested as increased insulin response to glucose stimulation

Next, we tested if blocking IL-1 β signalling can enhance β -cell function in cultured

human islets. As shown in Figure 5-5, blocking IL-1 β action by treatment with anakinra markedly enhanced β -cell function in cultured human islets as demonstrated by a greater insulin response to elevated glucose in anakinra-treated islets compared to non-treated cultured islets. Moreover, anakinra-treated islets cultured under elevated glucose conditions had higher islet insulin content (Figure 5-5b) than non-treated islets following culture although this did not reach the level of significance.

5.2.5 Enhanced β -cell function by treatment with anakinra restores impaired proIAPP processing in cultured human islets

To investigate if improved β -cell function by blocking IL-1 β signalling can improve impaired proIAPP processing in human islets during culture, (pro)hIAPP immunoreactive forms including proIAPP and its intermediate forms and mature hIAPP (Figure 5-6a) were identified and measured in the anakinra-treated and non-treated islet lysates following 7-day culture in normal (5.5 mmol/l) or elevated (11.1 mmol/l) glucose concentrations. Mature hIAPP was the major (pro)hIAPP immunoreactive form detected in freshly isolated human islets with very low levels of proIAPP intermediate forms also detectable (Figure 5-6b, c). However, in cultured human islets, proIAPP intermediate forms were the major forms present with low levels of mature hIAPP also detectable, indicating that islet culture causes impaired proIAPP processing (Figure 5-6b, c). Importantly, the major proIAPP intermediate form present in cultured human islets was identified to be the NH₂-terminally unprocessed proIAPP (Figure 5-6d), suggesting that impaired proIAPP processing during culture occurs mainly at its NH₂-terminal cleavage site.

Interestingly, enhanced β -cell function by treatment with anakinra restored impaired proIAPP processing during islet culture manifested as markedly reduced levels of partially processed proIAPP and increased levels of mature hIAPP in both human islets cultured in normal and elevated glucose conditions (Figure 5-6b, c). Thus, mature hIAPP was the major form present in cultured human islets treated with anakinra. Reduced level of partially processed proIAPP detected in anakinra-treated human islets was mainly due to the lower level of NH₂-terminally unprocessed proIAPP (Figure 5-6d). ProIAPP levels present in cultured human

islets were comparable in anakinra-treated and non-treated islets (Figure 5-6b, c).

Protein levels of both PC1/3 and PC2, two key enzymes responsible for proIAPP processing (Figure 1-1), were reduced in lysates from cultured islets compared to those from freshly isolated islets. However, both PC2 and PC1/3 protein levels remained unchanged during culture in the presence or absence of anakinra (Figure 5-7), suggesting that improved proIAPP processing in response to treatment with anakinra is likely not due to its effects on the protein expression of these enzymes.

5.2.6 Exposure to exogenous IL-1 β potentiates impaired proIAPP processing in cultured human and mouse islets

To directly examine the effects of IL-1 β on proIAPP processing, human and mouse islets were treated with recombinant IL-1 β (2 ng/ml) in the presence or absence of anakinra. As expected, islet culture led to impaired proIAPP processing in human islets, which was potentiated by treatment with IL-1 β (Figure 5-8a). Pre-treatment with anakinra restored impaired proIAPP processing mediated by IL-1 β manifested as increased levels of mature hIAPP in anakinra-treated islets compared to islets treated with IL-1 β alone (Figure 5-8a). Similarly, exposure to exogenous IL-1 β significantly increased levels of proIAPP and its partially processed forms in both wild-type and hIAPP-expressing mouse islets during culture compared to non-treated islets (Figure 5-8b). Moreover, similar to our findings in human islets, treatment with anakinra resulted in increased levels of mature IAPP and reduced unprocessed and partially processed proIAPP forms in these islets (Figure 5-8b).

5.2.7 Improved proIAPP processing by treatment with anakinra markedly reduces amyloid formation in human islets during culture

Impaired proIAPP processing has been shown to contribute to amyloid formation (365,466). Thus, we examined if restoring impaired proIAPP processing in anakinra-treated islets can reduce aggregation of endogenously produced hIAPP during culture. Only a small proportion of pre-culture human islets contained hIAPP oligomers, which significantly increased following culture (Figure 5-9a). Similarly, thioflavin S-positive islets were not detectable in

freshly isolated human islets but were detectable after 7 days culture (Figure 5-9b). Interestingly, amyloid formation was reduced in anakinra-treated human islets as shown by a lower number of A11- and thioflavin S-positive islets and islet amyloid area to total islet area as compared to non-treated cultured islets (Figure 5-9). In line with these findings, treatment with anakinra significantly reduced exogenous IL-1 β -induced amyloid formation in cultured human islets as shown by a lower number of thioflavin S-positive islets and decreased islet amyloid area than cultured human islets treated with IL-1 β alone (Figure 5-10).

5.3 Discussion

While growing evidence suggests that amyloid formation may contribute to islet inflammation and β -cell death (491,492,515), the underlying mechanisms have yet to be identified. Our studies in Chapters 3 and 4 have shown that the pro-inflammatory cytokine IL-1 β may play a key role in mediating amyloid-induced β -cell apoptosis. It is important to note that β -cells are particularly sensitive to the effects of IL-1 β due to high expression of its receptor, IL-1R type 1 (IL-1R1), on β -cell membrane (516,532). In this chapter, we performed mechanistic studies using cultured human and hIAPP-expressing transgenic mouse islets to directly test if endogenously formed hIAPP aggregates induce Fas-mediated β -cell apoptosis by increasing islet IL-1 β production and if blocking IL-1 β signalling by treatment with the clinically approved IL-1R antagonist anakinra, a recombinant, non-glycosylated form of the naturally produced IL-1R antagonist, can protect β -cells from the cytotoxic effects of hIAPP aggregates. We further examined if IL-1 β induced β -cell dysfunction can impair proIAPP processing and thereby potentiate amyloid formation and β -cell death in cultured human islets.

Our findings showed that exogenously applied hIAPP aggregates induce IL-1 β expression in dispersed mouse islet cells, which was associated with increased β -cell Fas upregulation and activation of the Fas apoptotic pathway resulting in a markedly higher number of Fas, active caspase-8, and TUNEL positive β -cells in hIAPP-treated islet cells compared to non-treated islet cells. Similarly, endogenous amyloid formation in human islets during culture closely correlated with increased islet IL-1 β immunoreactivity, suggesting that aggregation of biosynthetic hIAPP induces islet IL-1 β production. Moreover, amyloid-induced islet IL-1 β

production was associated with increased β -cell Fas upregulation, caspase-8 activation and apoptosis in cultured human islets. Importantly, blocking IL-1 β signalling by treatment with anakinra markedly reduced β -cell Fas expression, caspase-8 activation, and apoptosis in both dispersed mouse islet cells exposed to synthetic hIAPP aggregates and amyloid forming human islets during culture. Our data further showed that prevention of amyloid formation either by siRNA-mediated suppression of hIAPP expression or treatment with the amyloid inhibitor Congo red markedly reduced islet IL-1 β production in human islets during culture compared to non-treated islets. Collectively, our findings indicate that IL-1 β plays a major role in amyloid-induced β -cell Fas upregulation and activation of the Fas-mediated apoptotic pathway.

Our studies further suggest that IL-1R antagonists may provide an effective approach to reduce β -cell toxicity in conditions associated with islet amyloid formation such as type 2 diabetes, islet culture and transplantation. In support, recent studies have demonstrated that treatment with IL-1R antagonists can enhance β -cell survival and function in type 2 diabetes (507,509,512) and islet grafts transplanted into animal models of type 1 diabetes (534,535). For example, administration of IL-1R antagonist has been shown to promote islet graft survival and counteract the low dose STZ-induced diabetes in a mouse model of type 1 diabetes (536). Moreover, a clinical trial has shown that treatment with anakinra improves glycemic control in patients with type 2 diabetes (537).

The findings of our studies may also be applicable to patients with Alzheimer's disease where formation of β -amyloid plaques has been shown to correlate with overexpression of IL-1 molecules (538). In support, the amyloidogenic A β peptides, an important neurotoxic factor in the pathogenesis of Alzheimer's disease, has been shown to activate NLRP3 inflammasome leading to release of IL-1 β , which contributes to inflammation and tissue damage in Alzheimer's disease (539). Therefore, blocking IL-1 β signalling by treatment with IL-1R antagonists has been proposed as an approach to prevent the progression of Alzheimer's disease (540).

The cellular source of IL-1 β in islets has yet to be identified and it may be islet β -cells, non- β -cells or non-islet cells including endothelial, ductal or recruited immune cells (514). For example, the recent report from Westwell-Roper *et al.* (515) suggests that hIAPP aggregates trigger localised islet inflammatory response via promoting IL-1 β release from resident

macrophages in hIAPP-expressing transgenic mouse islets. However, under culture conditions used in our study (7 days), it is less likely that increased IL-1 β immunoreactivity associated with aggregation of endogenously produced hIAPP is attributable solely to macrophage IL-1 β release, because resident macrophages comprise only about 0.5% of islet cells (10 to 15 per islet), which are lost dramatically (>90%) over the course of islet culture for more than 7 days (541). Thus, it is likely that in addition to islet resident macrophages, other cells including β -cells also contribute to the elevated islet IL-1 β levels detected in cultured human islets. Indeed, our studies consistently show that IL-1 β immunoreactivity co-localises with islet β -cells and overlaps with areas of amyloid formation. In support, Maedler *et al.* (481) reported that islet β -cells themselves are the likely source of islet IL-1 β expression in human islets cultured under elevated glucose conditions.

In addition to culturing human islets in high glucose conditions (11.1 mmol/l) at which we observe significant amyloid formation, we also cultured human islets at a normal glucose concentration (5.5 mmol/l) at which human islets have minimal or no amyloid formation, to assess any potential effects of amyloid-independent IL-1 β production during islet culture on β -cell Fas expression. Non-treated human islets cultured under normal glucose had low levels of β -cell Fas expression, caspase-8 activation, and apoptosis, indicating that islet culture *per se* can induce some levels of IL-1 β production resulting in Fas expression and apoptosis, albeit to a much less extent than that mediated by amyloid formation. Therefore, these findings also have implications in clinical islet transplantation where blocking IL-1R signalling may enhance β -cell viability in human islet grafts during pre-transplant islet culture at normal glucose conditions.

Furthermore, exposure to elevated glucose (20-30 mmol/l) has been shown to induce both islet IL-1 β production and release as well as β -cell Fas upregulation associated with β -cell dysfunction (480,481). Accordingly, it has been proposed that elevated glucose in type 2 diabetes may promote islet IL-1 β release thereby contributing to progressive β -cell loss and impaired insulin secretion in those patients. Thus, in order to assess the amyloid-independent effects of elevated glucose on islet IL-1 β production in our experimental model, we compared the levels of islet IL-1 β following culture under elevated (11.1 mmol/l) glucose with or without prevention of amyloid formation. Inhibition of amyloid formation by siRNA-mediated suppression of hIAPP

expression or treatment with the amyloid binding dye Congo red significantly reduced islet IL-1 β levels compared to untreated islets cultured at same glucose conditions (11.1 mmol/l), indicating that amyloid formation (and not elevated glucose) is the major factor inducing islet IL-1 β production at the glucose concentration used in these studies.

One of the key findings of our studies in this chapter was that blocking IL-1 β signalling by anakinra treatment reduced hIAPP aggregation and the extent of amyloid formation in human islets during culture. This led us to the notion that IL-1 β , in addition to its role in mediating amyloid-induced β -cell Fas upregulation, may also indirectly increase amyloid toxicity by potentiating amyloid formation. In type 2 diabetes, impaired processing of hIAPP precursor, proIAPP, has been implicated in islet amyloid formation due to increased secretion of proIAPP and/or partially processed proIAPP forms, which have higher tendency to form hIAPP aggregates than mature hIAPP (346,348,542). Moreover, impaired processing of proIAPP, in particular at its NH₂-terminal cleavage site, potentiates hIAPP aggregation thereby increasing amyloid-induced β -cell death (365,466). Indeed, our data showed that treatment with IL-1 β potentiates impaired proIAPP processing in human islets during culture and that enhanced β -cell function by blocking IL-1 β signalling restores impaired processing of proIAPP. Treatment with anakinra resulted in lower release of immature (pro)hIAPP forms, specifically the N-terminally unprocessed proIAPP, from β -cells thereby reducing islet amyloid formation in cultured human islets. Treatment with IL-1R antagonists such as anakinra may therefore provide a new approach to reduce amyloid formation in both human islet grafts and type 2 diabetes, two conditions associated with β -cell dysfunction.

In summary, studies performed in this chapter provide direct evidence to show that IL-1 β plays a dual role in amyloid formation and its β -cell toxicity. We show that amyloid formation induces IL-1 β production in islets leading to Fas upregulation and activation of the Fas-mediated apoptosis. Our studies further demonstrate that IL-1 β impairs proIAPP processing thereby potentiates islet amyloid formation and that blocking IL-1R signalling restores impaired proIAPP processing and reduces amyloid formation.

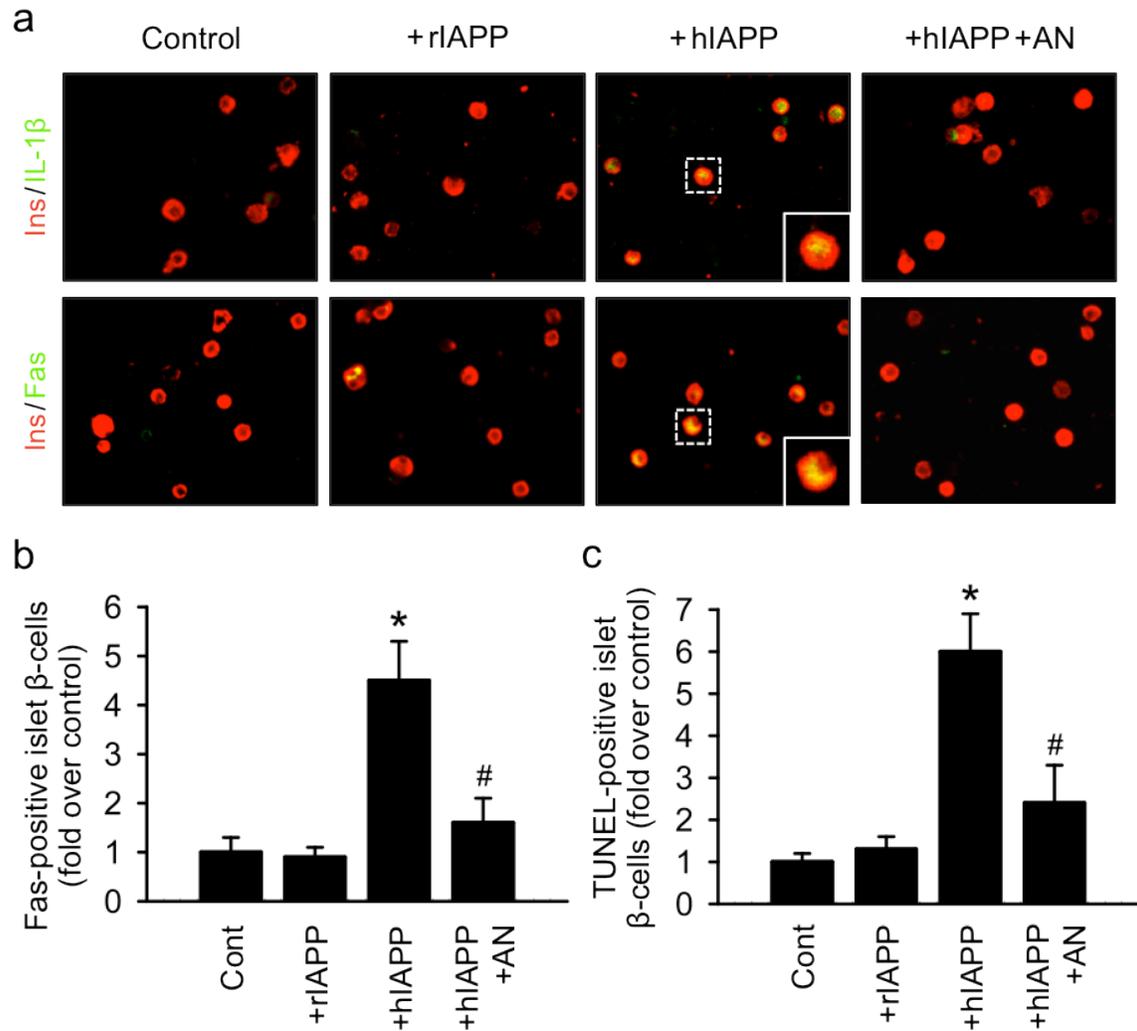


Figure 5-1. Treatment with the IL-1R antagonist markedly reduces β -cell Fas upregulation and apoptosis induced by aggregation of exogenously applied hIAPP.

Dispersed islet cells from C57BL/6 mice were treated with hIAPP (10 μ mol/l) or rIAPP (10 μ mol/l) alone or hIAPP with the IL-1R antagonist anakinra (+AN; 5 μ g/ml) for 12 h (to detect Fas and IL-1 β) or 24 h (to detect apoptosis). **(a)** Islet cells were immunolabelled for insulin and IL-1 β or insulin and Fas. The white squares denote regions enlarged in each image. The proportion of **(b)** Fas-positive or **(c)** TUNEL-positive islet β -cells (non-treated islet cells taken as control). Quantifications represent a minimum of ten microscopic fields each 100-150 islet cells. Results are expressed as means \pm SEM. *vs control group, #vs hIAPP-treated group ($p < 0.05$, one-way ANOVA)

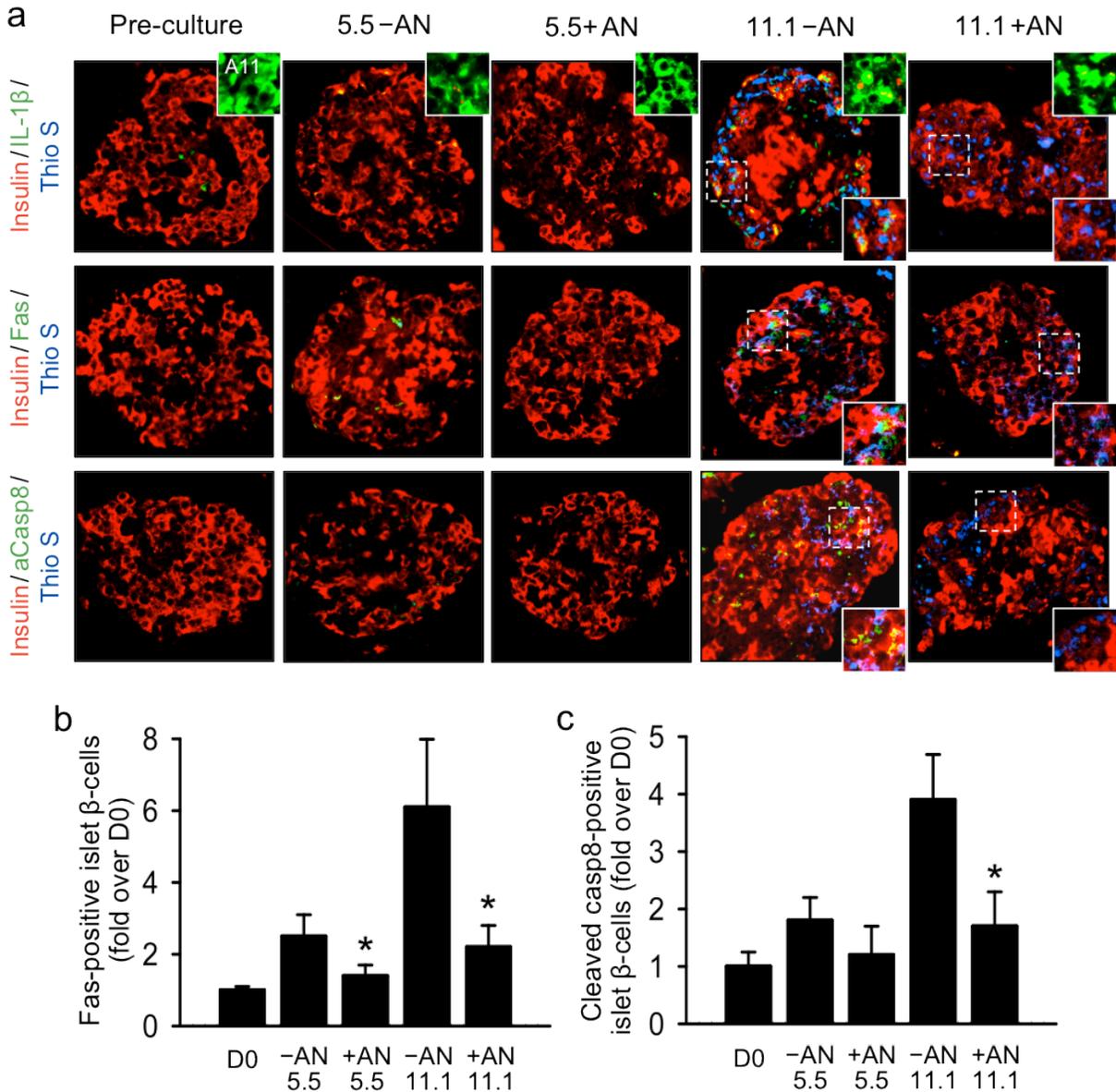


Figure 5-2. Amyloid formation in human islets during culture results in increased levels of islet IL-1 β production leading to β -cell Fas upregulation and caspase-8 activation, both of which are markedly reduced by treatment with IL-1R antagonist.

(a) Pre-culture and 7-day cultured human islets in low (5.5 mmol/l) or elevated (11.1 mmol/l) glucose in the presence or absence of the IL-1R antagonist anakinra (10 μ g/ml) were immunolabelled for insulin, thioflavin S (Thio S), and IL-1 β or Fas or cleaved caspase-8 (aCasp8). Inserts show smaller hIAPP aggregates detected by immunolabelling for insulin (red) and A11 (green). The white squares denote regions enlarged in each image. The proportion of (b) Fas-positive or (c) cleaved caspase-8-positive islet β -cells in each condition. Results are expressed as means \pm SEM of eight independent studies from eight human islet preparations (n=30-35 islets per condition in each study). *vs corresponding non-treated group ($p < 0.05$, one-way ANOVA)

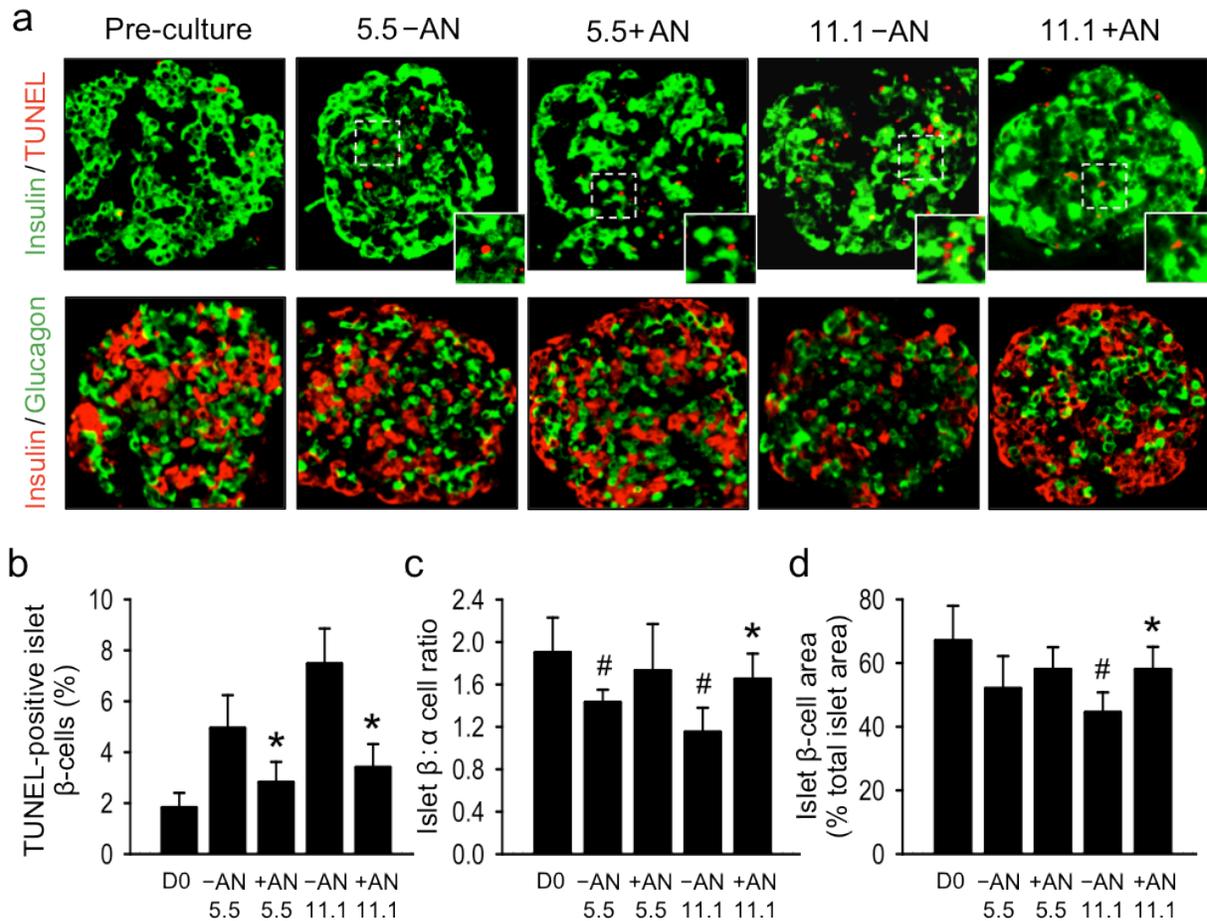


Figure 5-3. Reduced amyloid-induced β -cell Fas upregulation and caspase-8 activation by treatment with IL-1R antagonist is associated with increased β - to α -cell ratio and β -cell area in human islets during culture.

Human islets were cultured in CMRL (5.5 or 11.1 mmol/l glucose) with or without the IL-1R antagonist anakinra (10 μ g/ml) for 7 days. **(a)** Paraffin-embedded sections from pre-culture and 7-day cultured anakinra-treated and non-treated human islets were immunolabelled for insulin and TUNEL or insulin and glucagon. The white squares denote regions enlarged in each image. The proportion of **(b)** apoptotic islet β -cells, **(c)** islet β - to α -cell ratio, and **(d)** β -cell area to total islet area were quantified in a total of about 50 islets per condition for each human islet preparation. Results are expressed as means \pm SEM of eight independent studies from eight human islet preparations. *vs corresponding non-treated group, #vs day 0 group ($p < 0.05$, one-way ANOVA)

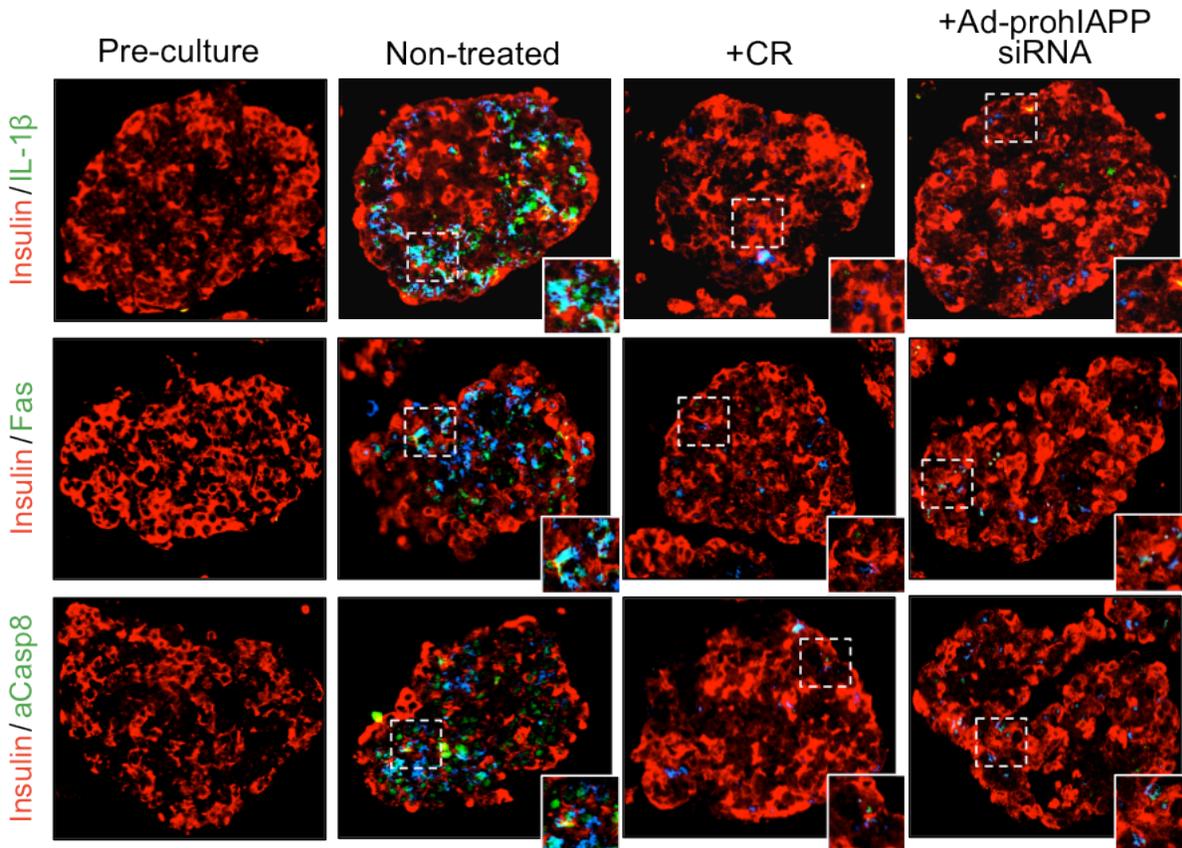


Figure 5-4. Inhibition of islet amyloid formation in cultured human islets with Ad-prohIAPP-siRNA or Congo red markedly reduces IL-1 β levels, Fas expression, and caspase-8 activation.

Human islets transduced overnight with Ad-prohIAPP-siRNA (+Ad-prohIAPP) and non-transduced islets with or without Congo red (+CR; 100 μ mol/l) treatment were cultured for 7 days in CMRL containing 11.1 mmol/l glucose (to potentiate amyloid formation). Paraffin embedded islet sections were immunolabelled for insulin/thioflavin S (Thio S)/IL-1 β , insulin/Thio S/Fas, and insulin/Thio S/cleaved caspase-8 (aCasp8). The white squares denote regions enlarged in each image. Images are representative of three independent studies from three human islet preparations. In each study, 100 islets were used for control and Congo red conditions and 50 islets for Ad-hIAPP-siRNA condition.

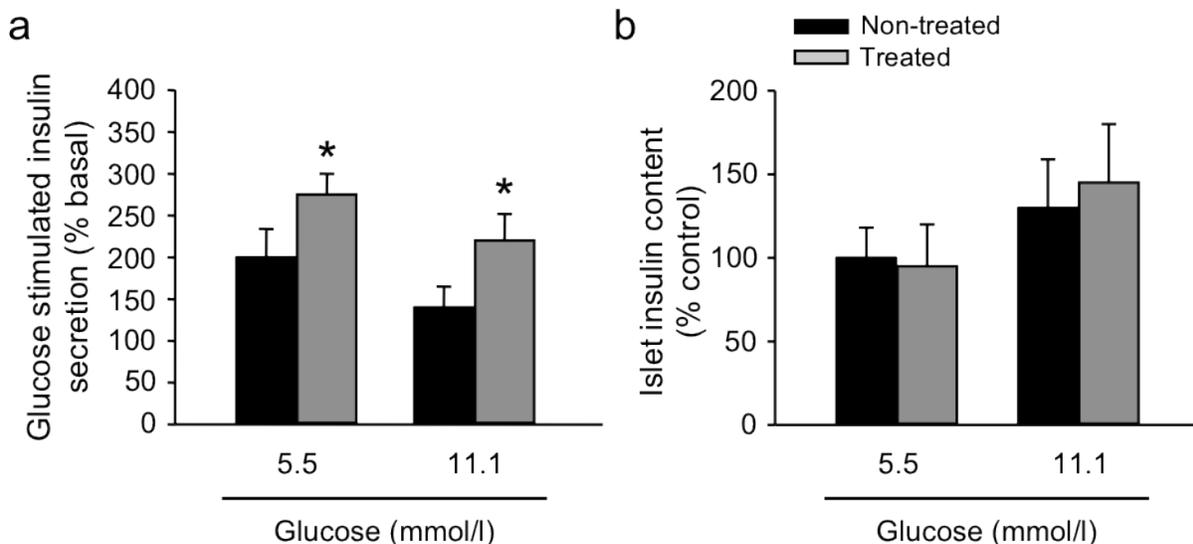


Figure 5-5. Blocking IL-1 β signalling in amyloid forming human islets during culture enhances β -cell function.

Human islets were cultured in CMRL (5.5 or 11.1 mmol/l glucose) with or without the IL-1R antagonist anakinra (10 μ g/ml) for 7 days. **(a)** Islet insulin response to glucose stimulation (16.7 mmol/l) and **(b)** islet insulin content in cultured anakinra-treated and non-treated human islets. Glucose stimulated insulin release is reported as percent increase over basal insulin release (1.67 mmol/l glucose). Islet insulin content is expressed as percentage of insulin content in non-treated islets taken as 100%. Results are expressed as means \pm SEM of six independent studies from six human islet preparations performed in duplicate. *vs corresponding non-treated group ($p < 0.05$, one-way ANOVA)

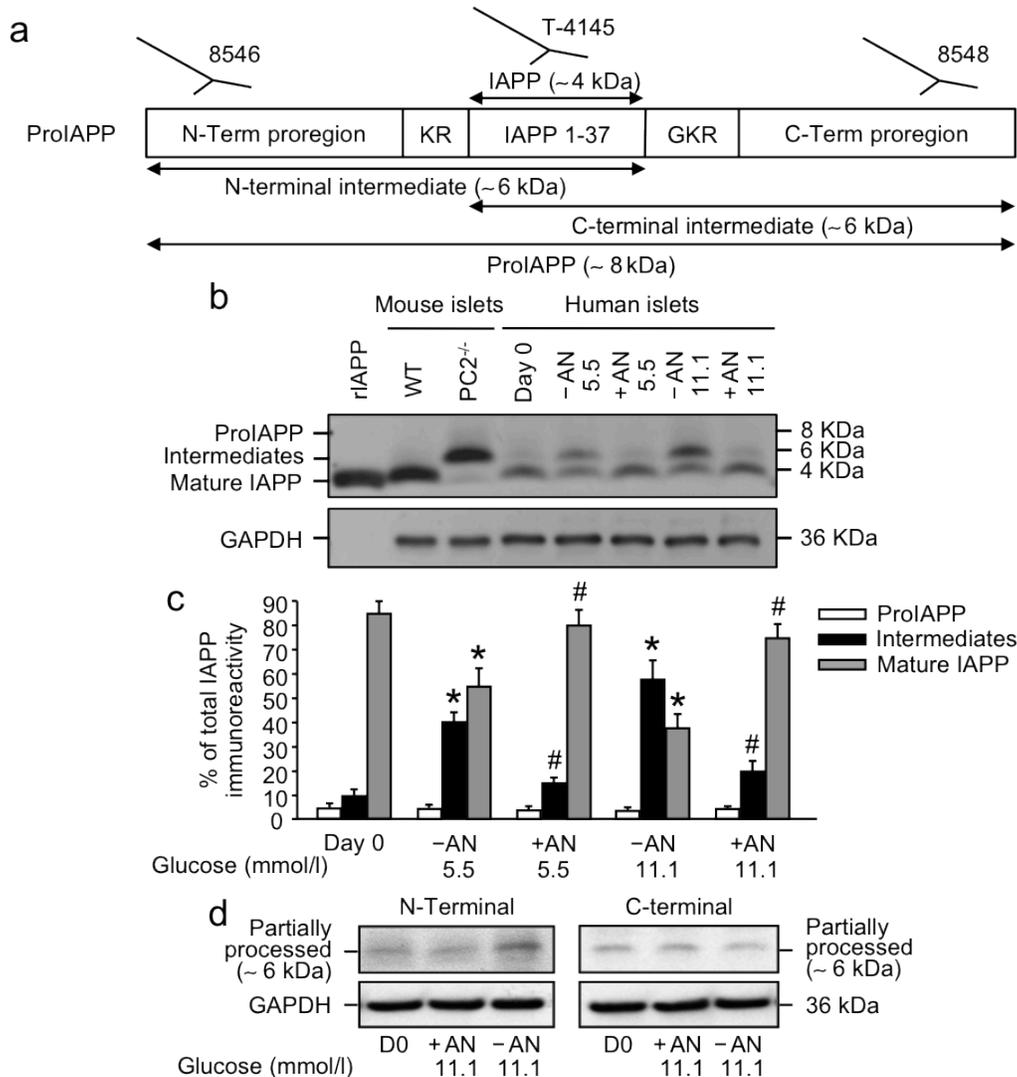


Figure 5-6. Enhanced β -cell function in anakinra-treated human islets restores impaired proIAPP processing.

(a) Schematic diagram demonstrating the antigen binding sites for each (pro)IAPP antibody. **(b)** The cellular protein levels of proIAPP, its intermediates and mature forms, were detected in the anakinra-treated (+AN; 10 μ g/ml) and non-treated (-AN) human islets following 7 days culture in 5.5 and 11.1 mmol/l glucose. Islet lysates from wild-type (WT) mice that mainly contain mature IAPP and from PC2 knockout mice that mainly contain NH₂-terminal unprocessed proIAPP and no mature IAPP (due to blocked proIAPP processing at its NH₂-terminus) are shown for comparison. **(c)** Densitometric analyses of immunoblots are presented as the percentage of each IAPP-immunoreactive molecular form with total IAPP immunoreactivity taken as 100%. **(d)** ProIAPP intermediate forms were detected in the anakinra-treated and non-treated human islet lysates using anti-sera specific to the NH₂- and COOH-terminal proIAPP, respectively. Results are expressed as means \pm SEM of six independent studies from six human islet preparations. *vs corresponding molecular form in freshly isolated islets (day 0). #vs corresponding molecular form in non-treated cultured islets ($p < 0.05$, two-way ANOVA)

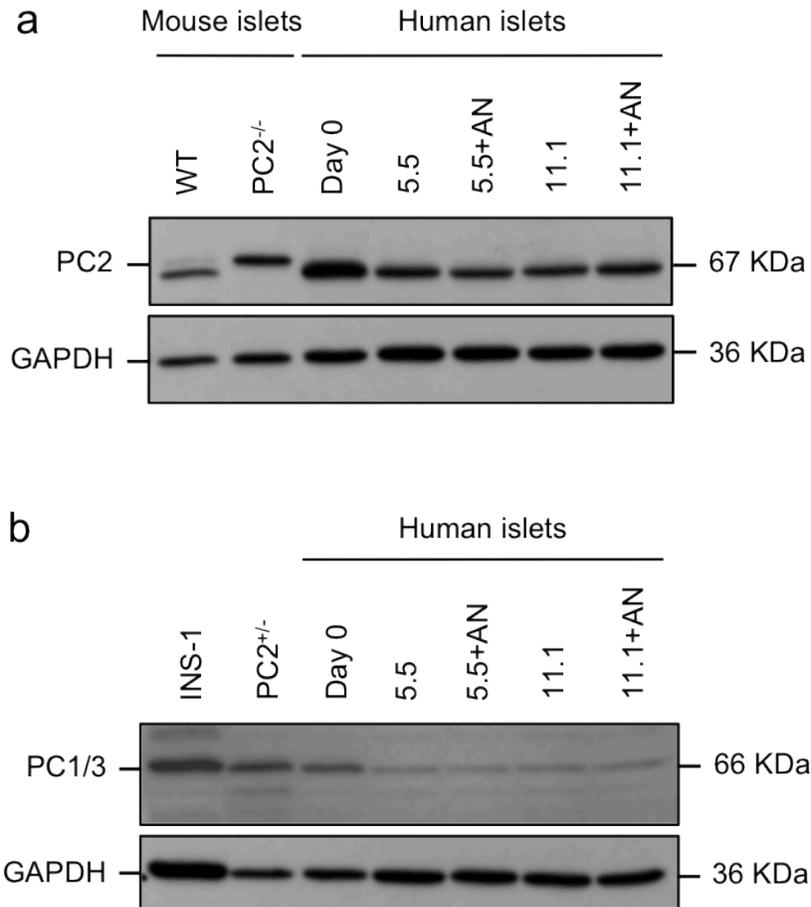


Figure 5-7. Treatment with IL-1 β antagonist has no detectable effect on PC2 and PC1/3 protein expression in cultured human islets.

The cellular protein levels of proIAPP processing enzymes **(a)** PC2 and **(b)** PC1/3 were detected in the anakinra-treated (+AN; 10 μ g/ml) and non-treated (-AN) human islets following 7 days culture in 5.5 and 11.1 mmol/l glucose. Immunoblots represent three independent studies from three human islet preparations.

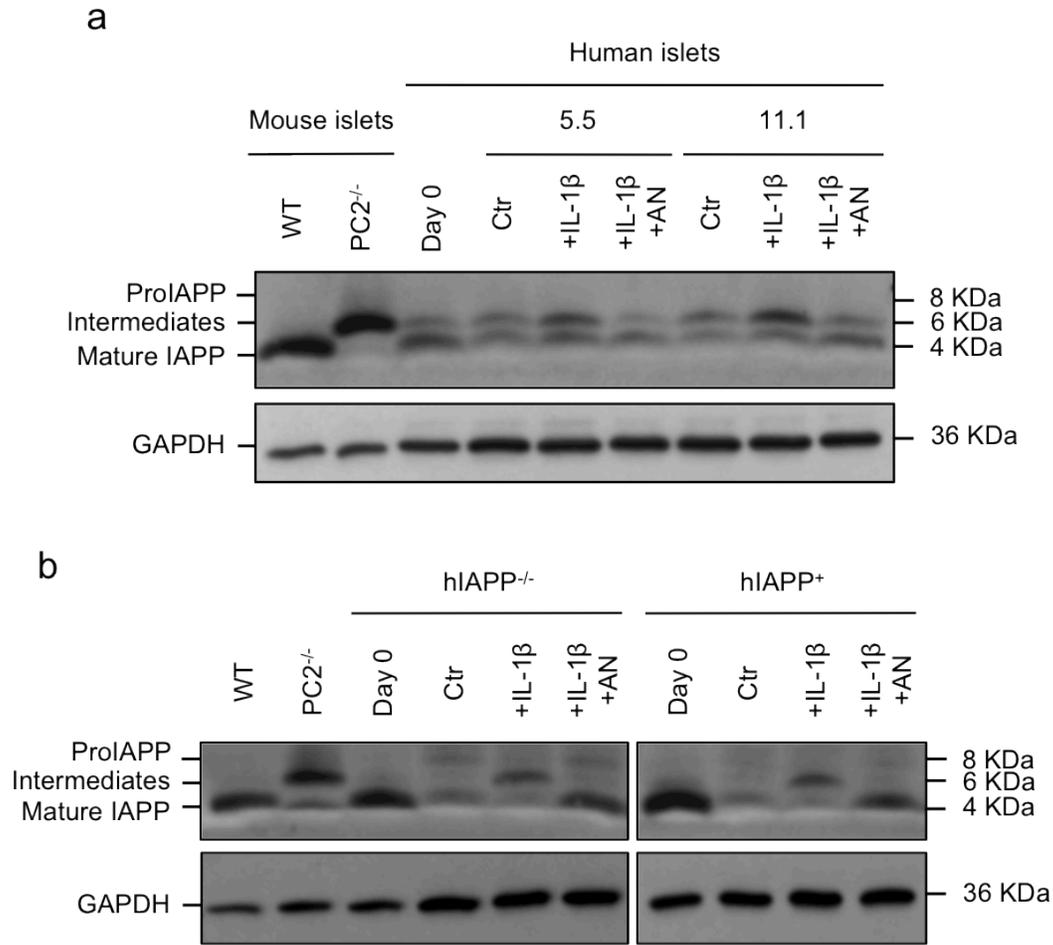


Figure 5-8. Treatment with IL-1 β potentiates impaired proIAPP processing in cultured human and mouse islets which is reversed by blocking IL-1R.

(a) Human islets were cultured in CMRL (5.5 or 11.1 mmol/l glucose) with or without synthetic IL-1 β (2 ng/ml) in the presence or absence of anakinra (10 μ g/ml) for 7 days. **(b)** Freshly isolated wild-type (hIAPP^{-/-}) and hIAPP-expressing transgenic mouse islets (hIAPP^{+/+}) were cultured in Ham's-F10 (16.7 mmol/l glucose) with or without synthetic IL-1 β (2 ng/ml) in the presence or absence of anakinra (10 μ g/ml) for 7 days. (Pro)IAPP forms were detected in islet lysates by Western blot followed by immunoblot using an antibody that recognises all (pro)IAPP forms. Immunoblots represent three independent studies from three human islet preparations and three mouse islet studies.

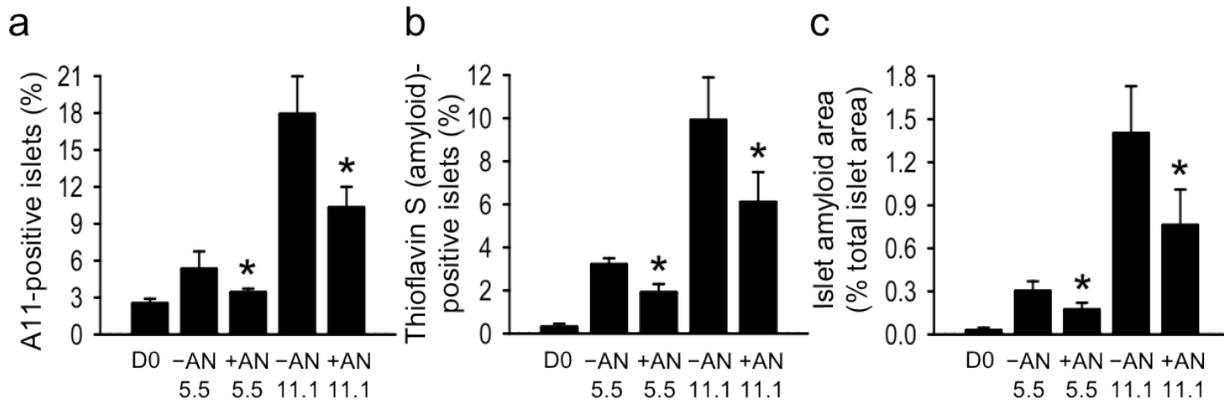


Figure 5-9. Treatment with IL-1R antagonist anakinra reduces both severity and prevalence of amyloid formation in cultured human islets.

Pre-culture and 7-day cultured anakinra-treated (+AN; 10 μ g/ml) or non-treated (-AN) human islets were immunolabelled for insulin and A11 or thioflavin S. The proportion of (a) A11-positive islets to the total number of islets, (b) thioflavin S-positive islets to the total number of islets, and (c) amyloid area to total islet area. Results are expressed as means \pm SEM of eight independent studies from eight human islet preparations (n=30-35 islets per condition in each study). *vs corresponding non-treated group ($p < 0.05$, one-way ANOVA)

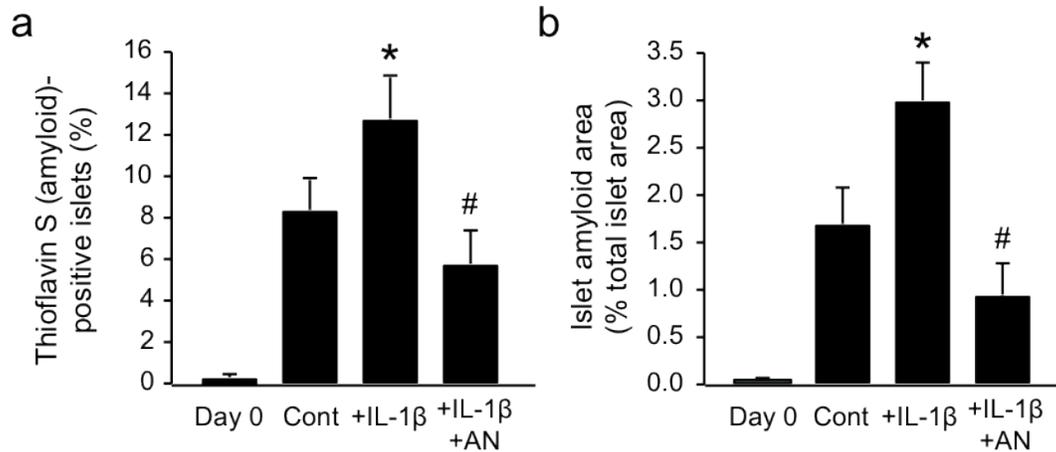


Figure 5-10. Treatment with anakinra reduces IL-1 β -induced amyloid formation in cultured human islets.

Human islets were cultured in CMRL (11.1 mmol/l glucose) with or without synthetic IL-1 β (2 ng/ml) in the presence or absence of anakinra (+AN; 10 μ g/ml) for 7 days. The proportion of **(a)** thioflavin S-positive islets to the total number of islets and **(b)** amyloid area to total islet area. Results are expressed as means \pm SEM of four independent studies from four human islet preparations (n=30-35 islets per condition in each study). *vs non-treated control group, #vs IL-1 β -treated group (p <0.05, one-way ANOVA)

Chapter 6: Enhancing β -cell function by GLP-1R agonists restores impaired proIAPP processing and reduces amyloid formation

6.1 Background

The finding that islet amyloid formation occurs in type 2 diabetes, cultured and transplanted islets (256,260,364,366,367,384), which are three conditions associated with β -cell dysfunction, led us to the notion that impaired β -cell function may initiate and/or potentiate islet amyloid formation likely due to impaired processing of proIAPP. In support of this idea, it has been shown that impaired proIAPP processing in isolated islets from hIAPP-expressing transgenic mice lacking PC2, the essential enzyme for proIAPP processing, leads to increased amyloid formation during culture (365). Moreover, impaired proinsulin processing has been reported in human islets following transplantation into patients with type 1 diabetes (543). Since proIAPP and proinsulin are processed in β -cell granules by the same enzymes, it is likely that proIAPP processing is also impaired in transplanted islets.

Our findings from studies presented in Chapter 5 show that exposure to IL-1 β can cause β -cell dysfunction, resulting in impaired proIAPP processing thereby inducing amyloid formation and β -cell death. Furthermore, our studies have demonstrated that blocking the IL-1 β signalling by treatment with IL-1R antagonist anakinra can improve β -cell function manifested as enhanced proIAPP processing, reduced islet amyloid formation and toxicity in cultured human islets. These findings indicate that enhancing β -cell function at early stages of amyloid formation may provide a feasible strategy to prevent islet amyloid formation and β -cell toxicity.

GLP-1 is a potent incretin hormone produced in the intestinal L-cells (544,545). The ability of GLP-1 to reduce apoptosis, stimulate proliferation, and enhance survival and function of islet β -cells is of significant clinical benefit in type 2 diabetes (546,547). However, native GLP-1 has a very short plasma half-life (1-2 min) and is rapidly degraded by the enzyme dipeptidyl peptidase IV following release from the L-cells (544,545,548). GLP-1R agonists exhibit actions similar to those of GLP-1 by directly binding to the GLP-1R on β -cells but with increased plasma half-life, making them more clinically useful (549). Exenatide is a GLP-1R

agonist with plasma half-life of about 2-4 hours when administered subcutaneously (549). Exenatide shares ~53% structural homology with GLP-1 and was the first GLP-1R agonist approved for clinical use in 2005 (550). Treatment with exenatide can effectively stimulate glucose-dependent insulin secretion, increase insulin synthesis, β -cell proliferation and neogenesis, and reduce β -cell apoptosis in transformed β -cell lines, animal models and isolated human islets (547,551-554). In addition to its effects in patients with type 2 diabetes, clinical studies indicate that treatment with GLP-1R agonists may improve survival and function of islet grafts in transplant recipients with type 1 diabetes (555,556).

Furthermore, recent studies have demonstrated that exenatide has anti-inflammatory properties against the deleterious effects of cytokines. In adipocytes, exenatide has been shown to induce expression of anti-inflammatory adipose hormones such as adiponectin and inhibit expression of pro-inflammatory adipokines (557). In an animal model of Parkinson's disease, systemic administration of exenatide has been reported to prevent loss of dopaminergic neurons by suppressing expression of TNF- α and IL-1 β (558). Importantly, studies have shown that treatment with exenatide reduces IL-1 β -induced β -cell death and dysfunction in transformed and primary islet β -cells (559-562). For example, Cechin *et al.* has shown that exenatide treatment reduces endogenous cytokine production and β -cell apoptosis in isolated human islets (563). However, the mechanisms mediating the protective effects of exenatide against cytokines are still not well understood. The findings from previous studies suggest that the cytoprotective effects of exenatide in β -cells may involve modulation of multiple pathways including activation of cyclic-adenosine monophosphate (cAMP)/protein kinase A (PKA), signal transducer and activator of transcription 3 (STAT3), and mitogen-activated protein kinase (MAPK) pathways (560,563) as well as inhibition of JNK and perforin/granzyme pathways (559,563).

In this study, we used human islets in culture as an *ex vivo* model of impaired proIAPP processing and islet amyloid formation, to test if enhancing β -cell function by GLP-1R agonists such as exenatide can restore proIAPP processing thereby reducing amyloid formation and its β -cell toxicity. We first tested if exenatide treatment can enhance β -cell function in human islets cultured under conditions used in clinical islet transplantation and elevated glucose concentration. Next, we detected immunoreactive forms of (pro)hIAPP and quantified severity

and prevalence of amyloid formation in freshly isolated and cultured human islets, to examine if exenatide-induced improvement in β -cell function can restore impaired processing of proIAPP and reduce amyloid formation. Finally, we assessed whether exenatide mediated reduction in islet amyloid formation can decrease amyloid-induced β -cell apoptosis and/or enhance β -cell proliferation; as well we investigated the potential pathways involved in this process.

6.2 Results

6.2.1 Treatment with exenatide markedly enhances β -cell function in cultured human islets

Islet insulin content and insulin release from the exenatide-treated and non-treated human islets were measured to assess if treatment with exenatide can improve β -cell function during islet culture. Exenatide-treated human islets had higher insulin release during culture than non-treated cultured islets, which was more profound following short-term culture (2 days) than long-term culture (7 days) (Figure 6-1a, c). Islet insulin content was not significantly different in 2-day cultured exenatide-treated and non-treated islets but was markedly higher in 7-day exenatide-treated islets (Figure 6-1b, d). Moreover, there was no detectable difference in basal insulin release (1.67 mmol/l glucose) between exenatide-treated and non-treated cultured islets but exenatide-treated islets had a greater insulin response to elevated glucose (Figure 6-1e) associated with their higher insulin content (Figure 6-1f).

6.2.2 Improved β -cell function with exenatide treatment restores impaired processing of proIAPP in human islets during culture

To assess the effects of improved β -cell function on proIAPP processing, (pro)hIAPP immunoreactive forms including proIAPP, its partially processed intermediates, and mature hIAPP were detected in the exenatide-treated and non-treated islet lysates following 7-day culture in normal (5.5 mmol/l) or elevated (11.1 mmol/l) glucose. Similar to our findings in Chapter 5, mature hIAPP was the major (pro)IAPP immunoreactive form detected in freshly isolated human islets with low levels of unprocessed and partially processed proIAPP also detectable (Figure 6-2a, b). Islet culture at both normal and elevated glucose conditions led to

impaired proIAPP processing manifested as increased protein levels of proIAPP intermediate forms and reduced mature hIAPP form (Figure 6-2a, b). The antisera against NH₂-terminus of proIAPP identified this elevated intermediate form to be the NH₂-terminally unprocessed proIAPP (Figure 6-2c).

Enhanced β -cell function by treatment with exenatide restored impaired proIAPP processing as demonstrated by markedly reduced levels of partially processed proIAPP in human islets cultured in normal and elevated glucose (Figure 6-2). The mature hIAPP was the main (pro)hIAPP form released from exenatide-treated islets (Figure 6-2a, b). Also, proIAPP levels were comparable in exenatide-treated and non-treated islets (Figure 6-2a, b). Improved proIAPP processing in exenatide-treated islets resulted in lower release of NH₂-terminally unprocessed proIAPP forms from those islets than non-treated cultured islets (Figure 6-2c). In contrast, there was no significant difference between the level of COOH-terminally unprocessed proIAPP intermediate form in exenatide-treated and non-treated islets (Figure 6-2c).

6.2.3 Enhanced proIAPP processing in exenatide-treated human islets is associated with reduced islet amyloid formation during culture

Next, we examined if improved proIAPP processing in exenatide-treated human islets can reduce formation of hIAPP aggregates. A11 (oligomer)- and thioflavin S (amyloid)-positive islets were absent in freshly isolated human islets but were present in 7-day cultured islets (Figure 6-3a, b). A11-positive, but not thioflavin S-positive, islets were detectable as early as 2 days after culture (Figure 6-3c, d). Interestingly, isolated human islets treated with exenatide had a markedly lower number of islets containing hIAPP oligomers compared to non-treated cultured islets as assessed by immunolabelling for insulin and A11 (Figure 6-3a, c). Furthermore, eight of ten exenatide-treated human islet preparations had lower amyloid prevalence (number of thioflavin S-positive islets) and severity (islet amyloid area to total islet area) than non-treated cultured islets whereas two human islet preparations had comparable levels of amyloid formation (Figure 6-3d, e).

6.2.4 Reduced islet amyloid formation in exenatide-treated human islets is associated with decreased caspase-3 activation and β -cell apoptosis

Islet β -cell apoptosis was assessed by caspase-3 activity in islet lysates and quantitative immunolabelling for insulin and cleaved (active) caspase-3 or TUNEL. Reduced amyloid formation in exenatide-treated human islets correlated with significantly lower caspase-3 activation in those islets as compared to non-treated cultured islets (Figure 6-4a, b). Furthermore, treatment with exenatide markedly reduced TUNEL-positive (apoptotic) β -cells in both 2-day and 7-day cultured human islets in normal and elevated glucose (Figure 6-4c, d, e). Despite the high rate of β -cell apoptosis, cultured human islets had a very low number of apoptotic α -cells with no significant difference between exenatide-treated and non-treated islets (Figure 6-5a, b).

6.2.5 Reduced amyloid formation and β -cell apoptosis in exenatide-treated human islets during culture is associated with increased islet β - to α -cell ratio and β -cell area

We tested if reduced amyloid formation and β -cell apoptosis is associated with higher β -cell mass in exenatide-treated islets as compared to non-treated cultured islets. Human islets were immunolabelled for insulin and glucagon following culture in normal or elevated glucose with or without exenatide (Figure 6-6a). There was no detectable difference in islet β - to α -cell ratio or islet β -cell area between 2-day cultured exenatide-treated and non-treated islets (Figure 6-6b, c). The progressive β -cell death in human islets associated with amyloid formation during 7-day culture decreased β -cell mass manifested as reduced islet β - to α -cell ratio and β -cell area as compared to pre-culture islets (Figure 6-6d, e). Treatment with exenatide markedly improved islet β - to α -cell ratio and preserved islet β -cell area during 7-day culture (Figure 6-6d, e).

6.2.6 Exenatide treatment reduces JNK activation induced by hIAPP aggregates formed in human islets during culture

We tested if amyloid formation in cultured human islets induces activation of JNK and examined the effects of exenatide on JNK activation as a potential mechanism by which exenatide may protect β -cells from amyloid toxicity. Immunolabelling for insulin and phospho (active)-JNK revealed that amyloid formation in cultured islets is associated with increased

number of phospho-JNK positive β -cells and that exenatide-treated islets had markedly lower phospho-JNK positive β -cells than non-treated cultured islets despite amyloid formation (Figure 6-7a). There was no significant difference between phospho-JNK protein levels in lysates from exenatide-treated and non-treated cultured islets by Western blot analysis (Figure 6-7b).

6.2.7 Reduced amyloid formation and toxicity is associated with increased β -cell PKB phosphorylation and proliferation rate in exenatide-treated human islets

We assessed phospho-PKB levels in the exenatide-treated islets as a potential mechanism by which exenatide may mediate its anti-apoptotic and proliferative effects in β -cells during amyloid formation in cultured human islets. Treatment with exenatide increased phospho-PKB levels in β -cells following 7-day culture at both 5.5 (minimal amyloid formation) and 11.1 mmol/l glucose (significant amyloid formation) conditions (Figure 6-8a). Increased phospho-PKB levels in exenatide-treated human islets were associated with enhanced β -cell proliferation during culture as assessed by immunolabelling for insulin and PCNA (a marker of cell proliferation). The β -cell proliferation rate was comparable in 2-day cultured exenatide-treated and non-treated islets but was higher in exenatide-treated islets at 5.5 and 11.1 mmol/l glucose following 7-day culture (Figure 6-8b, c). These findings suggest that treatment with exenatide enhances β -cell proliferation in amyloid-forming cultured human islets.

6.3 Discussion

In this chapter, we examined if improving β -cell dysfunction by treatment with exenatide, a GLP-1R agonist, can protect islet β -cells from the cytotoxic effects of amyloid as a potential therapeutic approach to preserve β -cells in type 2 diabetes. Using human islets in culture as an *ex vivo* model of endogenous islet amyloid formation and impaired β -cell function, we demonstrated that enhanced β -cell function by treatment with exenatide can restore impaired proIAPP processing, resulting in the lower release of immature (pro)hIAPP forms from β -cells thereby reducing hIAPP aggregation and amyloid toxicity. Moreover, human islets in these studies were cultured under conditions used in clinical islet transplantation. Thus, treatment with exenatide may provide a feasible approach to preserve β -cells during pre-transplant islet culture.

Consistent with the previous studies which showed that GLP-1 and GLP-1R agonists can improve β -cell function (546,564), our studies showed that exenatide-treated human islets had higher insulin secretion than non-treated islets cultured under elevated (11.1 mmol/l) glucose conditions. Interestingly, the effect of exenatide on islet insulin release was more profound in 2-day cultured islets compared to 7-day cultured islets. One possible explanation for the decrease in exenatide-induced insulin release following 7-day culture with elevated glucose is down-regulation of the GLP-1R on β -cells. GLP-1R mRNA and protein are highly expressed in β -cells under normal conditions (545), but exposure to elevated glucose has been shown to reduce expression of GLP-1R leading to decreased insulin response to GLP-1 in β -cells (565). Accordingly, reduced GLP-1R expression has been observed in pancreatic sections from patients with type 2 diabetes (566). Similarly, exposure to apoptotic stimuli such as palmitate has been shown to decrease GLP-1R expression in transformed and mouse islet β -cells (567).

Moreover, 7-day exenatide-treated islets had comparable basal insulin release but greater insulin response to elevated glucose following incubation in exenatide-free buffer, indicating that the effects of exenatide on β -cells are maintained after its removal. Enhanced β -cell insulin release in response to elevated glucose in 7-day cultured human islets might be due to higher islet insulin content and/or increased β -cell insulin secretion. The former is supported by our finding that cultured human islets treated with exenatide had higher insulin content compared to non-treated islets. Also, a previous study reported that treatment with GLP-1 increases intracellular insulin content in isolated human islets (546). However, there was no significant difference between islet insulin content in 2-day exenatide-treated or non-treated islets, which may be due to the higher insulin release in 2-day cultured islets as compared to 7-day cultured islets and insufficient time for β -cells to produce more insulin to compensate for increased insulin release.

In line with our data shown in Chapter 5, islet culture was associated with impaired proIAPP processing manifested as increased levels of proIAPP and its intermediate forms and reduced mature hIAPP form. Moreover, impaired processing of proIAPP in cultured human islets is likely not due to reduced protein levels of proIAPP processing enzymes PC1/3 or PC2 as shown in Chapter 5.

The key finding of the studies in this chapter is that improved β -cell dysfunction with GLP-1R agonists such as exenatide can restore impaired proIAPP processing in cultured human islets. Exenatide treatment markedly enhanced proIAPP processing, resulting in lower cellular levels of NH₂-terminally unprocessed proIAPP and higher mature hIAPP. The effects of exenatide on proIAPP appear to be mainly on its processing rather than its expression since only a modest increase in proIAPP protein levels was observed in exenatide-treated islets compared to non-treated islets following culture at both normal or elevated glucose conditions. Furthermore, exenatide treatment reduced immature (pro)hIAPP to hIAPP ratio released from human islets during culture. The major immunoreactive form released from exenatide-treated islets was the mature hIAPP whereas the partially processed proIAPP form was the major immunoreactive form released from the non-treated cultured islets. Similarly, treatment with exenatide has been shown to improve islet graft dysfunction and reduce serum proinsulin to insulin ratio in islet transplant recipients with type 1 diabetes (568).

Importantly, improved processing of proIAPP in exenatide-treated human islets was associated with a lower number of islets containing A11-positive hIAPP oligomers or thioflavin S-positive amyloid deposits as well as reduced islet amyloid area to total islet area compared to those in non-treated cultured islets. These findings indicate that enhancing β -cell function at early stages of islet culture may have a significant impact on reducing amyloid formation and toxicity. Similarly, early therapeutic interventions to improve β -cell function in patients with type 2 diabetes and islet recipients with type 1 diabetes may therefore slow down the process of hIAPP aggregation and prevent amyloid-induced β -cell death. In support, two recent studies have demonstrated that treatment with GLP-1R agonists such as exenatide and liraglutide reduces formation of A β oligomers and amyloid plaques in the brain of mouse models of Alzheimer's disease (569,570).

Increased insulin secretion from β -cells by GLP-1R agonists also increases release of hIAPP that is co-secreted with insulin (571,572), raising the possibility that GLP-1R agonists may potentiate amyloid formation in human islets. However, we observed reduced amyloid formation in exenatide-treated islets compared to non-treated islets. This is likely because exenatide-treated human islets release mainly mature hIAPP rather than immature (pro)hIAPP.

Accordingly, previous studies have shown that elevated hIAPP level *per se* such as its overexpression in hIAPP-expressing mice is not sufficient for amyloid formation (573), whereas elevated production and release of immature (pro)hIAPP potentiates hIAPP aggregation (365,466). In contrast to our findings, treatment with exendin-4 (exenatide analogue) in hIAPP-expressing transgenic mouse islets, which form amyloid much faster than cultured human islets, was shown to potentiate amyloid formation during short-term (2 day) culture with elevated glucose (16.7 mmol/l) (572). This finding indicate that factors such as the level of hIAPP released from islets (human versus transgenic mice), glucose conditions that islets are exposed to, and the period of islet culture may play important roles in the net effect of GLP-1R agonists on amyloid formation.

Reduced amyloid formation in exenatide-treated islets correlated with markedly lower β -cell apoptosis in human islets during 2- or 7-day culture at both normal and elevated glucose conditions. Furthermore, apoptosis was much lower in α -cells compared to β -cells during islet culture and treatment with exenatide had no detectable effect on α -cell survival. Reduced β -cell apoptosis resulted in a higher islet β - to α -cell ratio and β -cell to total islet area in 7-day exenatide-treated islets compared to non-treated cultured islets, indicating that exenatide treatment preserves β -cell mass during islet culture. In line with these findings, a previous study showed that GLP-1 reduces β -cell apoptosis in human islets *ex vivo* (546). Similarly, exenedin-4 treated hIAPP-expressing transgenic mouse islets, despite their higher amyloid formation, had lower β -cell apoptosis and higher islet β -cell area than non-treated cultured islets, indicating that GLP-1R agonists protect islet β -cells from amyloid toxicity even after formation of hIAPP aggregates (572).

Previous studies have shown that hIAPP-induced β -cell death may be mediated by activation of the JNK pathway (439,490). A study by Zhang *et al.* (439) has demonstrated that treatment with synthetic hIAPP aggregates can induce activation of the JNK pathway in transformed β -cells. Similarly, JNK activation has been shown to mediate islet amyloid-induced β -cell death in cultured hIAPP-expressing transgenic mouse islets (490). Consistent with these earlier studies in transformed and transgenic mouse islet β -cells, our studies showed that amyloid formation in cultured human islets is associated with increased phospho-JNK levels in β -cells.

Conversely, blocking the JNK signalling using specific JNK inhibitor results in reduced β -cell apoptosis in hIAPP-expressing transgenic mouse islets to levels similar to those in non-transgenic islets (490). In our study, treatment with exenatide resulted in reduced JNK activation as shown by lower phospho-JNK immunoreactivity in exenatide-treated islets compared to non-treated islets, which was associated with decreased amyloid formation and β -cell apoptosis. These data indicate that in addition to its effects on reducing amyloid formation, anti-apoptotic effects of exenatide against toxic islet amyloid may also be mediated by inhibition of the amyloid-induced activation of JNK pathway.

In Chapter 5, we showed that IL-1 β plays an important role in mediating β -cell toxic effects of amyloid. Interestingly, a previous study has reported that exendin-4 prevents activation of JNK pathway induced by IL-1 β thereby protecting transformed and primary islet β -cells from IL-1 β -induced apoptosis (559). This suggests that the protective effects of exenatide may be through suppressing multiple mediators of apoptotic mechanism(s) associated with amyloid formation. We could not detect any difference between phospho-JNK protein levels in lysates from exenatide-treated and non-treated islets by Western blot, likely because in whole-islet lysates, α -cells that form a significant proportion of cells in human islets may mask the changes in β -cell phospho-JNK levels induced by hIAPP aggregates. Interestingly, elevated JNK phosphorylation and suppression of its activation by exenatide were recently reported in a mouse model of Alzheimer's disease (570), raising the idea that different amyloidogenic proteins may share similar mechanisms of toxicity which can be prevented by the effects of exenatide.

Recent studies from our research group have demonstrated that formation of hIAPP aggregates in cultured human and hIAPP-expressing transgenic mouse islets is associated reduced PKB phosphorylation (Y. Zhang *et al.* unpublished data). Since previous studies have shown that anti-apoptotic effects of GLP-1 involve activation of the PKB signalling (551,574), we detected phospho (active)-PKB immunoreactivity in exenatide-treated and non-treated amyloid-forming human islets. Interestingly, following 7-day culture, phospho-PKB levels were higher in exenatide-treated islet β -cells compared to non-treated islets, indicating that exenatide treatment may restore amyloid-induced reduction in PKB phosphorylation. In support, a previous study has shown that synthetic hIAPP aggregates can reduce β -cell phospho-PKB levels and that

treatment with exendin-4 restores hIAPP-induced reduction of PKB phosphorylation in transformed β -cells (575). Taken together, these data suggest that the PKB signalling may play a role in β -cell protective effects of exenatide during amyloid formation in cultured islets.

Finally, the rate of β -cell proliferation was slightly higher in 7-day exenatide-treated islets than non-treated cultured islets, indicating that exenatide treatment may enhance β -cell proliferation (likely via PKB signalling) during islet culture despite amyloid formation. However, considering the very low rate of β -cell proliferation in human islets during culture, changes in β -cell proliferation does not appear to play a significant role in preserving β -cell mass by exenatide in amyloid-forming human islets during culture.

In summary, our studies suggest that improved β -cell function by treatment with GLP-1R agonists such as exenatide can restore impaired proIAPP processing thereby reducing hIAPP aggregation and its β -cell toxicity in cultured human islets. Thus, GLP-1R agonists may provide an effective therapeutic approach to reduce amyloid formation in type 2 diabetes and human islet grafts for transplantation.

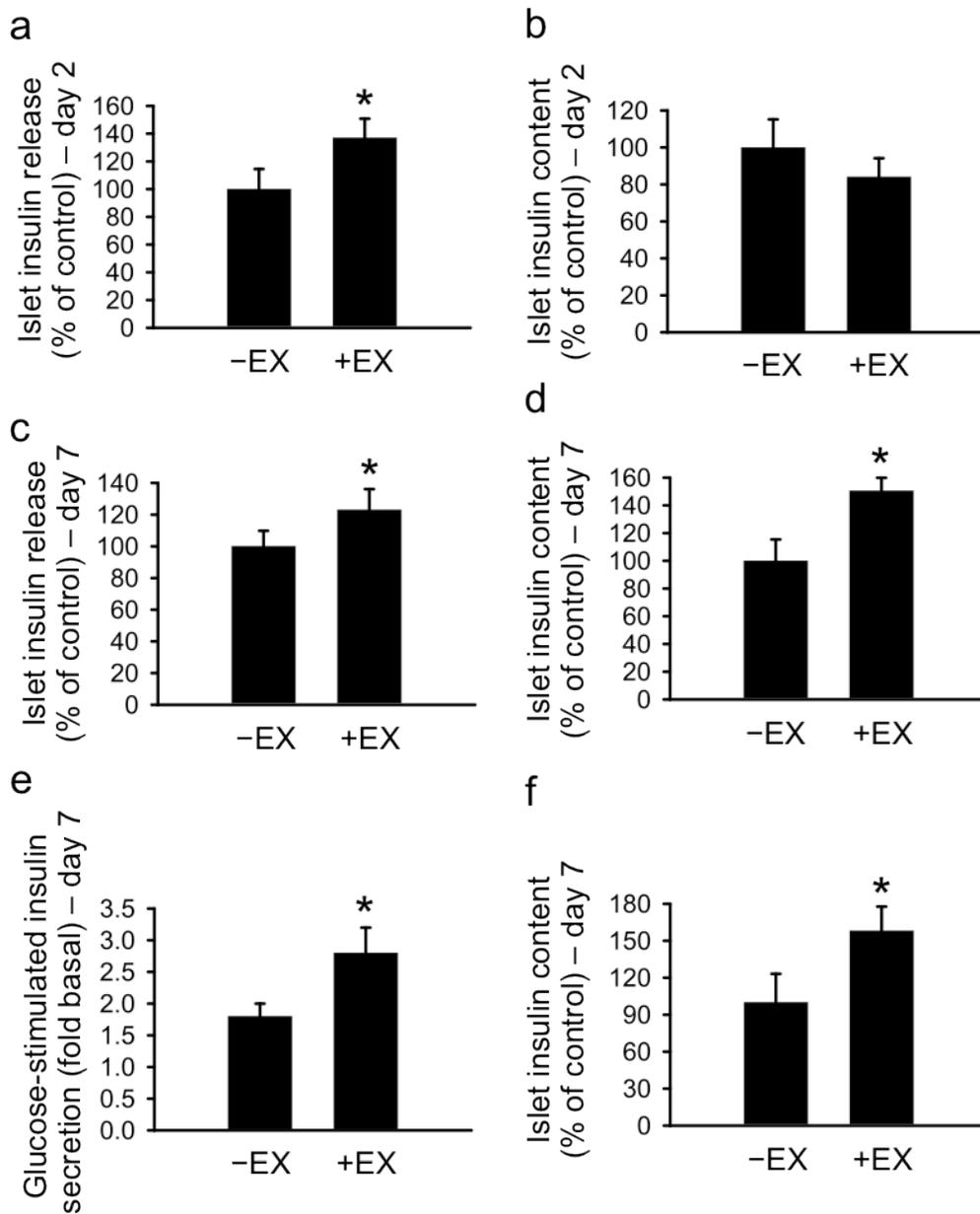


Figure 6-1. The exenatide-treated human islets have higher insulin content and insulin response to elevated glucose than non-treated cultured islets.

Human islets were cultured in CMRL (5.5 or 11.1 mmol/l glucose) with or without exenatide (10 nmol/l) for 2 or 7 days. **(a, c)** Insulin release and **(b, d)** islet insulin content were measured in the culture medium and islet lysates following culture. **(e)** Islet insulin response to glucose stimulation (16.7 mmol/l), and **(f)** islet insulin content in 7-day cultured (11.1 mmol/l glucose) exenatide-treated and non-treated human islets. Islet insulin release and insulin content are expressed as percentage of non-treated (control) islets taken as 100%. Glucose stimulated insulin release is reported as fold increase over basal insulin release (1.67 mmol/l glucose). Results are expressed as means \pm SEM of ten independent studies from ten human islet preparations performed in duplicate. *vs non-treated group ($p < 0.05$, Student's *t*-test)

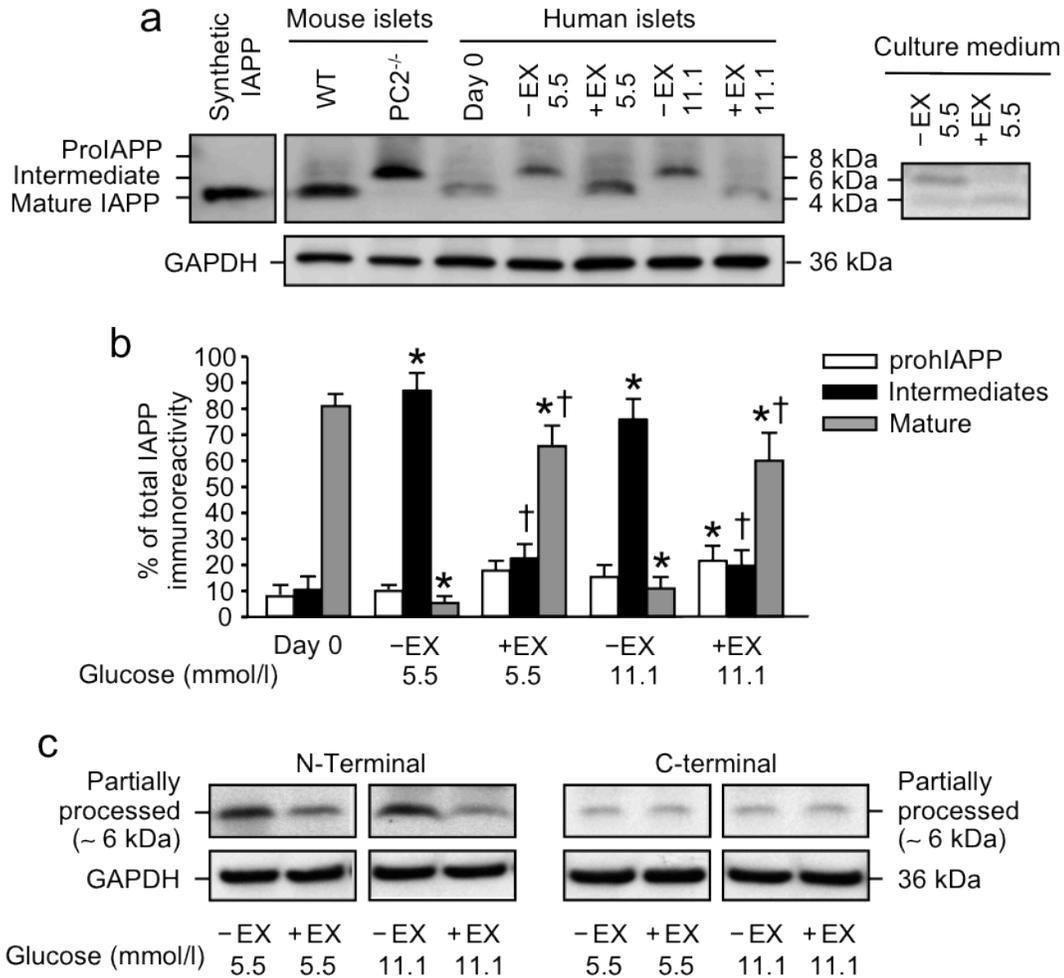


Figure 6-2. Improved β -cell function by treatment with exenatide restores impaired processing of proIAPP in human islets during culture.

(a) The cellular protein levels of proIAPP, its intermediates and mature forms, were detected in the exenatide-treated (+EX; 10 nmol/l) and non-treated (-EX) human islets following 7 days culture in 11.1 mmol/l glucose by Western blot on islet lysates (left) or culture medium (right) followed by immunoblot using an antibody that recognises both mature and immature forms of IAPP. **(b)** Densitometric analyses of immunoblots are presented as the percentage of each IAPP-immunoreactive molecular form with total IAPP immunoreactivity taken as 100%. **(c)** ProIAPP and its intermediate forms were detected in the exenatide-treated and non-treated human islet lysates using anti-sera specific to the NH₂- and COOH-terminal proIAPP, respectively. The immunoblots are representative of five independent experiments from five human islet preparations. Densitometric data are expressed as means \pm SEM. *vs corresponding molecular form in freshly isolated islets (day 0), #vs corresponding molecular form in non-treated cultured islets ($p < 0.05$, two-way ANOVA)

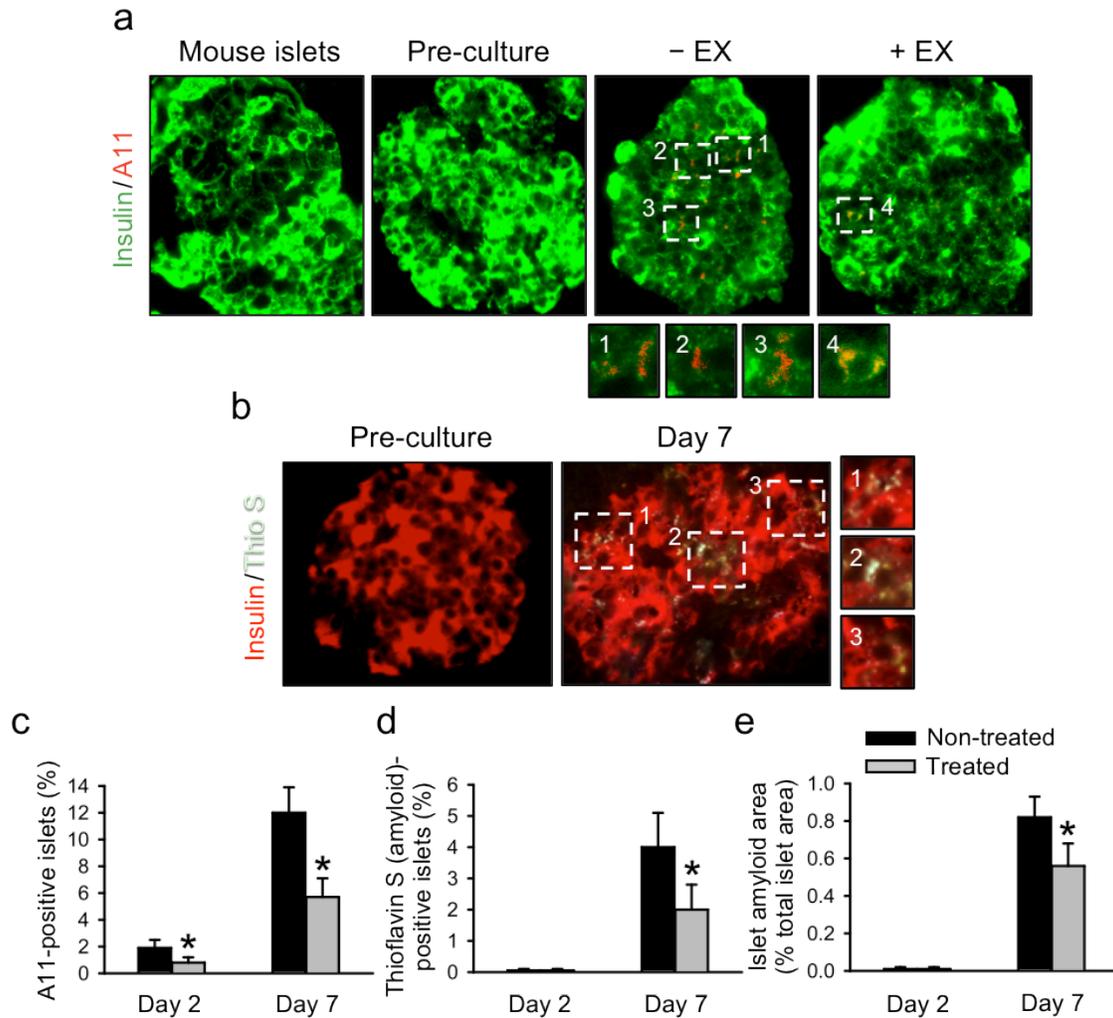


Figure 6-3. Enhanced proIAPP processing in the exenatide-treated human islets is associated with reduced islet amyloid formation during culture.

(a) Formation of hIAPP oligomers was detected in freshly isolated and 7-day cultured exenatide-treated (+EX; 10 nmol/l) and non-treated (-EX) human islets by immunolabelling for insulin and A11. Paraffin-embedded sections from 7-day cultured wild-type mouse islets that do not express hIAPP were used as control. (b) Amyloid formation was detected in freshly isolated human islets and following 7-day culture by immunolabelling for insulin and thioflavin S staining. The white squares denote regions enlarged in each image. The proportion of (c) A11 (hIAPP oligomer)-positive or (d) thioflavin S (amyloid)-positive human islets to total number of islets in each condition; and (e) Islet amyloid area / total islet area. The proportion of hIAPP oligomer or amyloid-positive islets is expressed as the ratio of islets containing hIAPP oligomer or thioflavin S-positive areas to total number of islets in each condition (50 islets). Islet amyloid area is reported as the percentage of thioflavin S-positive area to total islet area in each islet in about 50 islets per condition. Results are expressed as means \pm SEM of ten independent studies from ten human islet preparations (four for oligomer studies). *vs corresponding non-treated group ($p < 0.05$, Student's *t*-test)

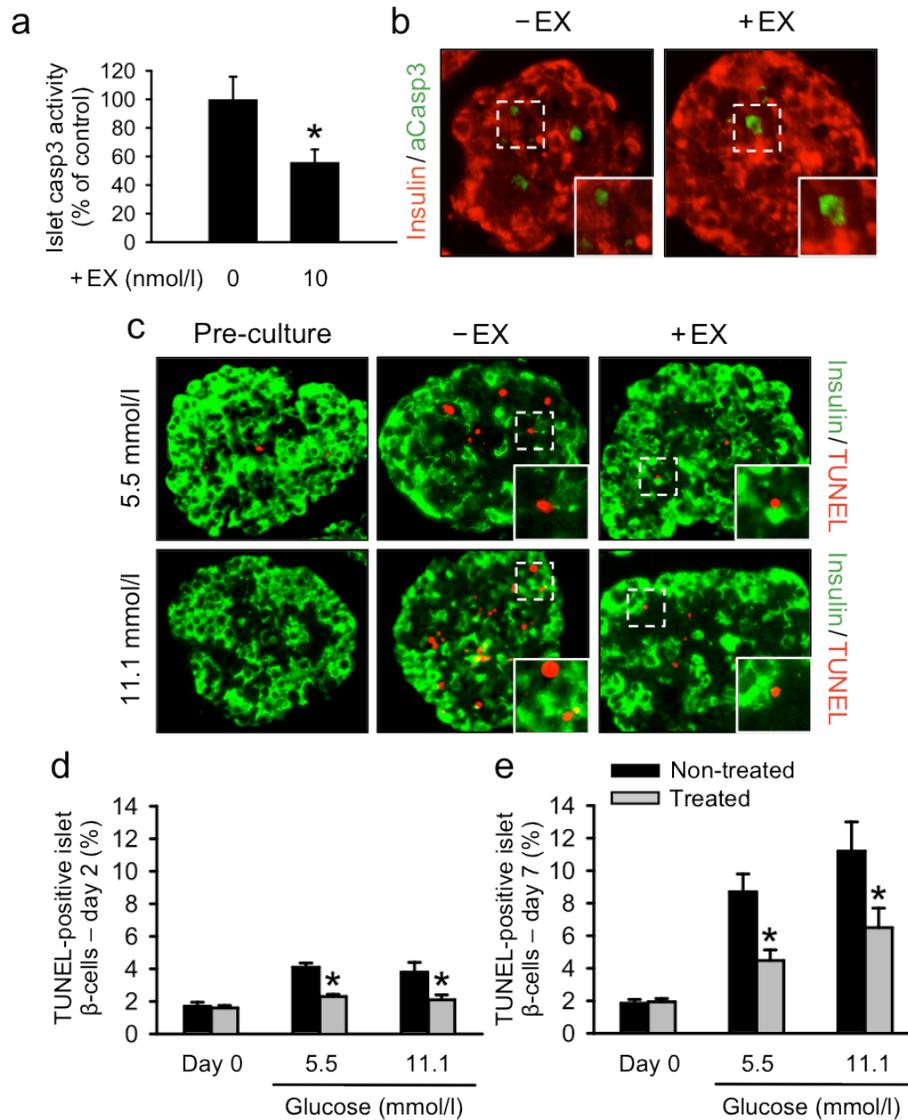


Figure 6-4. Reduced amyloid formation in exenatide-treated human islets during culture is associated with decreased β -cell apoptosis.

Human islets were cultured in CMRL (5.5 or 11.1 mmol/l glucose) with or without exenatide (10 nmol/l) for 2 or 7 days. **(a)** The cellular levels of active (cleaved) caspase-3 were quantified in the exenatide-treated and non-treated islet lysates using ELISA. Paraffin-embedded sections from 7-day cultured exenatide-treated and non-treated islets were immunolabelled for **(b)** insulin and cleaved caspase-3 (aCasp3) or **(c)** insulin and TUNEL. **(d, e)** The proportion of apoptotic β -cells was quantified in each condition. The white squares denote regions enlarged in each image. The caspase-3 activity is expressed as percentage of non-treated (control) islets taken as 100%. The proportion of apoptotic β -cells was calculated by manual counting of double insulin and TUNEL-positive cells in each islet in a total of 50 islets per condition for each human islet preparation. Results are expressed as means \pm SEM of ten independent studies (five for caspase-3 ELISA studies) from ten human islet preparations performed in duplicate. *vs corresponding non-treated group ($p < 0.05$, Student's *t*-test)

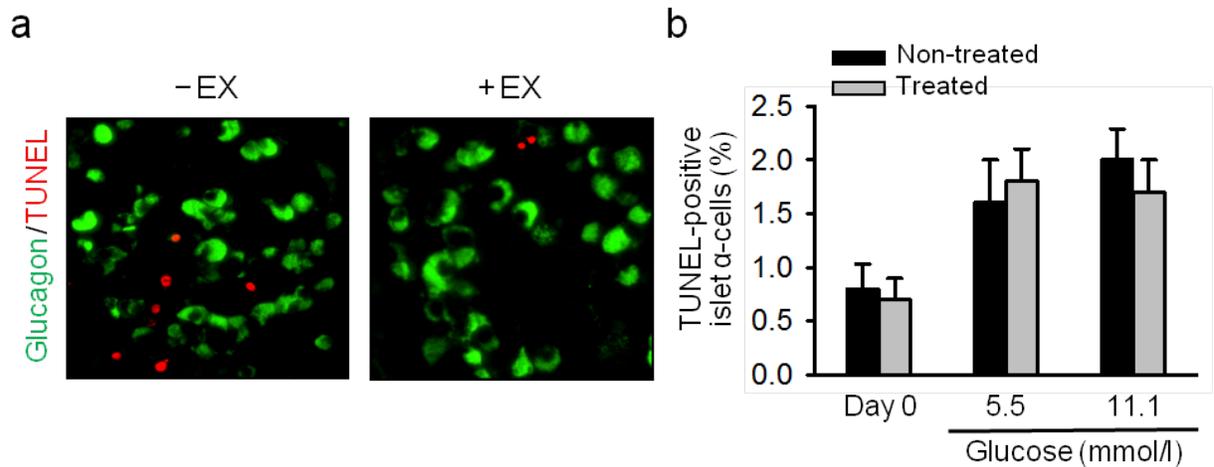


Figure 6-5. Treatment with exenatide has no effect on α -cell apoptosis in human islets during culture.

Human islets were cultured in CMRL (5.5 or 11.1 mmol/l glucose) with or without exenatide (10 nmol/l) 7 days. **(a)** Paraffin-embedded sections from cultured exenatide-treated and non-treated islets were immunolabelled for glucagon and TUNEL. **(b)** The proportion of apoptotic α -cells was quantified in each condition. The proportion of apoptotic α -cells was calculated by manual counting of double glucagon and TUNEL-positive cells in each islet in a total of 50 islets per condition for each human islet preparation. Results are expressed as means \pm SEM of five independent studies from five human islet preparations performed in duplicate. *vs corresponding non-treated group ($p < 0.05$, Student's t -test)

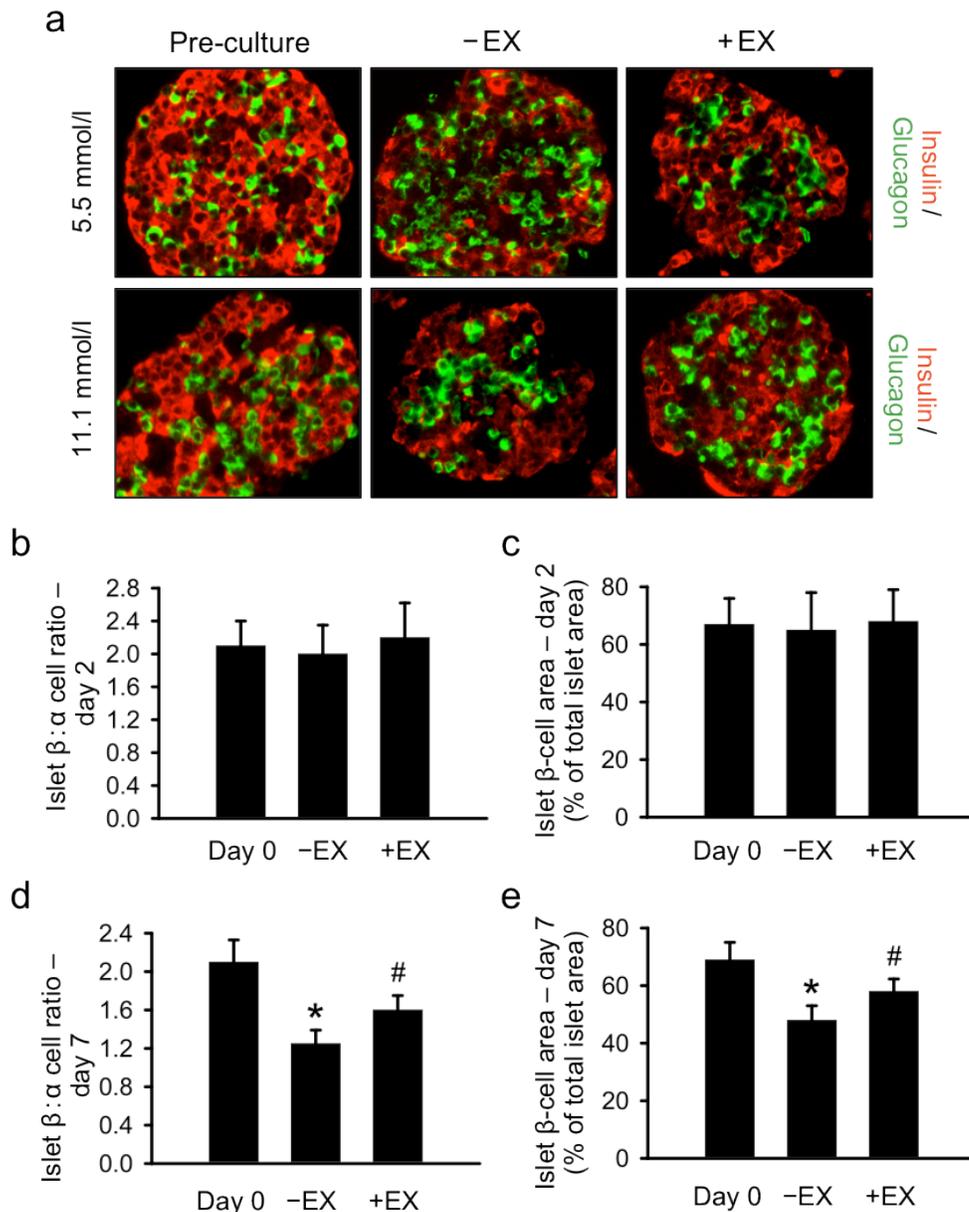


Figure 6-6. Reduced β -cell apoptosis in the exenatide-treated human islets is associated with increased islet β - to α -cell ratio and β -cell area to total islet area during culture.

(a) Paraffin embedded sections from freshly isolated or 7-day cultured human islets in 5.5 or 11.1 mmol/l glucose with or without exenatide (10 nmol/l) were double immunolabelled for insulin and glucagon. Quantification of (b, c) islet β - to α -cell ratio and (d, e) β -cell area to total islet area after 2 or 7 days culture with or without exenatide. Data are expressed as the ratio of insulin / glucagon-positive cells and insulin-positive islet area to total islet area, respectively, in each islet in a total of about 50 islets per condition. Results are expressed as means \pm SEM of ten independent studies from ten human islet preparations performed in duplicate. *vs day 0 group, #vs non-treated cultured group ($p < 0.05$, one-way ANOVA)

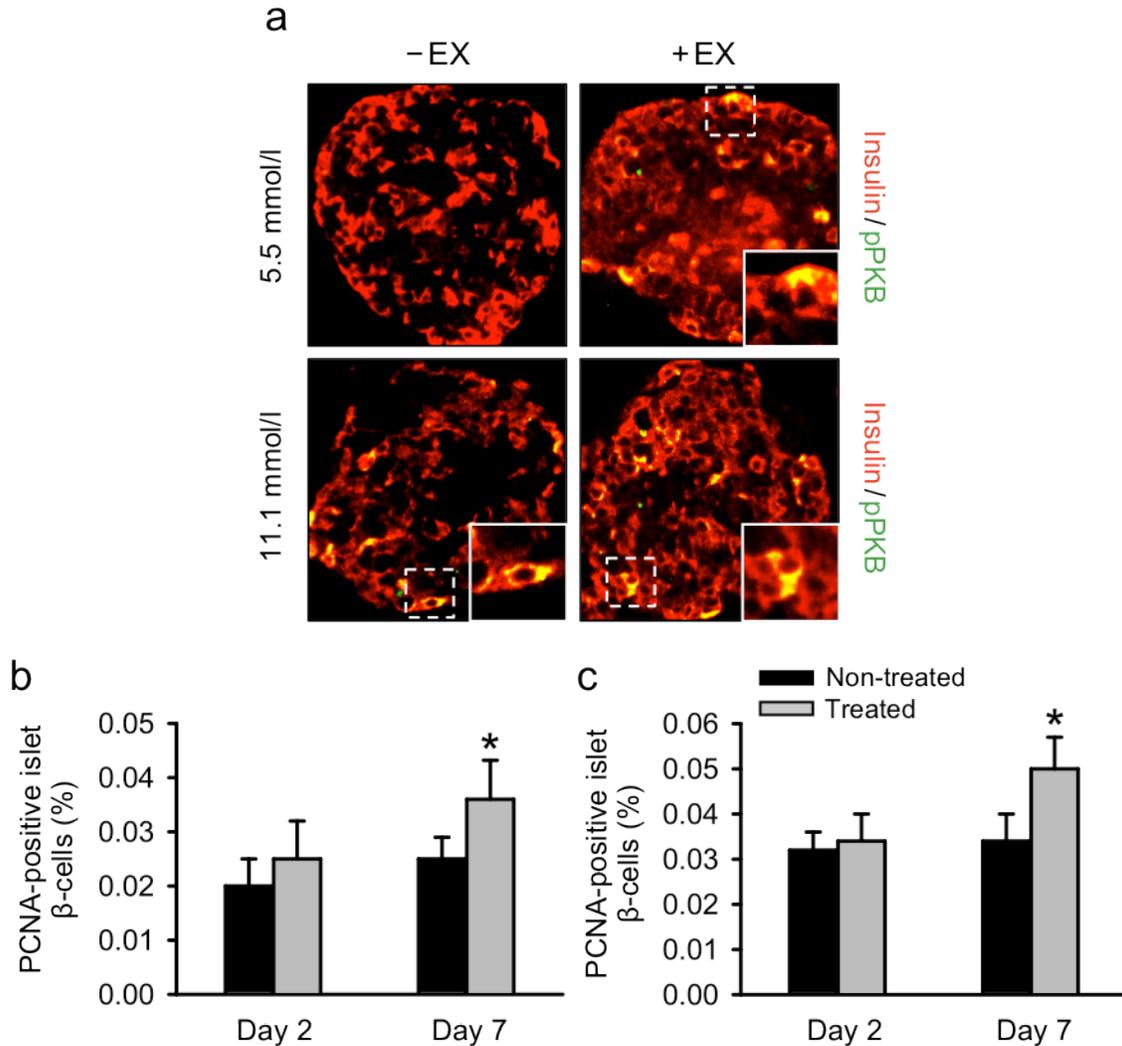


Figure 6-8. Increased islet β -cell PKB activation and proliferation in the exenatide-treated cultured human islets.

(a) Double immunolabelling of cultured human islets for insulin and phospho (active)-PKB following 7-day culture in 5.5 or 11.1 mmol/l glucose with or without exenatide (10 nmol/l). The white squares denote regions enlarged in each image. The proportion of PCNA-positive (proliferative) β -cells was assessed by quantitative insulin and PCNA immunolabelling of paraffin-embedded sections from exenatide-treated (10 nmol/l) and non-treated human islets following 2- or 7-day culture in (b) 5.5 mmol/l or (c) 11.1 mmol/l glucose. The proportion of PCNA-positive β -cells was quantified by manual counting of double PCNA and insulin positive-cells and total number of insulin-positive cells in each islet in about 50 islets per condition. Results are expressed as means \pm SEM of eight independent studies from eight human islet preparations performed in duplicate. *vs corresponding non-treated group ($p < 0.05$, two-way ANOVA)

Chapter 7: Conclusions and future directions

7.1 Conclusions

Islet amyloid formation is an important non-immune factor contributing to progressive β -cell dysfunction and death in type 2 diabetes and islet grafts in type 1 diabetes. Despite intensive investigations in the past, the molecular mechanism(s) underlying amyloid-induced β -cell death in primary islets are still unclear. The goal of this thesis project was to identify the molecular mechanism(s) by which toxic hIAPP aggregates induce β -cell death. Figure 7-1 presents a schematic overview of the proposed mechanism by which hIAPP aggregates induce the Fas-mediated apoptotic pathway leading to β -cell death. It also includes therapeutic interventions that can target the important mediators in this process to protect β -cells from amyloid toxicity. Based on our findings, we conclude that the Fas apoptotic pathway plays an important role in mediating the cytotoxic effects of islet amyloid. Thus, modulating the key steps in the Fas-mediated apoptotic pathway may provide a potential therapeutic approach to preserve β -cells in conditions associated with islet amyloid formation such as in type 2 diabetes, cultured and transplanted islets.

Studies in Chapter 3 demonstrated that aggregation of exogenously applied and endogenously produced hIAPP in islets upregulate Fas, which is not normally present in β -cells, resulting in increased β -cell death. Conversely, treatment with the amyloid inhibitor Congo red prevented formation of hIAPP aggregates and reduced hIAPP-induced β -cell Fas upregulation and apoptosis. Similarly, our results showed that adenovirus siRNA-mediated suppression of hIAPP expression protects β -cells from hIAPP-induced Fas upregulation and apoptosis in cultured human and hIAPP-expressing mouse islets. Importantly, β -cell specific deletion of Fas significantly reduced amyloid-induced β -cell death in hIAPP-expressing mouse islets as shown by lower levels of active caspase-3 and apoptotic β -cells, resulting in higher islet β - to α -cell ratio compared to hIAPP transgenic mouse islets expressing Fas. Taken together, these findings revealed a key mechanism by which endogenously produced hIAPP aggregates can cause β -cell death in conditions associated with islet amyloid formation. Moreover, we demonstrated that

blocking amyloid-induced Fas expression may be used as a feasible approach to prevent β -cell death induced by toxic hIAPP aggregates.

We next examined if Fas upregulation induced by hIAPP aggregates in cultured human or hIAPP-expressing mouse islet β -cells results in Fas and FasL interaction and activation of downstream caspase cascade and subsequent cell death. In Chapter 4, we showed that caspase-8, the key upstream enzyme in the Fas-mediated apoptotic pathway, promotes the Fas cell death signalling activated by the hIAPP aggregates. Treatment with synthetic hIAPP aggregates induced Fas upregulation, caspase-8 activation and apoptosis in human islet β -cells. However, inhibition of caspase-8 prevented hIAPP-induced Fas-mediated β -cell caspase-3 activation and apoptosis. Moreover, our data showed that blocking the Fas and FasL assembly using a Fas antagonist provides an effective strategy to prevent activation of the Fas apoptotic pathway despite hIAPP-induced upregulation of Fas in β -cells. Also, in line with our findings in Chapter 3, the amyloid inhibitor Congo red reduced caspase-8 activation in human islet β -cells by preventing hIAPP-induced β -cell Fas upregulation.

We further demonstrated that deletion of caspase-8 protected islet β -cells from the cytotoxic effects of endogenously secreted hIAPP aggregates. In hIAPP-expressing mouse islets with caspase-8 deletion, amyloid formation and β -cell Fas expression were comparable with those in islets expressing hIAPP and caspase-8, but β -cell apoptosis was markedly lower resulting in higher islet β -cell mass. Taken together, our studies provided evidence to show that amyloid-induced Fas upregulation promotes the Fas-FasL interaction and caspase-8 activation in islet β -cells and that inhibition or deletion of caspase-8 disrupts the caspase cascade triggered by the Fas activation induced by hIAPP aggregates thereby reducing amyloid β -cell toxicity.

In both Chapters 3 and 4, amyloid-induced β -cell Fas upregulation was associated with increased islet IL-1 β levels. Therefore, we investigated the potential role of IL-1 β in mediating hIAPP-induced Fas upregulation in human and mouse islet β -cells during culture. In Chapter 5, we showed that IL-1 β has a dual function in islet amyloid-induced β -cell toxicity. First, we demonstrated that IL-1 β plays an important role in mediating the β -cell toxic effects of hIAPP aggregates. As shown in Figure 7-2, our results indicated that hIAPP exerts its apoptotic effects

through stimulating islet IL-1 β production, which induces upregulation of Fas in β -cells and eventually causes β -cell apoptosis. Conversely, treatment with IL-1R blocker anakinra reduced amyloid-induced Fas upregulation and β -cell death. Second, we showed that exposure to IL-1 β causes islet β -cell dysfunction, resulting in impaired processing of proIAPP thereby potentiating islet amyloid formation and its β -cell toxicity. Accordingly, inhibition of IL-1 β signalling restored impaired proIAPP processing leading to reduced amyloid formation and β -cell death (Figure 7-3). Therefore, we concluded that targeting IL-1 β may provide an effective strategy to prevent amyloid formation, Fas upregulation and β -cell apoptosis in type 2 diabetes and human islet grafts.

In Chapter 6, we expanded our search for finding new strategies to protect islets from amyloid formation and toxicity. GLP-1R agonists have been previously shown to exert their protective effects on β -cells against different apoptotic stimuli and improve β -cell function. Thus, we tested the therapeutic potential of the GLP-1R agonist exenatide to prevent amyloid formation and amyloid-induced β -cell death in human islets. Treatment with exenatide reduced caspase-3 activation and β -cell apoptosis in parallel with improved β -cell survival and function manifested as increased islet β -cell proliferation, PKB activation, insulin content and response to elevated glucose in amyloid-forming human islets during culture. Moreover, enhanced β -cell function by exenatide treatment restored impaired proIAPP processing and reduced islet amyloid formation in cultured human islets. Taken together, our studies showed that GLP-1R agonists such as exenatide may provide an effective strategy to protect islet β -cells from amyloid toxicity both directly by reducing amyloid-induced β -cell death and indirectly by decreasing amyloid formation through improving β -cell function (Figure 7-3).

Finally, findings from our studies have potential clinical applications in human islet transplantation process. One of the advantages of clinical islet transplantation as a β -cell replacement therapy for treatment of type 1 diabetes is that modulation of islets is possible prior to transplantation into the recipient, thus bypassing the potential side effects of *in vivo* treatment or manipulation of islets. For example, prevention of islet amyloid formation and its toxicity at early stages of hIAPP aggregation during pre-transplant islet culture may provide an efficient method to enhance both survival and function of human islet grafts. In line with this notion, our

results suggest that addition of amyloid, Fas, caspase-8 inhibitors to islet culture medium may prevent amyloid-induced activation of the Fas apoptotic pathway in β -cells, thereby reducing islet β -cell loss. Moreover, reducing amyloid formation during culture by treatment with IL-1R antagonists or GLP-1R agonists may significantly enhance long-term survival and function of human islet grafts (Figure 7-3). Importantly, pharmacotherapies used in this thesis are already on market, therefore our proposed strategies can be easily translated into clinical setting.

Overall, studies in this thesis provided proof-of-principle of the novel concept that islet amyloid-induced β -cell death is mediated, at least partially, through upregulation of Fas via IL-1 β and activation of the Fas apoptotic pathway initiated by caspase-8. Prevention of this process may therefore protect islet β -cells from amyloid toxicity leading to the better islet survival and function during pre-transplant culture *in vitro* as well as in type 2 diabetes and islet grafts *in vivo*.

7.2 Future directions

7.2.1 The role of the Fas Type II pathway in mediating hIAPP-induced β -cell death

In this thesis, we explored the role of Fas apoptotic pathway in mediating hIAPP-induced β -cell death. Our studies demonstrated that exogenously applied and endogenously secreted hIAPP aggregates induce upregulation of Fas in β -cells via islet IL-1 β release, which leads to Fas-FasL interaction, caspase-8 and -3 activation, ultimately resulting in β -cell apoptosis. As shown in Figure 1-5, this sequence of events leading to β -cell apoptosis is the characteristics of the major (Type I) pathway in the Fas cell death receptor signalling. However, the role of the alternative (Type II) pathway, which involves a cross-talk mechanism to activate the mitochondrial-mediated (intrinsic) apoptotic pathway, following hIAPP-induced Fas activation remains to be elucidated. Previous studies have demonstrated that the mitochondrial-mediated apoptotic pathway contributes to β -cell apoptosis in the presence of cytotoxic stimuli such as pro-inflammatory cytokines (576,577). Similarly, intra- and/or extra-cellular hIAPP aggregates may activate mitochondrial-mediated apoptotic pathway in β -cells through IL-1 β -dependent pathway(s). Thus, further mechanistic studies may reveal the potential role of the Fas Type II pathway and possible contribution of the cross-link between extrinsic and intrinsic apoptotic pathways in hIAPP-induced β -cell death.

7.2.2 FLIP as a target for preventing amyloid-induced Fas apoptotic pathway

Our results indicate that following activation of the Fas apoptotic pathway by hIAPP aggregates, a caspase cascade initiated by caspase-8 is a crucial step in mediating β -cell apoptosis. cFLIP is the key regulator of caspase-8. High levels of cFLIP can compete with caspase-8 and block the Fas-mediated apoptotic pathway whereas low levels of cFLIP promote the Fas-mediated pathway towards apoptosis (437). Thus, the cellular levels of cFLIP may play an important role in modulating amyloid-induced Fas-mediated β -cell apoptosis.

cFLIP appears to be a promising therapeutic target for preventing amyloid-induced β -cell apoptosis for two reasons. First, cFLIP can attenuate the apoptotic signalling at early stages by blocking the activation of the caspase cascade. Second, a previous study has shown that in addition to its anti-apoptotic role, elevated cFLIP levels can enhance β -cell proliferation by switching the Fas signalling from apoptosis to proliferation thereby preserve β -cell mass during islet culture (503). Modulation of cFLIP may therefore provide a potential strategy to prevent β -cell death evoked by toxic hIAPP aggregates.

In Chapter 4, we proposed that hIAPP aggregates may reduce expression of cFLIP thereby promote the Fas signalling towards apoptosis. Although we did not detect any significant difference between the cFLIP protein levels in islet lysates from hIAPP-expressing transgenic mice and wild-type mice following culture, this may be due to the limitations of the Western blot method that we used as discussed before. Future studies employing methods such as immunolabelling detection of β -cell FLIP expression and overexpression/inhibition of β -cell cFLIP in *ex vivo* experimental models of islet amyloid formation may therefore help to identify the role of cFLIP in hIAPP-induced Fas-mediated β -cell death.

7.2.3 Understanding the network of apoptotic signalling pathways activated by hIAPP

We demonstrated that the Fas apoptotic pathway plays an important role in mediating amyloid-induced β -cell death through activation of caspases. Previous studies have reported that caspases interact with other apoptotic signalling pathways (439,441,490). For example, increased hIAPP-induced caspase-8 activity has been associated with the activation of JNK and p38

pathways (439,441,490). Moreover, amyloid-induced JNK activation in cultured hIAPP-expressing transgenic mouse islets was linked to increased expression of caspase-3 activation (490). Similarly, our data in Chapter 6 showed that endogenously formed hIAPP aggregates induce JNK activation in cultured human islets, which correlated with increased hIAPP-induced caspase-3 activation and β -cell apoptosis. However, the regulation of amyloid-induced β -cell apoptosis through these interactions is not well understood. Therefore, another potential avenue of research from studies presented in this thesis is to investigate the inter-relationship between these apoptotic pathways, which may reveal better therapeutic targets to protect β -cells from islet amyloid toxicity.

7.2.4 The effects of blocking IL-1 β signalling on amyloid formation and toxicity *in vivo*

One of the key findings of studies in this thesis was that amyloid-induced Fas upregulation is mediated by IL-1 β secreted from islets. Moreover, we showed that prevention of IL-1 β signalling in an *ex vivo* model of islet amyloid formation provides a potential approach to reduce amyloid formation and amyloid-induced β -cell death. Therefore, next step of our research will be to validate these findings in an *in vivo* setting in an animal model of amyloid-associated type 2 diabetes. Studies on hIAPP-expressing mice treated with an IL-1R antagonist will allow us to test if blocking IL-1 β action *in vivo* can reduce amyloid formation and protect islet β -cells from amyloid toxicity thereby improve hyperglycemia and delay the onset of diabetes.

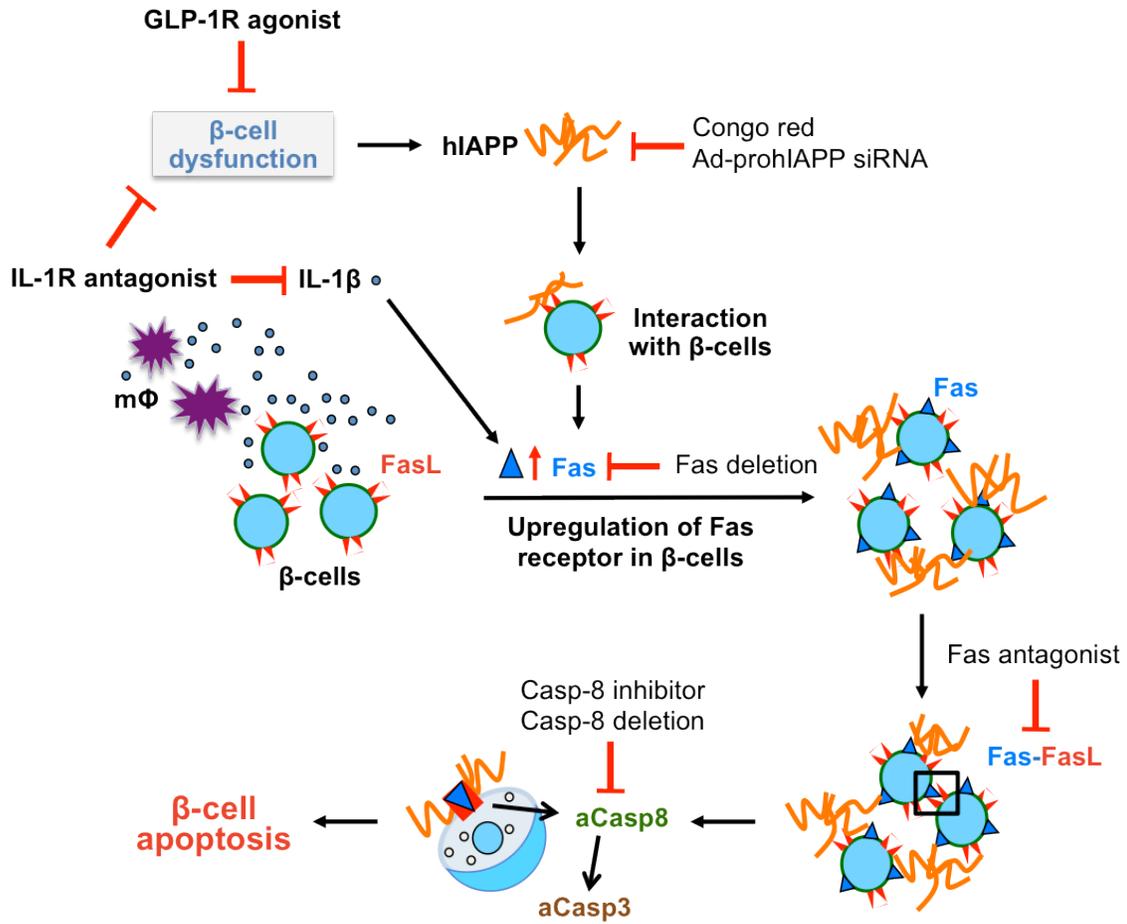


Figure 7-1. Overview of the proposed model for amyloid-induced β -cell apoptosis mediated by the Fas apoptotic pathway and therapeutic interventions to prevent this process.

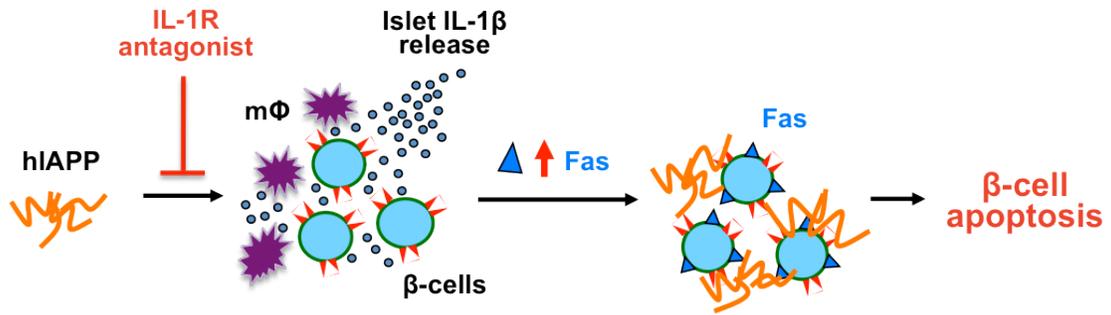


Figure 7-2. hIAPP aggregates induce islet IL-1 β release leading to β -cell Fas upregulation and apoptosis, which can be prevented by blocking the IL-1 β signalling using IL-1R antagonist.

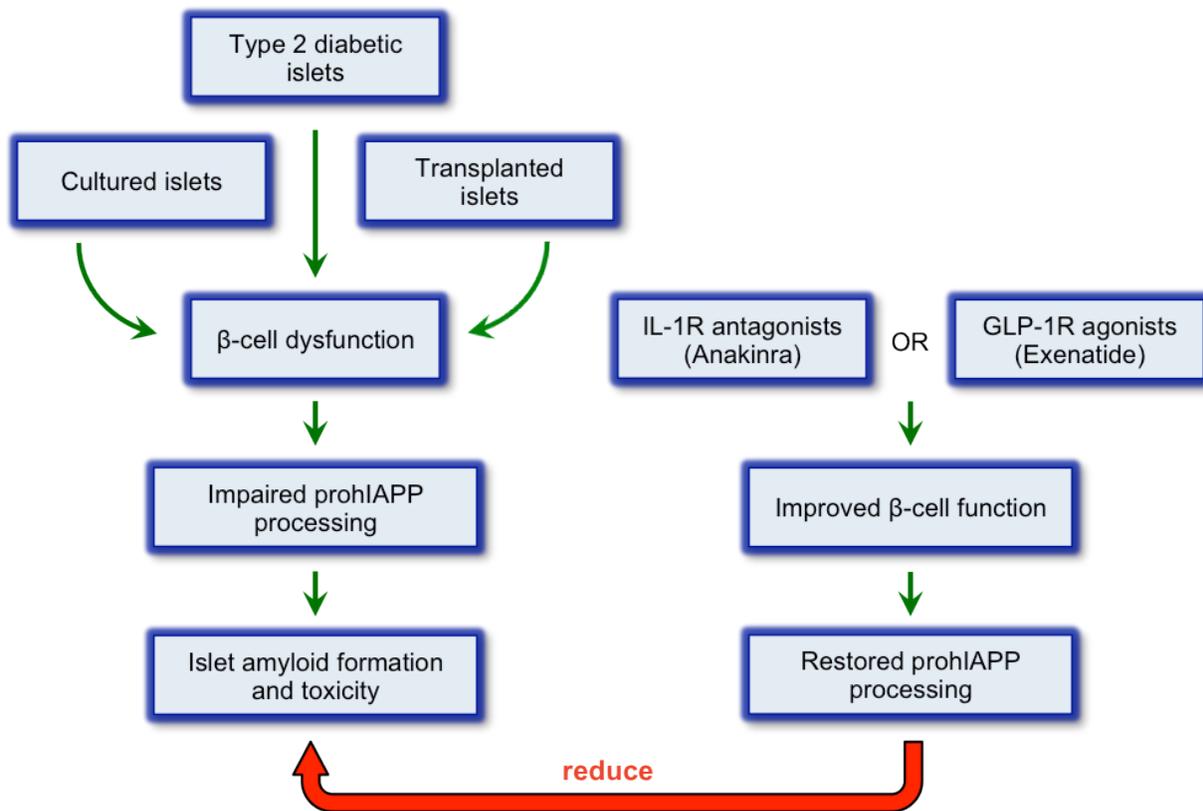


Figure 7-3. Proposed mechanism of how enhanced β -cell function by either IL-1R antagonists or GLP-1R agonists may prevent islet amyloid formation and toxicity.

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