The mechanisms of amyloid toxicity in pancreatic islets – Identifying the protective signaling pathways in islet α-cells

by

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Abstract

Type 2 diabetes (T2D) is characterized by peripheral insulin resistance, and progressive β-cell loss and dysfunction, associated with islet inflammation, leading to hyperglycemia. One of the factors that contribute to β-cell failure in T2D is islet amyloid deposition. Islet amyloid is formed by aggregation of a β-cell hormone, islet amyloid polypeptide (IAPP). Islet amyloid also forms during culture and following transplantation into patients with type 1 diabetes (T1D), potentially leading to islet graft failure. Amyloid triggers islet inflammation by promoting the production of proinflammatory cytokines, mainly interleukin-1 beta (IL-1β). Binding of IL-1β to interleukin-1 receptor-1 (IL-1R1), leads to activation of IL-1β signaling, upregulation of the Fas cell death receptor and activation of the Fas-mediated apoptotic pathway in β-cells. Interestingly, islet α-cells are much less susceptible than β-cells to the cytotoxic effects of amyloid and other β-cell apoptotic factors such as elevated glucose. The hypothesis of this MSc project is that lower expression of IL-1R1 and/or Fas receptor in islet α-cells than β-cells may protect α-cells from amyloid-induced apoptosis, leading to better survival of α-cells during amyloid formation in T2D.

Transformed mouse αTC1-6 and rat INS-1 β-cells were cultured without (control) or with exogenous hIAPP or IL-1β at two glucose concentrations (11.1 or 25 mmol/l). Fas and IL-1R1 were detected by double-immunolabelling for insulin or glucagon and each IL-1R1 or Fas. IL-1R1 immunoreactivity was detected in both αTC1-6 cells and INS-1 β-cells under basal condition and following treatment with hIAPP or IL-1β. IL-1R1 immunoreactivity was markedly higher in INS-1 β-cells than αTC1-6 cells in all conditions. Also, the number of Fas-positive cells was markedly lower in αTC1-6 cells than INS-1 β-cells following treatment with hIAPP or IL-1β. Finally, αTC1-6 cells had lower rate of apoptosis than INS-1 β-cells following treatment with hIAPP or IL-1β.

In summary, these data suggest that the higher susceptibility of β-cells to hIAPP aggregates or elevated IL-1β might be, at least partially, due to the higher expression of IL-1R1 and Fas in β-cells than α-cells.

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Dedication

To my father, Md. Mobarak Hossain for all the support and encouragement throughout my life. My father has greatly motivated me to pursue my dreams of higher education in a worldwide reputed university. He has always inspired me to focus on my studies and be confident. I had the courage to come abroad as an international student to pursue a master's degree from my father.

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CHAPTER 1: INTRODUCTION

1.1 Diabetes

1.1.1 Definition and prevalence of diabetes

Diabetes mellitus is the most common metabolic disorder which is characterized by hyperglycemia. Chronic hyperglycemia in diabetes is mainly caused by impaired insulin secretion, action or both [1]. Individuals with diabetes are often at risk of developing other health complications such as renal and cardiovascular diseases [2]. The global prevalence of diabetes has been increasing significantly over the past decade [3]. According to the International Diabetes Federation (IDF), approximately 151 million people were diagnosed with diabetes worldwide in 2000 [4]. It is predicted that the number of people living with diabetes will increase to about 642 million worldwide by 2040 [3]. The estimated number of individuals with pre-diabetes worldwide is about 316 million. According to Diabetes Canada, the number of Canadians diagnosed with diabetes was approximately 2.4 million (about 7% of total population) in 2009, and was increased to approximately 3.7 million in 2019 [5].

1.1.2 The role of endocrine pancreas in glucose homeostasis in health and diabetes

The majority of pancreas (approximately 98%) is composed of exocrine tissue. The endocrine portion of the pancreas forms only 1-2% of the total pancreatic mass and is called the "islets of Langerhans" [6]. The exocrine and endocrine tissue functions are devoted mainly to digestion and glucose homeostasis, respectively. Approximately, one million islets of Langerhans are present in a healthy pancreas, weighing about one gram [6]. The five major islet cell types include alpha (α), beta (β), delta (δ), pancreatic polypeptide (PP) and epsilon (ε) cells [7]. The endocrine cells in the islets, regardless of cell type, are closely associated with blood vessels [8].

The most abundant islet cell type is the islet β-cell that produces and secretes insulin which is the only glucose-lowering hormone in the body [7]. Insulin is a 51-amino acid peptide hormone that plays a crucial role in nutrient uptake into cells [9]. Under physiological conditions, hyperglycemia stimulates insulin release from islet β-cells, thereby promoting glucose uptake into the skeletal muscle, liver, and adipose tissues. Also, incretin hormones produced in the gut, including glucagon-like peptide-1 (GLP-1) and gastric inhibitory factor (GIP-1), promote

stimulated insulin secretion from β-cells [10]. Approximately 28-75% and 80% of islet cells are β-cells in humans and mice, respectively. Typically, the islet β/α-cell ratio is higher in mice as compared to humans [11]. In addition to β/α-cell ratio, there are some structural differences between mouse and human islets. In human islets, β-cells and α-cells are distributed mainly in a random manner, while in mice, β-cells mainly form the core of islets and are surrounded by non-β-cells in the periphery of islets [8].

Islet α-cells secrete glucagon which is a 29-amino acid peptide and is the major hormone that increases blood glucose [7]. Islet α-cells make up approximately 10-65% and 10-20% of the total islet cells in human and mice, respectively [11]. Glucagon is the main counter-regulatory hormone to insulin and its major role is to prevent hypoglycemia. In healthy individuals, glucagon promotes insulin secretion from β-cells by binding to glucagon receptors (G protein coupled receptors), expressed in β-cells. On the contrary, insulin inhibits glucagon secretion upon binding to the insulin receptor, which is abundant in α-cells [12]. Also, glucagon secretion has been shown to be inhibited by other β-cell components, such as zinc ions and gamma-aminobutyric acid (GABA) that activates GABA receptor [12]. However, in individuals with diabetes, α-cell sensitivity to elevated glucose can be impaired, leading to inappropriately elevated glucagon secretion [13]. Fasting hyperglucagonemia in people with diabetes contributes to increased rate of hepatic glucose output due to loss of hyperglycemia- and insulin-induced suppression of glucagon release from α-cells [14]. It is reported that α-cell hyperplasia also contributes to hyperglucagonemia, leading to hyperglycemia in diabetes [13].

The δ-cells form approximately 10% of the total number of islet cells in human and secrete somatostatin which acts as a negative regulator of both glucagon and insulin release [15]. The PP cells which secrete pancreatic polypeptide form only 1-2% of islet cell population are the least studied islet cell type [16]. In hyperglycemic conditions, PP cells have been shown to inhibit glucagon release in mice[12]. Lastly, ε-cells form about 1% of adult islet cells and produce the hormone ghrelin that induces hunger which is thought to play a role in growth hormone release from the pituitary gland [7]. In physiological conditions, islet cells contribute to the regulation of hormone release from other islet cells and work together to maintain normal blood glucose homeostasis **(Fig. 1)** [9].

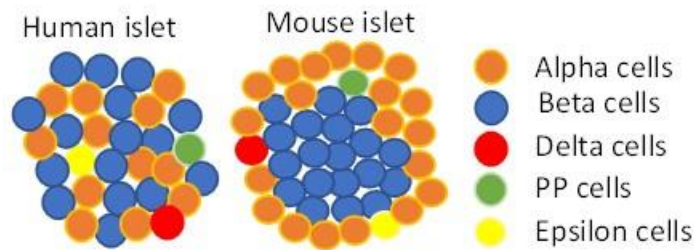


Figure 1. The differences between distribution of islet cells in human and mouse islets. Islet α-cells and β-cells are randomly distributed in human islets whereas in mouse islets β-cells are mainly located in the core of islets surrounded by α-cells.

1.1.3 The major types of diabetes

Diabetes has two major types including type 1 and type 2 diabetes.

Type 1 diabetes

Type 1 diabetes (T1D), also known as juvenile- or childhood onset diabetes accounts for 5-10% of all people with diabetes. The occurrence of T1D has been dramatically increasing among different geographic populations and racial groups over the past decades [17]. T1D is characterized by absolute insulin deficiency due to autoimmune-mediated destruction of β-cells, leading to hyperglycemia [18]. The "Search for diabetes in youth study" (SEARCH) in the United States reported a correlation between the incidence of T1D and age as well as sex [17]. Although T1D is most common before the age of 25, people from all age groups can develop T1D. Moreover, it is reported that T1D disproportionately affects males than females **(Table 1.1)** [19].

Type 2 Diabetes

Type 2 diabetes (T2D) is characterized by progressive β-cell failure and peripheral insulin resistance, leading to relative insulin deficiency and hyperglycemia. T2D has a higher prevalence in older individuals. There is a positive correlation between the age and the number of people diagnosed with T2D, possibly due to the age-associated decline in the body's ability to produce insulin and a decline in insulin sensitivity [20]. The Public Health Agency of Canada (PHAC) reported an increase in the prevalence of T2D in individuals aged ≥40 [21]. Importantly, T2D is also

progressively increasing in children and adolescents under the age of 18 [22]. Approximately 90% of all individuals with diabetes are diagnosed with T2D. **(Table 1.1)** [21].

Table 1.1: The differences between T1D and T2D (adapted from Diabetes Canada guidelines, 2018) [23]

Subject	T1D	T2D
Pathological characteristics	Autoantibodies, absolute insulin deficiency	Insulin resistance, relative insulin deficiency
Prevalence among types	5-10 %	95%
Causes	Autoimmune, genetic, and environmental factors	Genetic and environmental factors
Age of onset	Mainly in childhood to adulthood	Mainly in adulthood
Nutritional status	Usually underweight and undernourished	Obesity is common
β-cell autoantibodies	Present	Absent
Therapy	Insulin, islet transplantation	Lifestyle modification, oral medications, and insulin
Tendency of ketosis	Common	Rare

1.1.3.1. The pathogenesis of diabetes

1.1.3.1.1 The pathogenesis of type 1 diabetes

Progressive loss of islet β-cells is a hallmark of T1D. About 50-90% of pancreatic islets in individuals with T1D have no or very low number of β-cells, whereas the number of other endocrine cells remain mostly unchanged [24]. The size of pancreas is also significantly lower (20-50% reduction) in people with T1D than non-diabetic individuals, as reported by autopsy and radiographic studies [25]. During the progression of T1D, lack of inhibitory effects of insulin caused by significant β-cell loss, leads to changes in the function of other islet cell types, in particular α-cells [26]. Interestingly, studies performed in both human and rodent models of T1D have shown that there is a progressive decline in β-cell mass whereas, islet α-cell population remains unchanged or is increased in size (hypertrophy) and number (hyperplasia) [27]. Thus, autoimmune-mediated destruction of β-cells in T1D results in expansion of islet α-cells, leading to hyperglucagonemia, which plays an important role in hyperglycemia during diabetes [26].

The presence of islet autoantibodies is a characteristic of most patients with T1D [28]. Therefore, assessment of plasma islet-specific antibodies helps to monitor the progression of T1D in patients because the antibodies are typically detectable before the appearance of clinical signs and symptoms of diabetes such as hyperglycemia [28]. The autoantibodies associated with T1D that recognize autoantigens such as insulin (IAA), glutamic acid decarboxylase 65 (GADA), IA-2 antigen (IA-2A) and zinc transporter 8 (ZnT8A) are detected in approximately 70-80% of newly diagnosed patients with T1D [29]. Studies suggest that the presence of multiple autoantibodies is usually associated with increased risk of development of T1D. The progression of T1D also depends on the persistency of specific autoantibodies and their affinity for antigens [27].

Islet inflammation (insulitis) is an important pathological feature in T1D, which plays a key role in the progressive loss of β-cells [30]. Autoimmune mediated β-cell destruction by cytotoxic T lymphocytes occurs mainly by CD8+ T cells which triggers islet inflammation in T1D [30]. CD8+ T cells are present predominantly in the islets of patients with T1D during inflammation [31]. Neutrophils are also present in low numbers either in islets with insulitis or in the exocrine tissue of patients with T1D [32]. Moreover, during islet inflammation, endocrine cells produce and release various cytokines and chemokines that trigger activated immune cells to attack β-cells [30]. β-cell death is mainly mediated via apoptosis in both human patients and animal models with T1D [33].

1.1.3.1.2 The pathogenesis of type 2 diabetes

Insulin resistance

Insulin resistance is characterized by impaired insulin function in the peripheral tissues, leading to reduced glucose uptake in the skeletal muscle, liver, and adipose tissue. Insulin resistance in the peripheral tissues develops several years prior to the onset of hyperglycemia. The progressive insulin resistance in T2D is associated with reduced glycogen synthesis, increased gluconeogenesis in the liver and increased release of free fatty acids (FFAs) to the blood circulation due to lipolysis in the adipocytes [34].

Both genetic and environmental factors contribute to the progression of insulin resistance in patients with T2D. For example, a genetic mutation of the insulin receptor causes insulin resistance by changing its affinity for binding to insulin molecule. The environmental factors that promote insulin resistance in patients with T2D include, but are not limited to, sedentary lifestyle,

age, and poor diet. People living with obesity generally have varying degrees of insulin resistance but not all of them develop diabetes [35]. It also appears that genetic susceptibility plays a role in this process. As insulin resistance progresses, β-cells release more insulin to maintain normoglycemia [36].

Loss of β-cell mass and function

During early stages of insulin resistance in T2D, pancreatic β-cells expand and produce more insulin as a compensatory response to prevent hyperglycemia (hyperinsulinemic state) [36]. T2D occurs when β-cells fail to compensate for insulin resistance to maintain blood glucose within normal range [36]. The elevated insulin secretion due to progressive insulin resistance can gradually result in β-cell exhaustion and dysfunction, which further worsens hyperglycemia, eventually leading to reduced insulin levels in patients at later stages of the disease [37].

It has been reported that about 40% and 65% of total β-cell mass is lost in lean and obese patients with T2D, respectively, when compared to sex-, age- and body mass index (BMI) matched non-diabetic individuals [33]. Glucose is the main factor that stimulates insulin secretion from β-cells. Long-term exposure to high glucose concentrations is toxic to β-cells and promotes β-cell dysfunction and apoptosis, which is known as glucotoxicity [38, 39]. Also, studies have shown that while β-cell proliferation is promoted when β-cells are exposed to gradually increasing glucose concentration for a short period of time [40], long-term exposure to elevated glucose supresses β-cell proliferation in human islets [39]. Similar to patients with T1D, loss of insulin and hyperglycemia-induced suppression of glucagon release from α-cells contributes to hyperglycemia in patients with T2D [14]. Moreover, stimulatory effects of arginine on glucagon secretion are higher in human subjects with T2D as compared to non-diabetic individuals, suggesting that α-cell dysfunction may co-exist with β-cell dysfunction and contribute to hyperglycemia [41].

Lipotoxicity is another phenomenon that contributes to β-cell dysfunction and apoptosis when β-cells are exposed to elevated lipid levels during T2D. The activity of lipases such as adipose triglyceride lipase, hormone sensitive lipase and monoglyceride lipase, the key enzymes in lipolysis process, are increased in fat cells during insulin deficiency, leading to the breakdown of fat into free fatty acids (FFAs) and glycerol [42]. Treatment with exogenous palmitate, the most

abundant FFA in human plasma, has been reported to cause apoptosis in rat β-cells [43]. In obesity-associated T2D, combined effects of glucose and lipid toxicity causes a more rapid loss of β-cell mass and function and this phenomenon is known as glucolipotoxicity [44]. Similarly, elevated glucose and FFAs cause glucolipotoxicity in T1D [45]. While the effects of FFAs on the survival and function of β-cells are extensively studied, current knowledge on the effects of FFAs on islet α-cells is limited. It has been reported that exposure of α-cell lines to FFAs during culture, stimulates glucagon secretion in a time- and dose-dependant manner but inhibits α-cell proliferation [46].

β-cell dedifferentiation into other islet cell types also contributes to loss of β-cell mass in T2D. Transcription factors such as pancreatic and duodenal homeobox 1 (Pdx1), v-maf musculoaponeurotic fibrosarcoma oncogene homolog A (MafA), neurogenic differentiation 1 (NeuroD1), NK6 homeobox1 (Nkx6.1), and forkhead box protein O1 (FoxO1) maintains β-cell identity [47]. Conditions associated with T2D such as hyperglycemia, hyperlipidemia, oxidative stress and endoplasmic reticulum (ER) stress, promote β-cell dedifferentiation into other endocrine cell types [48].

Islet inflammation and β-cell dysfunction in type 2 diabetes

Islet inflammation is a known pathological feature of T1D, but growing evidence suggests that it also plays an important role in the pathogenesis and progression of T2D, although the underlying mechanism is different. Islet inflammation in T2D is associated with activation of islet resident macrophages [49–51]. In healthy individuals, islets contain a small number of macrophages that contribute to the maintenance of islet homeostasis [52] but a previous autopsy study has reported that the number of islet resident macrophages detected by CD-68 (a human macrophage marker) was increased in the islets from patients with T2D as compared to non-diabetic controls [51]. M1-like macrophages are responsible for secreting cytokines and chemokines and contribute to islet inflammation and islet cell dysfunction in T2D, whereas M2-like macrophages play a key role in maintaining the physiological functions of islet cells such as proliferation [53].

The elevated levels of cytokines, mainly interleukin-1 beta (IL-1β) is proposed to be a key mediator in the process of islet inflammation in T2D [53, 54]. Although IL-1β plays a major role in β-cell dysfunction, the production of other cytokines and chemokines such as interleukin-16 (IL-

16), tumor necrosis factor (TNF), the CXC chemokine (CXCL1) and chemokine (C-C motif) ligand 3 (CCL-3) are also induced by hyperglycemia and hyperlipidemia and are thought to contribute to islet inflammation [55]. Hyperlipidemia is reported as one of the major stimuli that activates toll-like receptor 4 (TLR4) signaling in macrophages. For example, palmitate induces chemokines such as C-C chemokine 2 (CCL2) and CXCL1 in β-cells by TLR4 activation [55]. The proinflammatory cytokines produced by macrophages promote the expression of inflammatory chemokines in β-cells in T2D **(Fig. 2)**.

Figure 2: The role of islet inflammation in the pathogenesis of T2D. Several factors, mainly hyperglycemia and hyperlipidemia, contribute to islet inflammation in T2D. Islet inflammation promotes the production of IL-1β and other inflammatory cytokines and chemokines in T2D, leading to progressive loss of β-cell mass and function.

IL-1β is initially synthesized as an immature protein called proIL-1β (31 kDa). ProIL-1β activates the inflammasome, which in turn activate caspase-1 that is required to cleave proIL-1β to mature IL-1β (17.5 kDa) [56]. The factors associated with T2D, such as elevated plasma glucose, FFAs, leptin and amyloid formation, contribute to IL-1β release from islet β-cells [57, 58]. The resident macrophages (mainly) and β-cells appear to be the two sources of IL-1β in islets during inflammation, leading to β-cell death and dysfunction in T2D [50].

A recent study performed on paraffin-embedded human islets has reported IL-1β immunoreactivity in islet α-cells from donors with both T1D and T2D [59]. Moreover, our lab has previously shown that treatment of α-cell and β-cell lines with IL-1β is associated with a higher rate of apoptosis in β-cells compared to α-cells [60], suggesting that α-cells may have cellular mechanism(s) that protects them against IL-1β; however, this has not yet been investigated.

1.1.3.2 The role of genetic and environmental factors in the development of diabetes

1.1.3.2.1. Genetic and environmental factors in type 1 diabetes

Both genetic and environmental factors contribute to the development of T1D. Some of human leukocyte antigen (HLA) and non-HLA regions are found to be indicative of higher risk for development of T1D. Polymorphisms within HLA regions are thought to indicate the inherited risk of developing T1D [29]. Additionally, first-degree relatives of patients with T1D were reported to have smaller pancreatic volume as assessed by MRI, suggesting that they might have a higher risk of developing T1D as compared to healthy individuals with no family history of T1D [61]. Environmental factors such as exposure to viruses and microbes during the early life are also thought to play a role in increasing the risk of developing T1D by promoting autoimmunity. For example, it has been reported that infants infected with rubella virus are at higher risk of developing T1D [62]. The other environmental factors that are known to contribute to the development of T1D include, but are not limited to, dietary factors such as feeding cow milk in infants less than 3-4 months old, vitamin D deficiency and exposure to environmental toxins [62].

1.1.3.2.2 Genetic and environmental factors in type 2 diabetes

Individuals with a family history of T2D are at a higher risk of developing T2D. For example, risk of developing T2D in monozygotic twins ranges from 55% to almost 100% [63, 64]. It has also been reported that offspring of parents with diabetes are at 3.5 to 6-fold higher risk of developing T2D, compared to offspring from non-diabetic parents [65]. Moreover, race and ethnicity are risk factors for development of T2D. For example, studies have reported that in the United States, minorities (black people, Hispanic people, native Americans, Asians, and Pacific Islanders) are at higher risk of T2D [66]. The prevalence of T2D is higher in non-Hispanic black population compared to white population of the same age [67]. Morbidity is higher in black women aged 55 or older with T2D. Also, reports show that one in every two Pima Indians have T2D [66].

The major environmental factors that potentiate the progression of T2D include diet, lifestyle, and economic status. Studies have revealed that high energy density foods are associated with higher risk of T2D compared to low energy density foods such as plant-based food [35]. Sugar sweetened beverages are thought to increase the risk of diabetes [68]. Also, the Mediterranean diet has been reported to reduce the risk of diabetes without causing weight loss.

Moreover, three or more cups of coffee and green tea intake per day are thought to reduce the risk of T2D whereas, alcohol intake and cigarette smoking are shown to increase the risk of T2D in a dose dependant manner [35, 69].

Low physical activity increases the risk of T2D as physical exercise improves insulin sensitivity and glycemic control [70]. Other environmental factors such as noise pollution (>10 dB) and fine particulate matter (≥10µg/m3) may promote development of T2D over years [35]. Also, getting sufficient sleep (7-8 hours per day) has been reported to reduce the risk of T2D [35].Besides, higher economical status may lower the risk of T2D if it is accompanied by appropriate diet and healthy lifestyle [35]. Finally, infection with hepatitis C virus has been reported to correlate with insulin resistance, and development of both T1D and T2D and cardiovascular diseases [71].

1.1.3.3 Signs, symptoms, and complications of diabetes (T1D and T2D)

Individuals may not show signs and symptoms during the pre-diabetes period where the blood glucose levels are higher than normal but lower than the diagnostic value of diabetes (see **Table 1.2**). The symptoms of diabetes become more prominent as the disease progresses. The classic symptoms of both T1D and T2D include polyuria, polydipsia and polyphagia [72]. In the insulin sensitive tissues such as skeletal muscle and adipose tissue, glucose cannot enter the cells in the absence of insulin. Therefore, during diabetes, varying degrees of insulin deficiency leads to a corresponding increase in blood glucose concentration. During polyuria, the body experiences loss of water which in turn leads to polydipsia. Reduced glucose uptake into the insulin sensitive tissues makes them nutrition deficient, leading to polyphagia [73]. Also weight loss commonly occurs in patients with T1D [74].

Hyperlipidemia including increased blood triglyceride and cholesterol levels in diabetes increases the risk of complications of diabetes such as stroke and myocardial infarction [74]. Diabetic ketoacidosis occurs due to excessive breakdown of FFAs into ketone bodies in the liver which may lead to coma or death if not treated [75]. Additionally, patients with diabetes are at higher risk of development of infections such as pneumonia, urinary tract, skin and soft tissue infections as well as post-surgery infections as high blood glucose level impairs immune responses to infections and wound healing process [75].

The chronic complications of diabetes affect several organ systems in the patients which potentially lead to morbidity and mortality. Chronic complications associated with diabetes can be grouped into vascular and non-vascular complications. The microvascular complications include retinopathy, neuropathy, and nephropathy. The macrovascular complications of diabetes include coronary artery, peripheral vascular, and cerebrovascular disease. Non-vascular complications include, but are not limited to, problems such as gastroparesis and skin disorders [75].

1.1.3.4 Diagnosis of diabetes (T1D and T2D)

Diagnosis of diabetes is made based on measurement of fasting or random blood glucose or by an oral glucose tolerance test (OGTT) [18]. Other tests that are commonly used for assessment of diabetes include glycated hemoglobin, urinary glucose and acetone breath tests [17]. To confirm the diagnosis of diabetes, two blood test measurements performed on two different days are required. It is recommended to repeat the same test as well as an alternative test for confirmation [76].

The diagnostic tests most commonly used in Canada for the diagnosis of diabetes are fasting and random plasma glucose test, two-hour oral glucose tolerance test and the glycated hemoglobin test [23]. The fasting blood glucose level above normal value (6.1-6.9 mmol/l) is considered to be indicative of pre-diabetes in individuals[17]. In addition to fasting and random blood glucose test, the two-hour glucose tolerance test is performed by giving 75 grams of oral glucose to the overnight fasted individual. In non-diabetic individuals, glucose levels typically fall back to normal range within 2 hours. However, in individuals with diabetes, glucose levels remain higher than normal after 2 hours. The diagnostic criteria for patients with pre-diabetes or diabetes are summarized in **Table 1.2** (adapted from Diabetes Canada report 2018). The glycated hemoglobin test (HbA1C) is usually performed in patients diagnosed with diabetes to monitor their glycemic control over time[1]. HbA1C test shows the average glucose level of patients over a period of 3 months prior to measurement [17] and it does not require any special preparation such as fasting [72].

Higher blood glucose levels lead to filtration of more glucose into the renal tubules and if the amount of glucose is over reabsorption threshold of kidney, the excess glucose will appear in

urine. Thus, non-diabetic individuals typically lose undetectable amounts of glucose in their urine whereas a person with diabetes loses various amounts of glucose in the urine based on the severity of diabetes. Finally, acetone breath test is used in patients with diabetes to detect ketoacidosis as the acetoacetic acid in the blood is converted to acetone and is released with exhaled air [17].

Table 1.2: Current diagnostic criteria for pre-diabetes and diabetes[23]

Condition	Blood glucose	Criteria
Normal	(4.4-5) mmol/l	Fasting plasma glucose No caloric intake for at least 8 hours
	≤ 7.7 mmol/l	Plasma glucose level two hours after 75 g oral glucose challenge
	≤ 5.7 %	HbA1C in adults
Pre-diabetes	(6.1-6.9) mmol/l	Fasting plasma glucose No caloric intake for at least 8 hours
	(7.7-11) mmol/l	Plasma glucose level two hours after 75 g oral glucose challenge
	(6-6.4) %	HbA1C in adults
Diabetes	≥ 7 mmol/l	Fasting plasma glucose No caloric intake for at least 8 hours
	≥ 11.1 mmol/l	Random at any time of the day without considering intervals since last meal
	≥ 11.1 mmol/l	Plasma glucose level two hours after 75 g oral glucose challenge
	≥ 6.5%	HbA1C in adults

1.1.3.5 Treatment strategies for diabetes

1.1.3.5.1 Treatment strategies for type 1 diabetes

Insulin therapy

Lifetime exogenous insulin therapy is used for the treatment for T1D patients. Discovery of insulin in 1922 was a significant accomplishment as it changed T1D from a fatal condition to a manageable disease [77]. Although subcutaneous insulin injections are the most common insulin delivery method, use of insulin pumps are also popular. However, insulin therapy comes with certain risks such as severe hypoglycemia caused by insulin overdose, which can be life

threatening. The patients with T1D have various degrees of hypoglycemia unawareness which contributes to up to 10% of mortality in those patients [77].

Whole pancreas or islet transplantation

Whole pancreas transplantation was first performed in 1966 as an approach to provide patients with diabetes an endogenous insulin source [78]. However, despite its success rate, whole pancreas transplantation has risk of complications such as graft rejection and need for lifetime immunosuppression therapy [79]. Islet transplantation, on the other hand, provides a less invasive surgical strategy in which islets are infused via the portal vein to provide an endogenous source of insulin for patients [80]. The collaborative Islet Transplant Registry has reported that morbidity risk is 20-fold lower in islet transplantation as compared to whole pancreas transplantation [77]. Also, reports from the Edmonton group indicate that most patients achieve insulin independence and maintain normal islet graft function after islet transplantation, but they eventually become insulin dependant after a few years and require another transplantation [81].

1.1.3.5.2 Treatment strategies for type 2 diabetes

Lifestyle modifications

In individuals recently diagnosed with T2D, normoglycemia typically can be achieved and maintained by a healthy lifestyle, proper diet and regular physical exercise [82, 83]. Obesity is often accompanied by insulin resistance and is one of the major risk factors for T2D. Individuals with obesity have higher levels of non-esterified fatty acids, glycerol and proinflammatory cytokines released from adipose tissue, leading to development of insulin resistance [84]. Regular physical exercise improves insulin sensitivity and glycemic control in people with T2D [85]. Additionally, psychological interventions to help reduce mental health problems such as stress and anxiety have been shown to help achieve better glycemic control in patients with T2D [35].

Pharmacological treatment approaches

The medications that are widely used for treatment of T2D include biguanides, thiazolidinediones, sulfonylureas, dipeptidyl peptidase 4 (DPP4) inhibitors, and glucagon-like peptide-1 (GLP-1) analogues. Among them, the most prescribed monotherapy in T2D is biguanides. Metformin is the most commonly prescribed drug for treatment of T2D. Metformin

improves insulin sensitivity and enhances glucose uptake in the peripheral tissues [86]. Metformin is also prescribed for treatment of obesity as it reduces weight and decreases serum triglycerides and LDL cholesterol levels [87]. Thiazolidinediones such as rosiglitazone also improve insulin sensitivity [88, 89] but is not commonly used anymore due to the risk of cardiovascular events such as myocardial infarction and heart failure [90] and potential weight gain in the patients [91]. Sulfonylureas such as glimepiride act by promoting insulin secretion from pancreatic β-cells by targeting the ATP-sensitive potassium channels in β-cells. However, sulfonylureas may cause β-cell exhaustion by increasing insulin release, leading to further β-cell dysfunction and failure. Additionally, sulfonylureas can cause hypoglycemia and weight gain in patients [92]. DPP4 inhibitors such as sitagliptin, inhibit DPP4, an enzyme that metabolizes incretin hormones such as GLP-1 and gastric inhibitory peptide (GIP), two hormones that are necessary for glucose homeostasis. DPP4 inhibitors have a lower risk of hypoglycemia and do not modify weight of patients [93]. GLP-1 analogues increase glucose stimulated insulin release from β-cells and decrease glucagon secretion from α-cells. GLP-1 also promotes delayed gastric emptying and weight loss [94]. Combination therapies such as biguanides and thiazolidinediones, biguanides and sulfonylureas, or biguanides and DD4 inhibitors are used based on the severity of diabetes and when single therapy is no longer sufficient to maintain normoglycemia in patients with T2D.

1.1.4 The role of α-cells in diabetes associated hyperglycemia

Insulin plays an important role in controlling α-cell function. Therefore, relative or absolute insulin deficiency in patients with diabetes leads to α-cell dysfunction [95]. Diabetes-associated impairment in the endocrine activity of pancreas is therefore not limited to β-cell dysfunction in insulin synthesis and release. Increased fasting plasma glucagon and impaired α-cell suppression during postprandial state also contribute to hyperglycemia during diabetes [9]. Reduced α-cell suppression of glucagon release in diabetes is likely due to the resistance of α-cells to the inhibitory action of insulin and/or impaired glucose sensing in α-cells [14]. It is reported that α-cell dysfunction is present in parallel with β-cell dysfunction in diabetes [9, 96]. The imbalance in plasma concentrations of these two pancreatic hormones is more prominent after a mixed meal such as carbohydrate, fat, and protein [97]. The paradoxical glucagon response characterized

by elevated glucagon secretion after a carbohydrate meal is observed in diabetes, further contributing to post-prandial hyperglucagonemia [9].

Findings suggest that β/α cell ratio decreases after onset of T2D due to decreased islet β-cell mass while α-cell mass remains unchanged or increased, resulting in hyperglycemia [26], which corresponds to duration of diabetes [95] **(Fig. 3)**. A study in human islets obtained from pancreatectomies of patients with normal glucose tolerance, impaired glucose tolerance, newly diagnosed T2D and longstanding T2D also revealed that activation of caspase-3 was very low in islet α-cells in all four groups whereas significantly high number of active caspase-3 positive β-cells were seen in the islets from longstanding T2D group [98]. Another study in European subjects with T2D showed that on average, the β/α ratio in individuals with T2D is 1.7 times lower than non-diabetic individuals which is mostly due to progressive β-cell loss whereas α-cell mass remains constant [95].

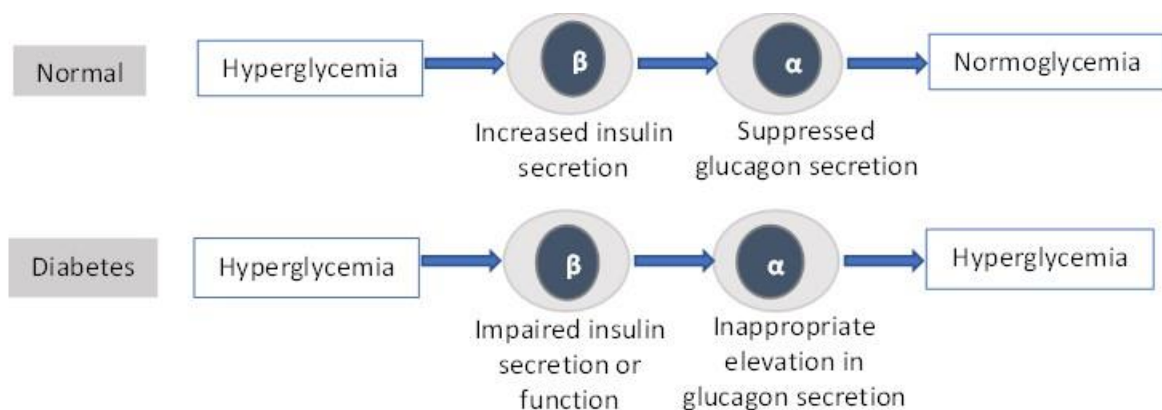


Figure 3: The response of islet α-cells and β-cells to hyperglycemia in health and diabetes. In healthy individuals, hyperglycemia is associated with β-cell production and secretion of insulin, leading to suppression of glucagon release from α-cells. In diabetes, hyperglycemia persists as insulin secretion and/or action is impaired and glucagon secretion is elevated inappropriately.

1.2. Islet amyloid and islet amyloid polypeptide

1.2.1 History and discovery

Islet amyloid was discovered in 1901 by two independent scientists Opie and Weichselbaum and was initially named "islet hyalinization" because of its similarity to the hyaline substances [99–101]. Although islet hyalinization was not unique to diabetes, it was correlated with diabetes during early discovery and this association was reported to be more profound in elderly individuals. Later, amyloid was also observed in a small number of non-diabetic individuals but its occurrence and intensity were much higher in patients with T2D [102]. Initially, insulin and proinsulin, which are the major secretory components of β-cells, were thought to constitute islet amyloid deposits as amyloid is also of polypeptide hormonal origin. However, further studies showed that amyloid deposits were mainly formed by aggregation of islet amyloid polypeptide (IAPP, amylin). In 1986, the Westermark group extracted IAPP fibrils from amyloid-rich insulinoma by using concentrated formic acid and named it "insulinoma amyloid peptide" [103]. Later, the Cooper group also purified IAPP from amyloid-rich pancreas of patients with T2D and named it "diabetes associated peptide" [104]. Finally, it was named "islet amyloid polypeptide (IAPP)" [105] or "amylin" [106].

1.2.2 Human islet amyloid polypeptide

Human islet amyloid polypeptide (hIAPP) is a β-cell hormone that is the major component of islet amyloid deposits [107]. Human IAPP is co-produced, co-stored, and co-secreted with insulin (molar ratio ~1:100; IAPP: insulin) from the β-cells in response to β-cell secretagogues, such as elevated glucose [97, 98]. Glucose-stimulated insulin and IAPP release from β-cells occur simultaneously. Human IAPP belongs to the calcitonin family which also contains calcitonin, calcitonin gene related peptide (CGRP), adrenomedullin and intermedin. Human IAPP shares approximately 50% identity with CGRP [105]. The physiological role(s) of IAPP are still not completely understood. The proposed physiological roles for IAPP are discussed in the later section. IAPP molecule has a random coil structure [110]. IAPP forms at least one transient amphipathic helix in the NH_2-terminal region [111] and the terminal part of NH_2 region forms a rigid ring structure [112]. The helical part of IAPP structure is presumed to be important in the receptor

binding and also plays a key role in the pathological transformation of amyloid fibrils [113]. IAPP forms a disulfide bond similar to other peptides in the calcitonin family [110].

Studies have demonstrated that small hIAPP aggregates named oligomers are the major toxic form of amyloid, which causes β-cell death whereas large mature hIAPP fibrils are thought to be relatively inert [114]. In addition to T2D, hIAPP aggregation occurs in human islets during culture [115] and following transplantation into human patients and mouse models of T1D [116]. Amyloid deposits formed by aggregation of hIAPP in the islets of individuals with T2D are typically found extracellularly adjacent to islet β-cells [113]. However, small hIAPP aggregates are also found intracellularly in the membrane encircled compartments. Therefore, it appears that initial hIAPP aggregates form inside β-cells followed by their rapid propagation, leading to membrane disruption, cell rupture and extracellular amyloid formation [113].

1.2.3 Localization and clearance of islet amyloid polypeptide

hIAPP is localized in the halo region of the β-cell secretory granules whereas hexameric insulin is mainly located in the core of β-cell granules [117]. Islet α-cells do not express IAPP but very low levels of IAPP has been reported in rodent δ-cells, sensory neurons, and human and rodent gastrointestinal tract [118] . The plasma concentration of IAPP is very low as compared to insulin (about 1-2% of insulin) [113]. IAPP is mainly eliminated from the body in urine [119] . To-date, two enzymes are known to contribute to degradation of IAPP in the body, including insulin degrading enzyme (IDE) and neprilysin. IDE is a Zn^{2+}-metalloprotease present in the cell membrane, cytosol, mitochondria, endosomes and peroxisomes of various cells [120]. Incubation with an IDE inhibitor has been shown to inhibit degradation of IAPP and promote IAPP aggregation in insulinoma cells [121]. *In vitro* studies have shown that neprilysin, a type-2 zinc containing metalloprotease present in islet cells, also contributes to amyloid degradation [122].

1.2.4. The physiological roles of islet amyloid polypeptide

1.2.4.1 The effects of islet amyloid polypeptide on insulin and glucagon secretion

IAPP has inhibitory effects on insulin secretion [123] which might be due to promoting glycogenolysis and lactate formation [124]. *In vitro* studies in rat islets have shown that IAPP can suppress insulin secretion. Accordingly, IAPP knockout ($IAPP^{-/-}$) male mice have increased

glucose-stimulated insulin release compared to wild-type mice ($IAPP^{+/+}$) during intravenous and oral glucose tolerance test and develop T2D later in life [123]. Interestingly, studies on mouse models suggest that IAPP may have dual effects on insulin secretion including stimulation of basal insulin release and suppression of stimulated insulin secretion [123]. IAPP also appears to limit the degree of blood glucose clearance and inhibit glycogen synthesis in the isolated rat skeletal muscle [125]. Additionally, it has been reported that soluble IAPP may promote β-cell proliferation in isolated mouse islets in a glucose dependent manner by promoting β-cell proliferation at lower glucose levels and suppressing β-cell proliferation at elevated glucose levels [126].

The effects of IAPP on glucagon secretion are still not well understood. Some studies have indicated that IAPP secreted from β-cells along with insulin inhibits postprandial glucagon secretion [127]. While other studies have proposed that IAPP may have an indirect regulatory effect on glucagon. For example, it suppresses nutrient-induced glucagon release in diabetic rats [128]. Moreover, the inhibitory effects of IAPP on arginine-induced glucagon secretion were observed in rodents *in vivo* but not in isolated cultured islets [129].

1.2.4.2. The role of islet amyloid polypeptide in satiation

IAPP and its analogues are well known for their inhibitory effects on appetite in both human and experimental animal models [119, 120]. During post-meal conditions that blood glucose level is elevated, circulating IAPP concentration has been reported to rise from 3-5 pmol/l to 15-20 pmol/l in rats [132]. IAPP reduces appetite, delays gastric emptying and reduces gastric acid secretion [132]. Patients with T1D have pathologically fast gastric emptying which might be, at least partially, due to lack of IAPP production [133]. Pramlintide, an IAPP analogue, has been reported to slow down gastric emptying, reduce caloric intake and blunt postprandial hyperglycemia in both non-diabetic and human subjects with diabetes. Pramlintide has currently been approved for treatment of both T1D and T2D [134]. Exogenous administration of IAPP produces an immediate dose-dependent anorectic effect. An association between food consumption and rapid increase in plasma IAPP concentration has been reported in animals *in vivo* [135]. Lutz et al. showed that IAPP reduces appetite in rats by producing a satiation signal without taste modification and this effect of IAPP is similar to that observed with other satiating hormones such as cholecystokinin [121, 125].

1.2.4.3. The role of islet amyloid polypeptide in energy homeostasis

IAPP has been proposed to promote energy expenditure in rats. It has been shown that elevated IAPP levels in the brain are associated with lower weight gain [137]. Also, reduction of body fat was more profound in rats following long-term treatment with exogenous IAPP as compared to control group [137]. Moreover, IAPP treatment in rats enhanced leptin activity, a hormone that regulates energy balance by increasing energy expenditure, suppressing hunger, and reducing body weight. Co-administration of IAPP and leptin in rats resulted in reduced adipose tissue and body weight [138, 139]. Another recent study has revealed that IAPP enhances leptin signaling in rats by inducing the production of interleukin-6 (IL-6) in microglial cells [140]. IAPP in higher concentration failed to prevent weight gain in IL-6 knockout mice, further indicating that IL-6 plays a role in mediating the weight lowering effect of IAPP [140]. Additionally, higher basal plasma IAPP levels have been reported in high fat diet (HFD) fed obese rats compared to lean controls [141]. It is also reported that long-term peripheral and central administration of IAPP is associated with reduction of fat-induced body weight in rats [142]. Accordingly, treatment of rats with an IAPP antagonist resulted in increased weight gain [143]. However, the effects of IAPP on energy expenditure were observed by supra-physiological concentrations of IAPP infusion in rodents [144]. Therefore, these findings should be interpreted carefully, and further studies are required with endogenously produced IAPP to validate these findings in mice and humans.

1.2.4.4. The other roles of islet amyloid polypeptide

As a member of the calcitonin family, IAPP is possibly involved in the regulation of tissue calcification. Experimental results showed that IAPP has inhibitory effects on osteoclastic activity and bone resorption [134, 135]. It has also been reported that IAPP produces vasodilation in animal studies, although it is less effective compared to CGRP [147].

1.2.5. The receptors of islet amyloid polypeptide

The calcitonin family acts through seven transmembrane domain G-coupled receptors. However, to-date no specific receptor has been identified for IAPP [113]. Specific binding sites were identified at various parts of the brain, including nucleus accumbens and the area postrema outside the blood brain barrier, where it can bind to IAPP secreted from the islets. Binding sites for IAPP are also found in the renal cortex [148]. IAPP acts through a family of receptor activity

modifying proteins (RAMPS). Three different RAMPs were identified which are not receptors themselves and are single domain proteins [149]. RAMPs dimerize with calcitonin receptors and their interaction leads to the formation of complexes with the calcitonin receptors. As a result, high affinity IAPP receptors are formed [150]. The calcitonin receptors and RAMPs are expressed in mouse and rat brain.

1.2.6 Processing of ProIAPP to IAPP

Processing of IAPP precursor, proIAPP, to IAPP and proinsulin to insulin occurs in parallel inside the β-cell secretory granules. Unlike insulin and other calcitonin peptides that are expressed by genes located in chromosome 11 [151], hIAPP, a 37-amino acid neuroendocrine hormone, is expressed by the gene located in chromosome 12 [54, 140]. IAPP is initially synthesized as an 89-amino acid pre-proprotein, pre-proIAPP, which contains a 22-amino acid signal peptide that is cleaved at the N-terminus to form proIAPP [153]. Cleavage of the signal peptide and conversion of pre-proIAPP to proIAPP takes place in the endoplasmic reticulum (ER). ProIAPP translocates from the ER to Golgi and then to the secretory vesicles, where it is processed into mature IAPP.

ProIAPP and proinsulin are processed by the same enzymes including prohormone convertase enzyme 2 (PC2), prohormone convertase enzyme1/3 (PC1/3) and carboxypeptidase E (CPE) **(Fig 4)**. First, sixteen amino acids from the C-terminus of proIAPP are cleaved by PC1/3 at position Lys 50 and Arg 51. This step is followed by the cleavage of eleven amino acids from the N-terminus by PC2 at position Lys 10 Arg 11 [153]. In the absence of PC1/3, PC2 is able to cleave proIAPP at COOH-terminal region, and thus plays a major role in proIAPP processing. Then carboxypeptidase E (CPE) removes the basic residues from the COOH-terminal region of proIAPP. The terminal glycine at COOH-terminal end of proIAPP is subject to amidation [154]. Finally, mature IAPP is formed by addition of a disulfide bond between cysteine residues 2 and 7. Both amidation of the C-terminus and formation of disulfide bond are necessary for the biological activities of IAPP [141, 143–147]. **(Fig. 4)**

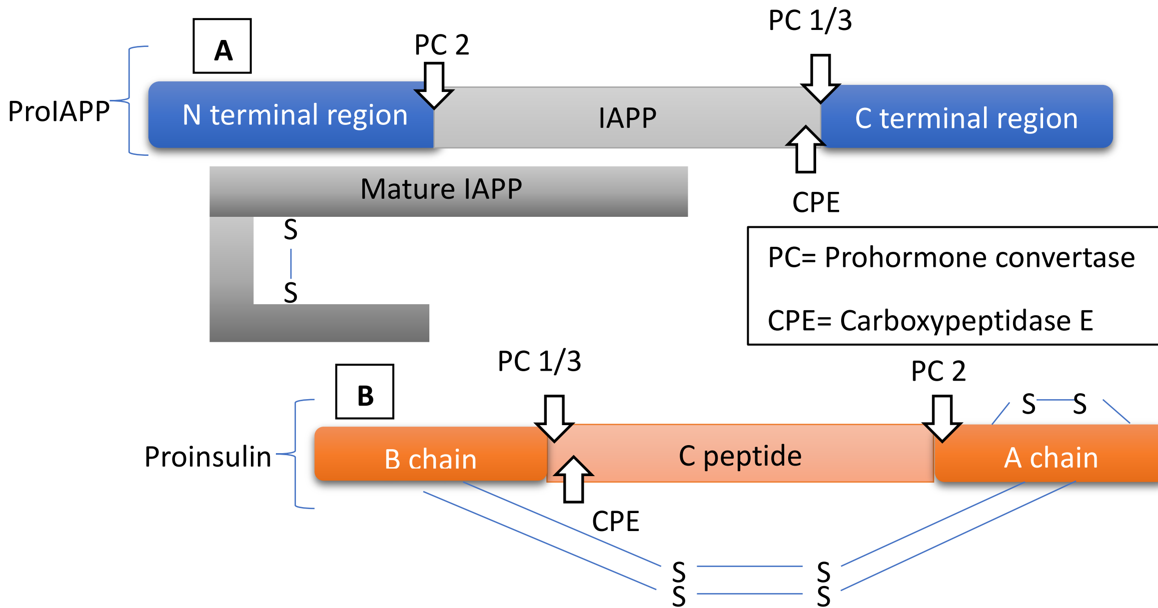


Figure 4: Processing of proIAPP and proinsulin. (A) ProIAPP is processed by prohormone convertases PC1/3 and PC2 at C-terminal and N-terminal region, respectively. The intermediate peptide is then processed by CPE for the formation of disulfide bond between cysteine 2 and 7. **(B)** Proinsulin is processed by the same enzymes as proIAPP. The B chain and A chain of insulin are cleaved by PC1/3 and PC2 at B chain-C peptide junction and A chain-C peptide junction, respectively. Two intramolecular disulfide bonds are formed between A and B chain and one within A chain [113].

1.2.7. Amyloid formation

The formation of amyloid occurs in three steps during the nucleation-dependent process. The first step is called the lag phase where the peptide is in monomeric form and aggregation is not initially supported, thus the step is called the rate-limiting step [148, 149]. During the lag phase, duration of nucleation of the monomeric peptide varies depending on the environmental conditions such as IAPP concentration, pH and temperature [113]. Studies have revealed that fibrils are not present during the lag phase, but small soluble oligomers can be present [160]. The second phase is the elongation phase which is also known as the growth phase. The growth phase is achieved once a critical nucleus of the monomeric peptides is formed that triggers aggregation. Fibril growth occurs and when it reaches a steady-state it is called plateau phase, also known as

saturation phase [160]. This process occurs spontaneously as long as the critical nuclei is present [150, 151]. **(Fig. 5)**

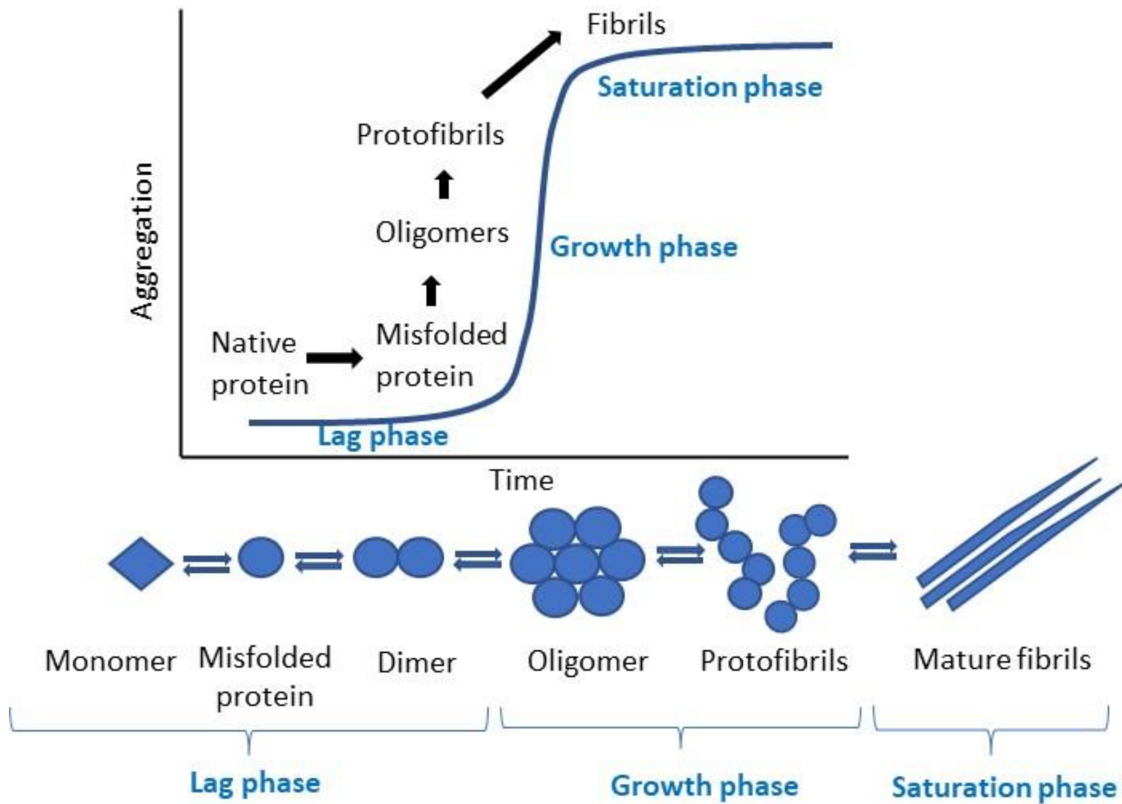


Figure 5: The nucleation dependant fibril formation process of IAPP. The lag phase involves misfolding of hIAPP. In the growth phase, oligomers are formed, and fibril growth is continued which facilitates the formation of protofibrils. In the saturation phase, fibril formation occurs at a constant rate.

1.2.8 The possible mechanisms of amyloid formation in type 2 diabetes

The autopsy studies have reported that islet amyloid forms in the pancreas of approximately 90% of patients with T2D [135]. It is still not well understood why soluble hIAPP forms toxic aggregates in T2D, but the proposed mechanisms of amyloid formation in T2D are as follows:

(1) Overproduction of IAPP in the β-cells along with insulin in T2D, associated with increased insulin demand, might result in IAPP aggregation and formation of toxic amyloid

deposits [164, 165]. However, studies have demonstrated that non-diabetic obese individuals with insulin resistance rarely develop amyloid deposits even though elevated IAPP is present [166]. Moreover, in studies with hIAPP-expressing transgenic mice, amyloid formation was only observed if a predisposing genetic or environmental factor such as diabetes background, obesity or high fat diet (HFD) was also present in addition to hIAPP overexpression [167]. These findings suggest that overexpression of hIAPP is an important factor, but not adequate alone for promoting amyloid formation in T2D [152].

(2) The amyloidogenic sequence of hIAPP facilitates its fibril formation in humans [168]**.** IAPP is the major component of islet amyloid deposition formed in patients with T2D. The other components of islet amyloid deposit include, but is not limited to, serum amyloid P component (SAP), apolipoprotein (apo) E, and the heparan sulfate proteoglycan perlecan [166]. Besides humans, toxic deposition of islet amyloid is also seen in some other mammalian species such as monkeys. However, rodents that are commonly used in diabetes research do not form islet amyloid because rodent IAPP lacks the amyloidogenic sequence that exists in human IAPP molecules. Human IAPP differs from rat and mouse IAPP only by six amino acids and five of those six amino acids are located in the fibrillogenic amino acid sequence (AA 20-29) [156, 157]. The presence of three proline residues in rat and mouse IAPP sequence keeps the protein in the soluble form and prevents its fibrillation. The proline residues in rodent IAPP sequence at amino acid positions 25, 28 and 29 prevent amyloidogenesis by acting as β-sheet breaker [170]. In addition to the fibrillogenic region 20-29, other amino acids in hIAPP molecule are also involved in fibril formation [156, 159]. For example, amino acid positions 1-19 in hIAPP molecule are involved in membrane interaction [160, 161] and hIAPP region 30-37 favors the fibril formation [174–176].

(3) Impaired processing of IAPP precursor, proIAPP, by islet β-cells in T2D leads to increased levels of unprocessed and partially processed proIAPP forms that have a higher tendency for aggregation compared to mature IAPP [164, 177]. *In vitro* studies in pancreatic β-cell lines (MIN6 cells) have shown that conditions associated with T2D such as hyperglycemia and hyperlipidemia can lead to reduced activation of PC2 and PC1/3, two major enzymes in proIAPP processing, resulting in impaired proIAPP processing and amyloid formation in T2D [178]. NH_2-

terminally extended proIAPP plays an important role in amyloid formation as it contains a heparin binding site which is removed during processing of proIAPP into mature IAPP [179]. **(Fig. 6)**

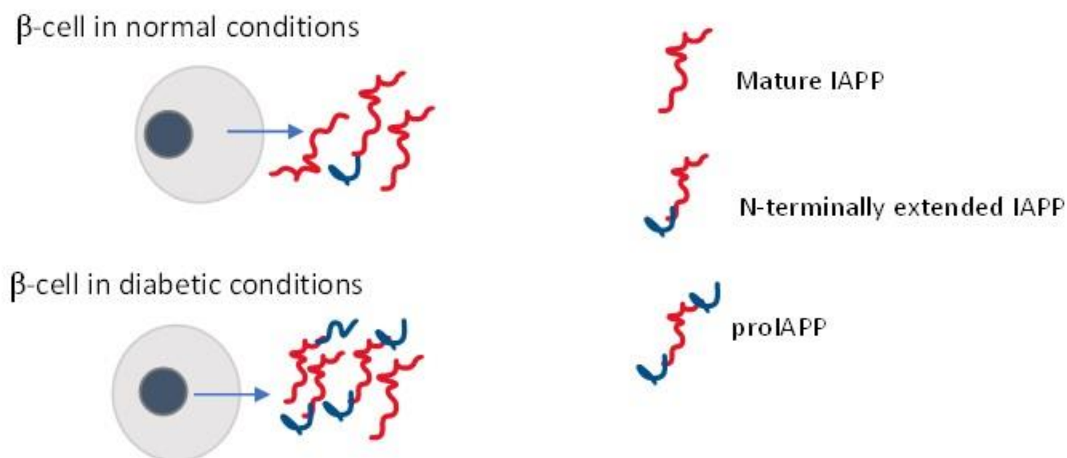


Figure 6: The proposed mechanisms for amyloid formation in patients with T2D. Increased demand for insulin in T2D associated with progressive insulin resistance results in higher release of insulin and proIAPP from β-cells. This leads to insufficient proIAPP processing time by PC1/3 and PC2, which mainly affects PC2 cleavage that happens in the Golgi and secretory granules, leading to higher cellular levels of N-terminally extended proIAPP. Both proIAPP and N-terminally extended intermediate forms of IAPP are prone to aggregation due to the presence of heparin binding sites in the N-terminal region.

(4) Mutation in the proIAPP gene may promote aggregation and has been shown to be associated with an early onset and more severe form of T2D. hIAPP amino acid positions 20-29 appear to be more prone to gene mutation. Findings from a study performed on a Japanese subpopulation suggests that mutations such as a glycine serine substitution, at position 20 (S20G) of hIAPP sequence, facilitates IAPP aggregation [164, 180]. However, other studies were not able to find a link between IAPP gene abnormality and T2D [181].

1.2.9. The role of amyloid in islet inflammation

Growing evidence from our lab and others suggest that amyloid formation in the pancreatic β-cells is associated with islet inflammation, leading to impaired β-cell function and increased β-cell apoptosis in human islets and animal models of amyloid-associated T2D [47, 49, 57, 171, 172]. Several studies have demonstrated that islet amyloid formation triggers activation of the pro-inflammatory cytokines, mainly IL-1β [49, 60, 184]. Studies from our lab have shown that amyloid formation in human and hIAPP-expressing mouse islets promotes IL-1β production, leading to β-cell Fas upregulation, activation of caspase-8, and apoptosis, all of which were markedly reduced by prevention of amyloid formation by adenoviral-siRNA suppression of hIAPP expression [49, 60, 184, 185]. IL-1β has two identified receptors including IL-1 receptor type 1 (IL-1R1), which promotes activation of IL-1β signaling, and IL-1 receptor type 2 (IL-1R2), which acts as a suppressor of IL-1 activity by competing for binding to IL-1β [56]. IL-1 receptor is expressed abundantly in the β-cell membrane, suggesting that islets are particularly susceptible to IL-1β-mediated toxicity [50]. **(Fig. 7)**

Interleukin-1 receptor antagonist (IL-1RA) is a natural inhibitor of IL-1β. The balance between IL-1β and IL-1RA plays a key role in the regulation of IL-1β signaling [58]. It was reported that treatment with synthetic IL-1RA in two models of T2D, high fat diet (HFD)-fed mice and Goto -Kakizaki (GK) rats, inhibited IL-1β signaling, improved hyperglycemia, increased insulin content, reduced proinsulin/insulin ratio and improved insulin sensitivity [186]. Also, clinical trials in patients with T2D have demonstrated that targeting IL-1 signaling by using recombinant IL-1RA improves insulin secretion and β-cell function without any detectable changes in insulin sensitivity [50].

Studies from our research group have reported that amyloid formation in human islets is associated with reduced IL-1RA/IL-1β ratio due to elevated IL-1β production and inhibition of IL-1RA production, which may contribute to β-cell dysfunction and apoptosis mediated by amyloid [60]. Accordingly, pharmacological blockade of IL-1R1 markedly reduced amyloid-induced β-cell toxicity in human islets and enhanced both β-cell function and survival [49, 60]. Moreover, an *in vivo* study from Westwell-Roper *et al*. showed that treatment with IL-1RA improved glucose tolerance in hIAPP-expressing mouse islets [187].

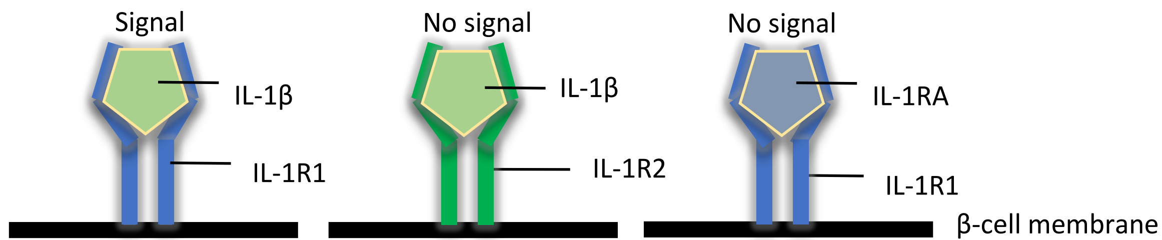


Figure 7: The two types of IL-1β receptors and their role in mediating IL-1β signaling. IL-1β binds to IL-1R1 which recruits IL-1R accessory protein to the site of interaction, resulting in activation of IL-1β signaling. On the other hand, binding of IL-1β to IL-1R2 leads to no signal. IL-1RA competes with IL-1β for binding to IL-1R1 and prevents IL-1β action.

1.2.10. Hyperglycemia-induced islet inflammation

It has been reported that T2D is associated with an increase in islet resident macrophages [13, 14, 54]. The possible mechanisms by which hyperglycemia induces macrophage infiltration in islets include (1) increased IL-1β mRNA levels in β-cells; and (2) secretion of chemokines from β-cells in response to IL-1β. IL-1β immunoreactivity has been reported in β-cells in pancreatic sections from patients with T2D and human islets cultured *ex vivo* with elevated glucose. However, IL-1β was not detectable in islets from healthy individuals or islets cultured in normal glucose [54]. Another study also revealed that in HFD-fed rats, normalization of plasma glucose was associated with reduced IL-1β production in islets, further suggesting the correlation between hyperglycemia and elevated IL-1β production [53]. The major source of hyperglycemia-induced IL-1β production in islets appears to be macrophages [54]. However, evidence suggests that β-cells also produce IL-1β [54]. For example, in HFD-fed rats, IL-1β immunoreactivity was observed in islet β-cells [54]. Since both hyperglycemia and amyloid promote islet IL-1β production, these two factors may have synergistic effects in individuals with T2D.

1.3. The major mechanisms of amyloid-induced β-cell death

Previous *in vitro* studies have suggested several possible mechanisms for β-cell apoptosis mediated by hIAPP aggregation. These mechanisms include activation of caspase pathways [190,] disruption of membrane integrity [191], formation of ion channel like structures [192] and interaction of β-cell components such as heparin sulphate proteoglycans with hIAPP fibrils [179]. Also, intracellular hIAPP aggregation *in vitro* contributes to β-cell toxicity by causing endoplasmic reticulum (ER) stress [193], oxidative stress [194] and disruption in the autophagy lysosomal pathway [195]. However, the contribution of these mechanisms to β-cell death in primary islets *in vivo* where endogenously produced hIAPP (pmol/l) is several fold lower than that used *in vitro* (µmol/l) has yet to be identified [196].

1.3.1 Mitochondrial stress

Cytotoxic oligomers that are developed in the ER and Golgi due to overexpression of proIAPP in conditions associated with T2D such as increased insulin demand, enter the cytosol after escaping the secretory granules [114]. From this location they can perforate membranes of other organelles such as mitochondria and cause oxidative stress and production of reactive oxygen species (ROS) [113, 197]. Toxic oligomer-mediated mitochondrial dysfunction is reported in hIAPP-expressing mice but does not occur in non-transgenic rodents that express non-fibrillogenic IAPP, suggesting that formation of hIAPP oligomers is associated with mitochondrial membrane disruption [114].

1.3.2 Endoplasmic reticulum (ER) stress

Increased insulin biosynthesis to compensate for the peripheral insulin resistance in T2D results in increased demand on the secretory machinery of β-cells, leading to β-cell exhaustion. Since insulin and hIAPP are co-synthesized and co-secreted, increased insulin production is associated with increased synthesis and secretion of IAPP from the β-cells. Increased IAPP biosynthesis and aggregation leads to development of ER stress [113]. Accordingly, *in vitro* studies from Butler P. et al, showed that overexpression of hIAPP in hIAPP-expressing rats was associated with ER stress and β-cell apoptosis [28, 29]. Also, translocation of C/EBP homologous protein /GADD153 (CHOP) which is a transcription factor in ER stress, in the nucleus from cytoplasm was

increased by six-fold in human islet section from patients with T2D, whereas CHOP-positive cell nuclei were not present in islet sections form obese or non-obese nondiabetic subjects [200].

1.3.3 Membrane disruption

In physiological conditions, IAPP aggregation is prevented inside the secretory granules by the interaction of IAPP with the components of the β-cell vesicles such as microcrystalline form of insulin [201], proinsulin and other processing intermediates of the vesicle such as zinc [202]. Insulin is a powerful inhibitor of IAPP aggregation [203]. More specifically, the B chain of insulin binds to the short segments of IAPP and inhibit IAPP aggregation inside the secretory granules [203]. Additionally, the IAPP: insulin ratio ranging between 1:5 and 1:100 inside the secretory vesicles has an inhibitory effect on hIAPP fibril formation [202]. Increased levels of unprocessed or partially processed proIAPP facilitates IAPP aggregation into amyloid deposits. A continuation of fibril growth leads to β-cell membrane disruption by formation of channels that allow ions to leak. Membrane permeabilization by IAPP can occur by formation of non-specific membrane pores [204].

1.4 The major apoptotic pathways in mammalian cells

1.4.1 The Fas-mediated (or extrinsic) apoptotic pathway

The Fas-mediated (extrinsic) apoptotic pathway is one of the two major apoptotic pathways in mammalian cells. The Fas-mediated apoptotic pathway is initiated by binding of the cell death receptor (Fas/CD95/APO-1), a transmembrane receptor protein that belongs to the tumor necrotic factor superfamily of receptors, to its ligand (FasL/CD95L; death ligand) [205]. The Fas-FasL interaction recruits caspase-8 (also known as FADD-like IL-1 converting enzyme or FLICE) to the death inducing signaling complex (DISC) via Fas-associated death domain (FADD) which then leads to activation of caspase-8, the major upstream caspase in the Fas apoptotic pathway, leading to activation of the downstream caspase, caspase-3 [184]. Incorporation of FLICE inhibitory protein (FLIP) instead of pro-caspase-8 prevents cleavage of pro-caspase-8 into active caspase-8 and thus FLIP acts as the endogenous regulator of Fas-mediated apoptosis [206]. The balance between caspase-8 and FLIP plays an important role in directing Fas-signaling either towards apoptosis or proliferation in the cells [207]. **(Fig. 8)**

1.4.2 The mitochondrial (or intrinsic) apoptotic pathway

The mitochondrial apoptotic pathway is also called intrinsic or cytochrome c apoptotic pathway. The pro-apoptotic and anti-apoptotic proteins of the B-cell lymphoma 2 (Bcl-2) family regulate the mitochondrial pathway. The mitochondrial apoptotic pathway is initiated by activation of the pro-apoptotic proteins such as Bcl-2-associated X protein (Bax) and Bcl-2 homologous antagonist/killer (Bak) [208]. Activation of Bax and Bak is associated with loss of mitochondrial membrane integrity, leading to the release of cytochrome c into the cytosol. Cytochrome c binds to apoptosis activating factor-1 (APAF-1) in the cytosol and forms a complex called the apoptosome [208]. Formation of the apoptosome results in activation of caspase-9, followed by activation of caspase-3, leading to β-cell death [209]. The mitochondrial apoptotic pathway can also be activated by the Fas-mediated apoptotic pathway. Caspase-8 indirectly activates caspase-9 by cleaving the Bcl-2 family protein, Bid, which translocates into the mitochondria and causes membrane disruption, leading to cytochrome c release [210]. **(Fig. 8)**

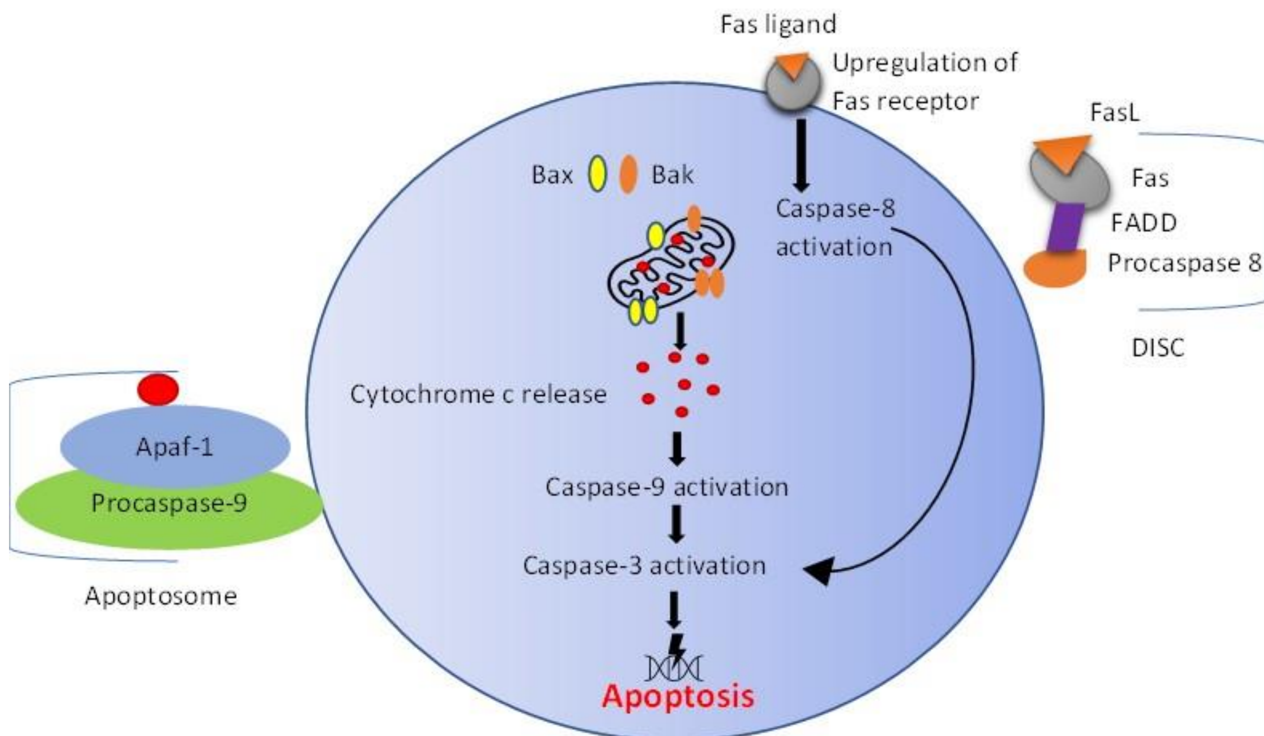


Figure 8: The Fas and mitochondrial apoptotic signaling pathways in mammalian cells. The Fas ligand binds to the Fas receptor and forms DISC, consisting of Fas, FADD and pro-caspase-8. Formation of DISC leads to cleavage of pro-caspase-8 to form active caspase-8, which in turn

activates caspase-3, resulting in apoptosis. In the mitochondrial apoptotic pathway, activation of Bax and Bak leads to permeabilization of mitochondrial membrane, resulting in the release of cytochrome c into the cytosol. Cytochrome c forms complexes with APAF-1 and pro-caspase-9, leading to the activation of caspase-9 which in turn activates caspase-3 and promotes apoptosis.

1.4.3 The role of the Fas-mediated apoptotic pathway in amyloid-induced apoptosis

β-cells consecutively express FasL, but Fas receptor is not normally expressed in β-cells at detectable levels [211]. Exposure to stressful conditions such as elevated glucose [211] , FFAs [212], leptin [213] and cytokines promotes Fas upregulation in β-cells, leading to apoptosis [171, 172]. The Fas-mediated apoptotic pathway appears to play an important role in β-cell apoptosis in both T1D and T2D [44, 45]. Our research group has shown that the Fas-mediated apoptotic pathway is the major signaling pathway mediating amyloid-induced β-cell apoptosis [184]. A study from the Marzban lab using human and hIAPP-expressing mouse islets demonstrated that Fas-positive and amyloid-positive islet areas are co-localized, suggesting a close correlation between amyloid formation and Fas upregulation [184]. Our group further showed that Fas upregulation by hIAPP aggregates results in the activation of the Fas-mediated apoptotic pathway manifested as activation of caspase-8 in β-cells in both human and hIAPP-expressing mouse islets [185]. Importantly, IL-1β, Fas, and caspase-8, were detected in the majority of islets with amyloid formation while islets with no detectable amyloid had very low levels of all three, indicating that amyloid formation induces upregulation of Fas and caspase-8 activation by promoting IL-1β production in islets [185]. Accordingly, inhibition of amyloid formation, markedly reduced Fas expression, caspase-8 activation, and β-cell apoptosis [3, 8, 184]. Moreover, deletion of Fas in hIAPP-expressing transgenic mice was associated with reduced amyloid-induced β-cell apoptosis [184].

One important finding from studies in our group was that during the process of amyloid formation in both human and hIAPP-expressing transgenic mouse islets, the majority of Fas-positive cells were β-cells and very few Fas-positive non-β-cells were seen [184]. Despite a significant increase in the number of apoptotic β-cells, α-cell area remained unchanged in both human and hIAPP expressing mouse islets during amyloid formation, resulting in reduced β:α cell ratio [184, 216]. Taken together, these findings suggest that amyloid formation mainly promotes apoptosis in β-cells. Thus, it appears that α-cells are likely to be equipped with cellular

mechanisms which protect them from amyloid toxicity. However, these mechanisms have yet to be identified.

1.4.4 The role of the mitochondrial apoptotic pathway in amyloid-induced apoptosis

The previous studies from our research group have shown that blocking Fas receptor in amyloid-forming human and hIAPP-expressing mouse islets was associated with a significant reduction in β-cell death, but did not completely inhibit hIAPP-induced β-cell apoptosis, suggesting that other apoptotic signaling pathway(s) may also contribute to amyloid-induced β-cell apoptosis [217]. Further studies showed that intracellular aggregation of hIAPP can induce β-cell apoptosis in hIAPP expressing mouse islets via activation of the mitochondrial apoptotic pathway manifested as activation of caspase-9. Moreover, adenoviral-mediated expression of prohIAPP in transformed INS-1 β-cells was associated with the release of cytochrome c from mitochondria, leading to caspase-9 activation and apoptosis [217]. One more important finding of these studies was that amyloid-mediated caspase 9 activation in hIAPP-expressing mouse islets was mainly specific to islet β-cells, while very low number of active caspase 9-positive islet non-β-cells were seen [217].

Also, islets from Bax and Bak or cytochrome c knockout mice that were transduced to express hIAPP, had lower β-cell apoptosis than transduced wild-type islets despite formation of comparable intracellular hIAPP aggregates. However, islet α-cell mass was comparable between wild-type and Bax, Bak or cytochrome c knockout mouse islets with intracellular hIAPP expression [217]. These findings further suggest that α-cells are protected against amyloid.

1.5. The effects of human islet amyloid polypeptide on islet α-cells

The current knowledge on the effects of amyloid formation on islet α-cells is very limited. The previous studies from our lab have demonstrated that islet α-cells are more resistant to amyloid-induced toxicity than β-cells [216]. Transformed and primary mouse islet α-cells had a higher survival rate than transformed and primary β-cells following treatment with the same concentrations of recombinant hIAPP [216]. Deletion of caspase-3 in hIAPP-expressing mice was associated with a significant decrease in amyloid-induced β-cell apoptosis whereas the proportion of apoptotic α-cells was low and comparable in hIAPP expressing mice with or without

caspase-3 expression [216]. Additionally, aggregates of exogenously applied hIAPP induced activation of caspase-8 in human islet β-cells but not α-cells [185].

The protective mechanisms underlying better survival of α-cells in diabetic conditions are still not well understood. In my MSc thesis project, we investigated whether different expression levels of IL-1R1 and/or Fas, two receptors involved in the IL-1β signaling pathway, which is the major pathway mediating amyloid-induced β-cell apoptosis, provides a protective mechanism in islet α-cells against β-cell apoptotic factors.

1.6 Hypothesis

The overall hypothesis of this project is that lower expression of Fas and/or IL-1R1 in islet α-cells compared to β-cells protects islet α-cells from amyloid-induced apoptosis, leading to better survival of α-cells than β-cells during amyloid formation in T2D.

1.6.1 The project aims

Aim 1: To test the difference(s) between IL-1R1 expression in transformed α-cells and β-cells under basal conditions and following treatment with elevated glucose in the presence or absence of hIAPP or IL-1β.

Rationale: A previous study from our lab has shown that mouse αTC1 cells have higher survival rate than mouse βTC3 cells following treatment with synthetic hIAPP. Moreover, the rate of apoptosis was markedly higher in dispersed mouse islet β-cells compared to α-cells when treated with increasing concentrations of exogenous hIAPP [218]. The mechanisms underlying the lower sensitivity of islet α-cells than β-cells to amyloid-mediated apoptosis are not clear. Previous studies from our lab have shown that islet amyloid formation in human and hIAPP-expressing mouse islets is associated with increased IL-1β immunoreactivity, which is closely correlated with Fas upregulation and β-cell apoptosis. Interestingly, those studies also revealed that IL-1β concentration required to induce apoptosis in αTC1 cells is markedly higher than that required to cause apoptosis in INS-1 β-cells [60]. Moreover, treatment of αTC1 cells with exogenous IL-1β increased IL-1RA immunoreactivity to a much lesser extent than that observed in transformed INS-1 β-cells [60]. Since IL-1β is a key mediator of amyloid-induced islet inflammation and β-cell

apoptosis, we examined if higher expression of IL-1R1 in β-cells than α-cells contributes to the higher sensitivity of β-cells to amyloid-induced apoptosis.

Aim 2: To examine the difference(s) between Fas receptor expression in transformed α-cells and β-cells under basal conditions and following treatment with elevated glucose in the presence or absence of hIAPP or IL-1β.

Rationale: Studies from our lab reported that treatment of both transformed and primary mouse and human islet β-cells with exogenous hIAPP promotes upregulation of Fas receptor and activation of the Fas-mediated apoptosis initiated by caspase-8 activation [216, 219]. Similarly, amyloid formation due to aggregation of endogenously produced hIAPP in both cultured human and hIAPP-expressing mouse islets was associated with Fas upregulation in islet β-cells, leading to the Fas-mediated apoptosis [184]. However, very low Fas-positive non-β-cells were observed in amyloid-forming human or hIAPP-expressing mouse islets during culture [184]. Since the majority of islet non-β-cells are α-cells, in aim 2 of this MSc project, we examined the difference between Fas expression in α-cells and β-cells before and after exposure to apoptotic factors such as elevated glucose and hIAPP or IL-1β.

CHAPTER 2: MATERIALS AND METHODS

2.1 Experimental models: Transformed cell lines

αTC1-6 cells: Mouse αTC1-clone 6 cells (αTC1-6), a pancreatic α-cell line which produces and secretes glucagon, were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia). αTC1-6 cells were isolated from the pancreas of mouse with adenoma, created in transgenic mice expressing the SV40 large T antigen oncogene under the control of the rat preproglucagon promoter [220]. αTC1-6 cells are more differentiated than their parenteral cell line (αTC1) which expresses glucagon and low levels of insulin [221].

INS-1 β-cells: INS-1 β-cells are a rat insulinoma β-cell line that secretes insulin in response to glucose and GLP-1 agonists. INS-1 β-cells were originally generated from an X-ray-induced insulinoma in rat [222]. In this study, INS-1 832/13 subclone, a derivative of INS-1 β-cells provided by Dr. C. Newgard (Duke University Medical Center, NC, USA) was used, which is more differentiated than INS-1 β-cells and has a higher glucose stimulated insulin secretion [222]. The INS-1 832/13 subclone was generated by stably transfecting the parenteral cells with the plasmid pCMV8/INS/IRES/Neo containing human cDNA for human insulin expression [222].

2.2 Cell culture conditions

Mouse αTC1-6 (passage #12) and rat INS-1 β-cells (passage #60) were cultured in DMEM (catalog no. 11885084; Gibco) and RPMI-1640 (catalog no. 11875093; Gibco), respectively, containing 10% FBS (vol./vol.), 1% penicillin and streptomycin, 1% sodium pyruvate (for INS-1 β-cells) and 1% glutamax, supplemented with 5.5 mmol/l or 11.1 mmol/l glucose. Cells were maintained by passaging every 3-5 days for a total of 8-9 passages. Briefly, when cell confluency reached about 70%, culture medium was removed and cells were incubated with 4 ml of 0.25% trypsin-EDTA (catalog no. 25200072, Gibco) for 1-2 minutes, followed by addition of 6 ml medium to inactivate trypsin. Cells were centrifuged for 5 minutes at 1200 rpm, supernatant was removed, and cells were resuspended in 10 ml medium. A portion of the cell suspension (1:5 dilution) was transferred to a new T-75 flask with fresh medium and incubated at 37°C incubator with 95% air and 5% CO2 as detailed in **Table 2.1**. For experiments, αTC1-6 cells and INS-1 β-cells were then seeded (150,000-180,000/well; 400 µl medium/well) in 8-well chamber slides (BD

Biosciences, Mississauga, ON, Canada) and cultured either in 11.1 mmol/l or 25 mmol/l (elevated) glucose for 48 hours prior to the start of treatments to ensure adhesion of cells and allow recovery before starting the treatments.

Table 2.1: Culture condition for αTC1-6 cells and INS-1 β-cells

Subject	αTC1 (clone 6)	INS-1 (832/13)
Source	ATCC (CRL-2350)	Provided by Dr. C. Newgard (Duke University Medical Center, NC, USA)
Derived from	Mouse	Rat
Culture medium	DMEM	RPMI-1640
Culture Conditions	37°C, 95% Air, 5% CO_2	37°C, 95% Air, 5% CO_2

2.3 Treatment studies with hIAPP or IL-1β

Cells were treated with 10 µmol/l non-fibrillogenic recombinant rIAPP (catalog no. 4030201; Bachem, Torrance, CA, USA), 10 µmol/l fibrillogenic recombinant hIAPP (catalog no. 4030200; Bachem, Torrance, CA, USA) or 1 ng/ml IL-1β (R&D Systems, Minneapolis, Minnesota) [60] as described below in **Table 2.2**. Lyophilised rIAPP or hIAPP were dissolved in 1,1,1,3,3,3-hexafluoroisopropanol (HFIP; 1 µg/1 µl) and incubated for one hour at room temperature. Aliquots (50 µg or 100 µg) were then frozen (−80°C) overnight and lyophilised the next day. Aliquotes of lyophilized rIAPP and hIAPP peptides were stored at -20 ˚C until used. Peptides were prepared fresh by disolving in either RPMI-1640 (for INS-1 β-cells) or DMEM (for αTC1-6 cells) culture medium for each study to get a final concentration of 20 µmol/l. rIAPP or hIAPP were added into the wells containing seeded cells at a final concentration of 10 µmol/l by diluting it 1:2 with culture media. Human recombinant IL-1β (R&D Systems, Minneapolis, Minnesota) was added to the seeded cells at a final concentration of 1 ng/ml [49].

For *in vitro* hIAPP treatment studies, 10 µmol/l hIAPP was used based on the previous studies from our lab which have shown this is the optimal concentration for inducing β-cell apoptosis by exogenously applied hIAPP [218]. The same concentration of rIAPP which does not form aggregates was used as a control in all studies. IL-1β concentration used in this study (1 ng/ml) was chosen based on previously published work from the lab for αTC1 and INS-1 β-cells.

In those studies, IL-1β at either 0.2 or 1 ng/ml concentration promoted apoptosis in INS-1 β-cells. However, IL-1β only at higher concentration (1 ng/ml) induced apoptosis in αTC1-6 cells [60]. Therefore, for this project, 1 ng/ml IL-1β concentration was used to assess IL-1R1 and Fas expression in αTC1-6 cells and INS-1 β-cells.

Fas upregulation was assessed 8-12 hours after treatment with synthetic hIAPP or IL-1β [184]. The time points were chosen based on the previously published studies from our lab which demonstrated that treatment with synthetic hIAPP (10 μmol/l) induces Fas-upregulation in INS-1 β-cells between 8-12 hours [184]. To assess expression of IL-1R1 and apoptosis, cells were treated with hIAPP or IL-1β for 24 hours, the optimal time point for detection of amyloid-induced β-cell apoptosis [218].

Table 2.2: Treatment conditions for αTC1-6 cells and INS-1 β-cells in 8-well chamber slides

11.1 mM glucose	11.1 mM glucose +10 μM rIAPP	11.1 mM glucose +10 μM hIAPP	11.1 mM glucose +1 ng/ml IL-1 β
25 mM glucose	25 mM glucose +10 μM rIAPP	25 mM glucose +10 μM hIAPP	25 mM glucose +1 ng/ml IL-1 β

2.4 Immunolabeling of cells

Cells were fixed in 4% paraformaldehyde (PFA) for 30 minutes at room temperature, followed by blocking in 2% normal goat and/or donkey serum (40 μl/condition, Vector Laboratories, Burlingame, CA, USA) for 30 minutes in a humid chamber at room temperature. Cells were then incubated with mouse anti-glucagon or guinea pig anti-insulin and each rabbit anti-Fas or anti-IL-1R1 primary antibodies (30 μl/condition) overnight at 4°C as listed in **Table 2.3.** For double immunostaining with glucagon or insulin and Fas, cells were incubated with Alexa 594-conjugated anti-mouse or Texas red anti-guinea pig and Alexa 488-conjugated anti-rabbit secondary antibodies, respectively, for 1 hour in a dark humid chamber. For double immunostaining for glucagon or insulin and IL-1R1, cells were incubated with Alexa 594-conjugated anti-rabbit or Texas red anti-guinea pig and Alexa 488-conjugated anti-goat, respectively, for 1 hour. Details about the secondary antibodies are listed in **Table 2.4**. For double immunostaining for glucagon or insulin and TUNEL, after incubation with primary and secondary

antibodies, cells were incubated for 30 min in a humid chamber at 37°C with TUNEL reaction mixture (Roche Diagnostics, Laval, QC, Canada).

Micrographs were taken using Zeiss epifluorescence inverted microscope (model: Axio observer, Carl Zeiss) and quantified by blind manual counting of Fas-positive and TUNEL-positive αTC1-6-cells or INS-1 β-cells in each condition. Four microscope fields per condition (20X magnification) each containing 300-400 cells were quantified. The mean intensity of IL-1R1 immunoreactivity was quantified using ImageJ software (version 1.53t) with the FIJI package. The mean fluorescence intensity was calculated by fluorescence intensity (integrated intensity) divided by the total area of cells on each slide.

Table 2.3: The list of primary antibodies

Antibodies	Host	Target	Immunogen	Company	Dilution
Glucagon (polyclonal)	Rabbit	Mouse, Rat	Human glucagon	Dako A056501	1:400
Glucagon (monoclonal)	Mouse	Human, Mouse, Rat, Guinea pig, Rabbit	Polymerized porcine glucagon	Sigma G2654	1:500
Insulin (polyclonal)	Guinea pig	Human, Mouse, Rat	Porcine pancreatic insulin	Dako A0564	1:250
Fas (polyclonal)	Rabbit	Human, Mouse, Rat	A synthesized peptide derived from human FAS (C-terminal amino acids)	Invitrogen PA5-115214	1:200
IL-1RI (polyclonal)	Rabbit	Human, Mouse, Rat	Recombinant human interleukin-1 receptor type 1 protein (18-320AA)	Invitrogen PA5-97866	1:200

Table 2.4: The list of secondary antibodies

Antibodies	Target	Host	Company	Dilution
Alexa 594	Rabbit	Donkey	Jackson laboratories; 711-515-152	1:200
Alexa 594	Mouse	Donkey	Invitrogen; A21203	1:200
Texas Red	Guinea pig	Goat	Jackson laboratories; 106-076-003	1:200
Alexa Flour 488	Rabbit	Goat	Invitrogen; A11034	1:200
Alexa Flour 488	Goat	Donkey	Invitrogen; A11055	1:200

2.5 Statistical analysis

All Fas and TUNEL studies were repeated three times (n= 3 independent studies). IL-1R1 studies were repeated two times (n= 2 independent studies). Statistical analyses were performed using two-way ANOVA. The treated groups were compared with the control groups within the cell lines. Comparisons were also made between the corresponding experimental groups in αTC1-6 cells and INS-1 β-cells. A *p*-value of $p<0.05$ was considered as significant.

CHAPTER 3: RESULTS

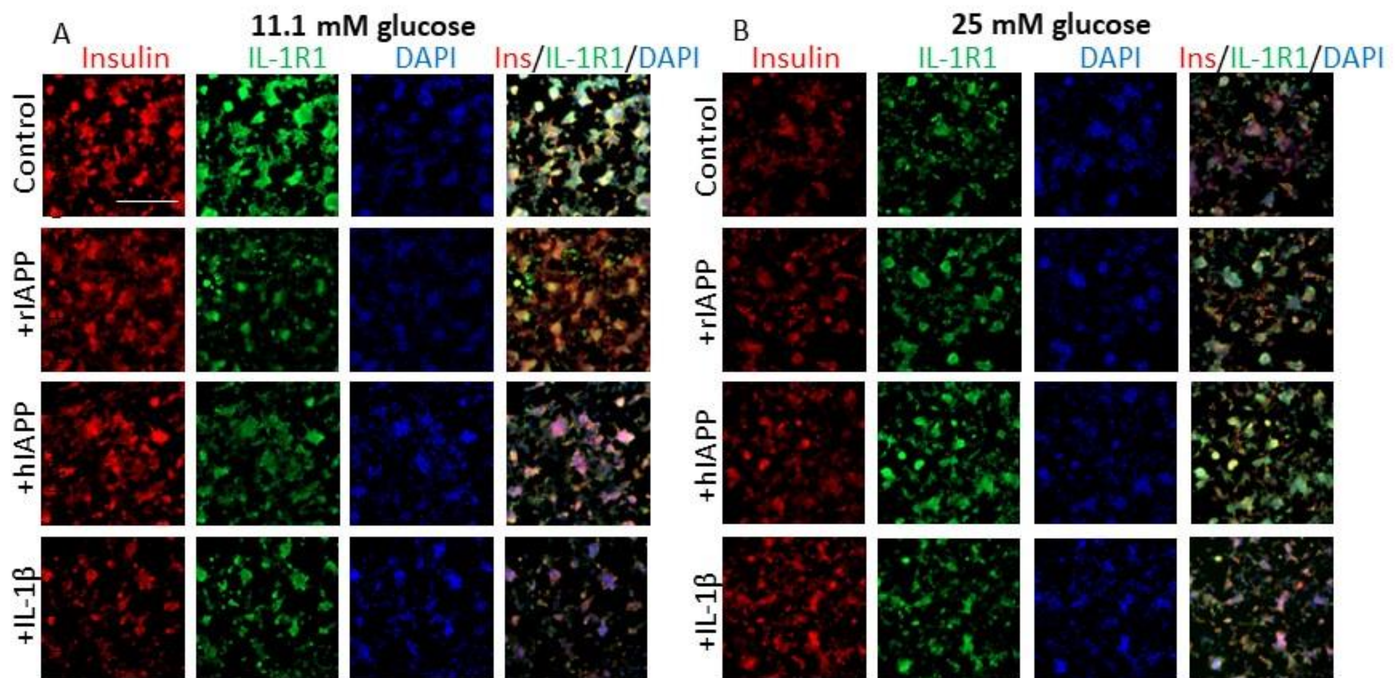
3.1 IL-1R1 immunoreactivity is lower in αTC1-6 cells compared to INS-1 β-cells in basal conditions and following exposure to hIAPP or IL-1β.

In aim 1, we examined the immunoreactivity of IL-1R1 in αTC1-6 cells and INS-1 β-cells cultured either in 11.1 mmol/l or 25 mmol/l (elevated) glucose and following treatment with hIAPP or IL-1β. To compare IL-1R1 immunoreactivity between αTC1-6 cells and INS-1 β-cells, the cells were cultured without or with non-fibrillogenic rIAPP (10 µmol/l), fibrillogenic hIAPP (10 µmol/l) or IL-1β (1 ng/ml) for 24 hours (n=2 independent studies done in duplicate). Double immunostaining was performed for glucagon or insulin and IL-1R1 and the mean fluorescence intensity of IL-1R1 was quantified in αTC1-6 cells and INS-1 β-cells in each condition.

In non-treated INS-1 β-cells cultured either in 11.1 mmol/l or 25 mmol/l glucose, a strong IL-1R1 immunoreactivity was observed (**Fig. 9 A-C**). We did not observe any noticeable difference between IL-1R1 fluorescence intensity in INS-1 β-cells cultured in 11.1 mmol/l or 25 mmol/l glucose. Similarly, IL-1R1 immunoreactivity was observed in non-treated αTC1-6 cells cultured in either 11.1 mmol/l or 25 mmol/l glucose and there was no noticeable difference between IL-1R1 fluorescence intensity in αTC1-6 cells cultured in 11.1 mmol/l or 25 mmol/l glucose (**Fig. 10 A-C**). However, when INS-1 β-cells and αTC1-6 cells were compared, IL-1R1 fluorescence intensity was noticeably lower in non-treated αTC1-6 cells as compared to INS-1 β-cells cultured in either glucose concentration (**Figs 9, 10**). Treatment with rIAPP, which was used as a control non-fibrillogenic peptide along with fibrillogenic hIAPP in all studies, did not change IL-1R1 immunoreactivity in either INS-1 β-cells (**Fig. 9**) or αTC1-6 cells (**Fig. 10**).

Treatment with hIAPP or IL-1β for 24 hours did not have any marked effect on IL-1R1 fluorescence intensity in INS-1 β-cells cultured in 11.1 mmol/l or 25 mmol/l glucose concentration, although the fluorescence intensity appeared to be slightly higher in all treatment groups at 25 mmol/l glucose (**Fig. 9 A-C**). Also, fluorescence intensity was comparable between non-treated and hIAPP or IL-1β treated αTC1-6 cells in both 11.1 mmol/l or 25 mmol/l glucose concentrations (**Fig. 10 A-C**). However, IL-1R1 fluorescence intensity was markedly lower in hIAPP or IL-1 β treated αTC1-6 cells as compared to corresponding treatment groups in INS-1 β-cells cultured either in 11.1 mmol/l or 25 mmol/l glucose concentration (**Figs 9, 10**). These findings

were consistent in two independent IL-1R1 experiments each performed in duplicate for all conditions.



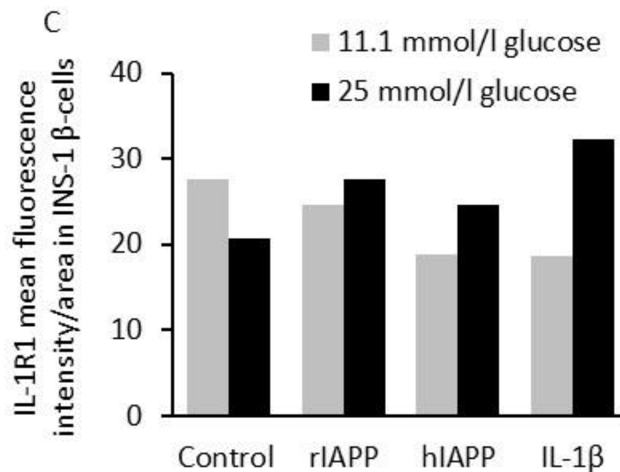


Figure 9: IL-1R1 immunoreactivity in INS-1 β-cells following treatment with hIAPP or IL-1β. INS-1 β-cells treated without or with rIAPP, hIAPP or IL-1β for 24 hours in **(A)** 11.1 mmol/l or **(B)** 25 mmol/l glucose were immunolabelled for insulin (red), IL-1R1 (green) and DAPI (blue); scale bar: 200 μm. **(C)** The mean fluorescence intensity of IL-1R1 was measured in non-treated INS-1 β-cells and following treatment with rIAPP, hIAPP or IL-1β at 11.1 mmol/l or 25 mmol/l glucose. Quantifications represent 4 microscopic fields per condition each containing 300-400 cells. Results are expressed as means of two independent studies (n=2), performed in duplicate for all conditions.

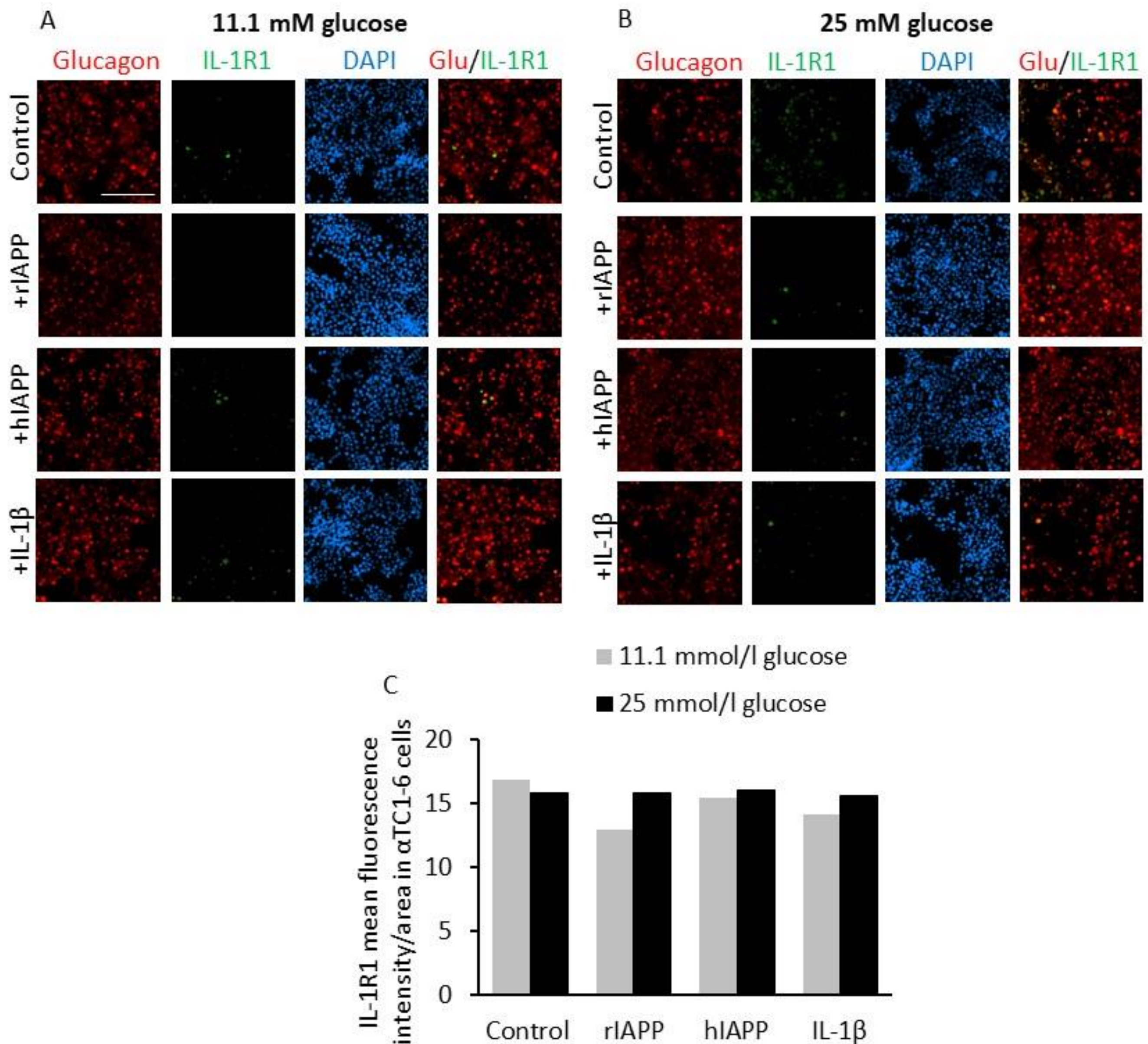


Figure 10: IL-1R1 immunoreactivity in αTC1-6 cells following treatment with hIAPP or IL-1β. αTC1-6 cells treated without or with rIAPP, hIAPP or IL-1β for 24 hours in **(A)** 11.1 mmol/l or **(B)** 25 mmol/l glucose were immunolabelled for glucagon (red), IL-1R1 (green) and DAPI (blue); scale bar: 200 µm. **(C)** The mean fluorescence intensity of IL-1R1 was measured in non-treated αTC1-6 cells and following treatment with rIAPP, hIAPP or IL-1β either in 11.1 mmol/l or 25 mmol/l glucose. Quantifications represent 4 microscopic fields per condition each containing 300-400 cells. Results are expressed as means of two independent studies (n=2), performed in duplicate for all conditions.

3.2 Fas expression induced by exogenous hIAPP or IL-1β is lower in αTC1-6 cells compared to INS-1 β-cells cultured in the same conditions.

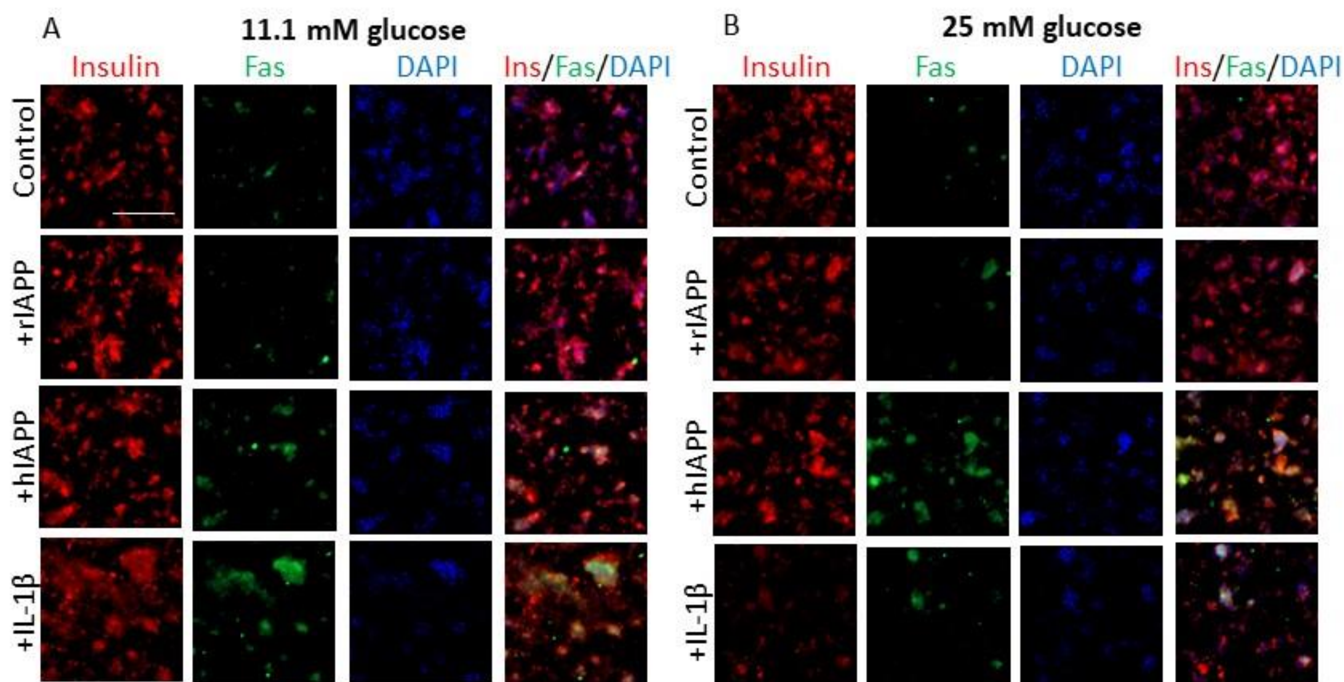
In aim 2, we examined if exposure to synthetic hIAPP or IL-1β can induce upregulation of Fas in αTC1-6 cells and INS-1 β-cells during culture either in 11.1 mmol/l or 25 mmol/l glucose. To compare proportion of Fas-positive αTC1-6 cells and INS-1 β-cells, the cells were cultured without or with non-fibrillogenic rIAPP (10 µmol/l), fibrillogenic hIAPP (10 µmol/l) or recombinant IL-1β (1 ng/ml) in 11.1 mmol/l or 25 mmol/l glucose for 8- and 12-hours (n=3 independent studies performed in duplicate). Double immunostaining was performed for glucagon or insulin and Fas, and the proportion of Fas-positive cells was quantified in αTC1-6 cells and INS-1 β-cells in each condition.

Only a few Fas-positive cells were detectable by immunolabelling in non-treated INS-1 β-cells cultured either in 11.1 mmol/l or 25 mmol/l glucose. Also, there was no detectable difference between the two glucose concentrations (**Fig. 11 A-C**). Similarly, very low number of Fas-positive αTC1-6 cells was detectable in non-treated control group at both 11.1 mmol/l and 25 mmol/l glucose (**Fig. 12 A-C**). Treatment of INS-1 β-cells with non-fibrillogenic rIAPP slightly increased the number of Fas-positive cells as compared to non-treated group but the increase was not statistically significant (**Fig. 11 A-C**). Also, αTC1-6 cells treated with rIAPP had comparable number of Fas-positive cells with non-treated group (**Fig. 12 A-C**).

Treatment with synthetic hIAPP significantly increased the number of Fas-positive INS-1 β-cells after 8- and 12-hours culture in either 11.1 mmol/l or 25 mmol/l glucose as compared to non-treated group (**Fig. 11 A-C**). There was no significant difference between the number of Fas-positive INS-1 β-cells in the hIAPP-treated group cultured either in 11.1 mmol/l or 25 mmol/l glucose **(Fig. 11 C)**. Treatment with hIAPP also significantly increased the number of Fas-positive αTC1-6 cells as compared to non-treated cells (**Fig. 12 A-C**). However, the number of Fas-positive αTC1-6 cells was markedly lower than that observed in INS-1 β-cells following treatment with hIAPP cultured in same glucose concentrations (**Figs 11, 12**).

Treatment with recombinant IL-1β resulted in a significant increase in the number of Fas-positive INS-1 β-cells after 8- and 12-hours as compared to non-treated group (**Fig. 11 A-C**). The number of Fas-positive INS-1 β-cells following treatment with IL-1β was comparable in 11.1

mmol/l or 25 mmol/l glucose concentrations. Additionally, there was no significant difference in the number of Fas-positive INS-1 β-cells treated with hIAPP or IL-1β **(Fig. 11 C)**. Similarly, the number of Fas-positive αTC1-6 cells was significantly higher following treatment with IL-1β as compared to non-treated group (**Fig 12 A-C**). There was no significant difference between the number of Fas-positive αTC1-6 cells cultured either in 11.1 mmol/l or 25 mmol/l following treatment with IL-1β (**Fig 12 A-C**). However, Fas expression in αTC1-6 cells following treatment with IL-1β was significantly lower than that of INS-1 β-cells cultured either in 11.1 mmol/l or 25 mmol/l glucose. (**Figs 11, 12**).



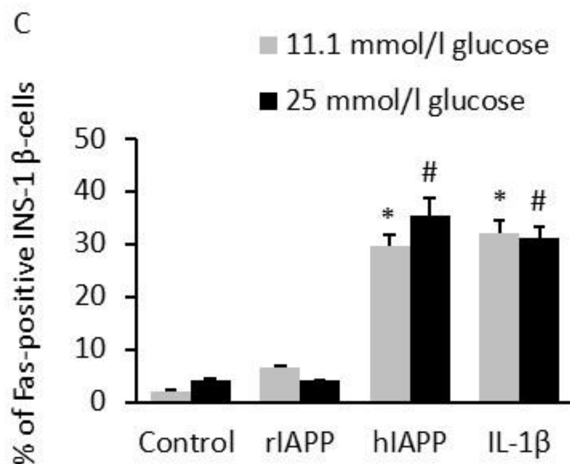


Figure 11: Fas upregulation in INS-1 β-cells following treatment with hIAPP or IL-1β. INS-1 β-cells treated without or with rIAPP, hIAPP or IL-1β for 8-12 hours in **(A)** 11.1 mmol/l or **(B)** 25 mmol/l glucose were immunolabelled for insulin (red), Fas (green) and DAPI (blue); scale bar: 200 µM. **(C)** The proportion of Fas-positive INS-1 β-cells in non-treated, rIAPP, hIAPP, or IL-1β treated cells cultured in 11.1 mmol/l or 25 mmol/l glucose. Quantifications represents 4 microscope fields per condition, each containing 300-400 cells. Results are expressed as means ± SEM of three independent studies (n=3). *vs control (11.1 mmol/l glucose); #vs control (25 mmol/l glucose); p<0.05, two-way ANOVA.

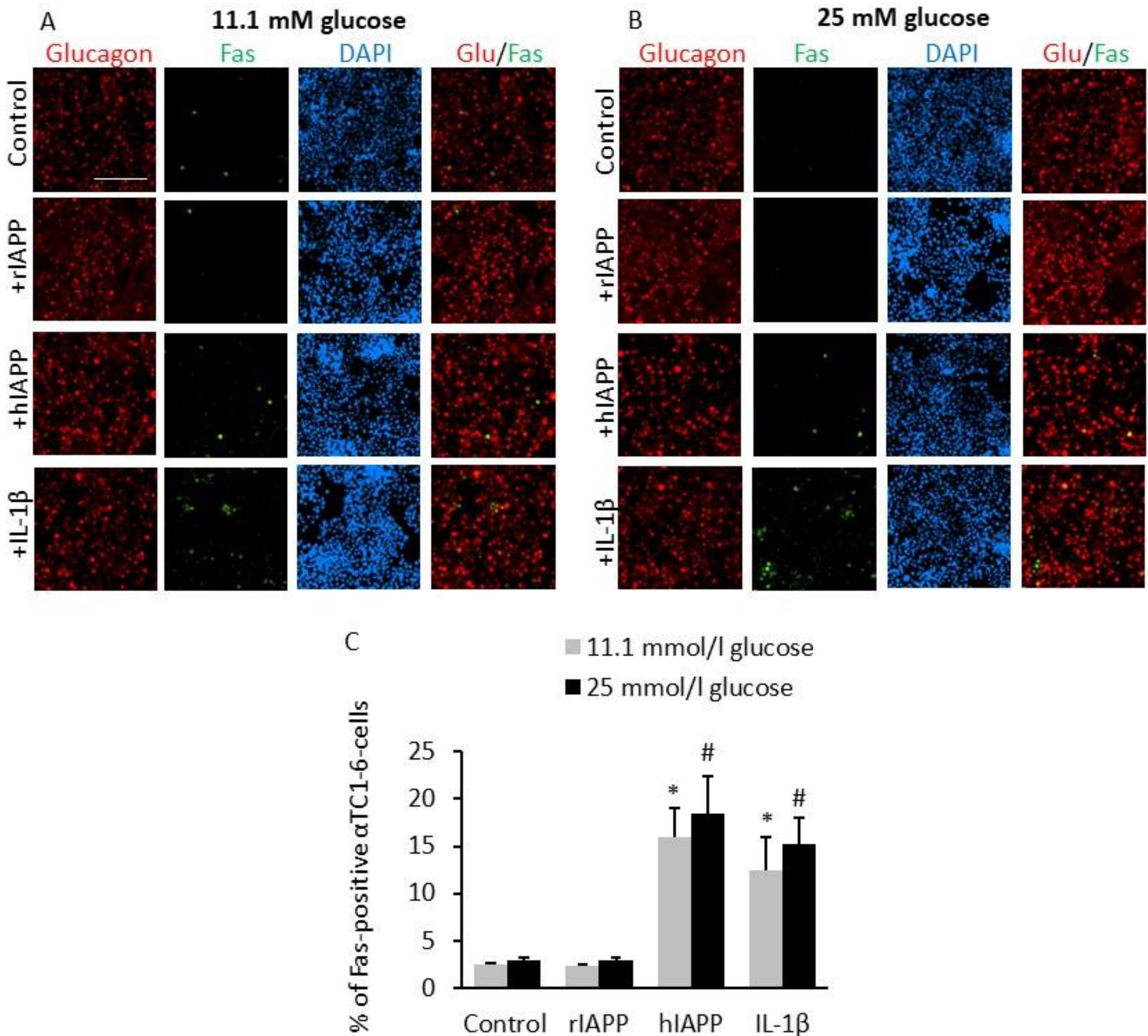


Figure 12: Fas upregulation in αTC1-6 cells following treatment with hIAPP or IL-1β. αTC1-6 cells treated without or with rIAPP, hIAPP or IL-1β for 8-12 hours in **(A)** 11.1 mmol/l or **(B)** 25 mmol/l glucose were immunolabelled for glucagon (red), Fas (green) and DAPI (blue); scale bar: 200 μM. **(C)** The proportion of Fas-positive αTC1-6 cells in non-treated, rIAPP, hIAPP, or IL-1β treated cells cultured in 11.1 mmol/l or 25 mmol/l glucose. Quantifications represents 4 microscope fields per condition, each containing 300-400 cells. Results are expressed as means ± SEM of three independent studies (n=3). *vs control (11.1 mmol/l glucose); #vs control (25 mmol/l glucose); *p*<0.05, two-way ANOVA.

3.3 αTC1-6 cells have lower rate of apoptosis than INS-1 β-cells following treatment with hIAPP or IL-1β.

We further examined the proportion of apoptotic αTC1-6 cells and INS-1 β-cells cultured either in 11.1 mmol/l or 25 mmol/l glucose without or with rIAPP, hIAPP or IL-1β treatment for 24 hours. Non-treated INS-1 β-cells had very few TUNEL-positive (apoptotic) cells at 11.1 mmol/l or 25 mmol/l glucose concentrations (**Fig. 13 A, C**). The number of TUNEL-positive αTC1-6 cells was also very low in non-treated group at either glucose concentration (**Fig 13 B, D**). INS-1 β-cells treated with non-fibrillogenic rIAPP had comparable rate of apoptosis with non-treated group (**Fig. 13 A C**). Similarly, treatment with rIAPP did not increase the proportion of apoptotic αTC1-6 cells (**Fig 13 B, D**).

The proportion of apoptotic INS-1 β-cells cultured in either 11.1 mmol/l or 25 mmol/l glucose was significantly higher than non-treated group following treatment with recombinant hIAPP for 24 hours but there was no significant difference between the proportion of apoptotic INS-1 β-cells treated with hIAPP at either 11.1 mmol/l or 25 mmol/l glucose (**Fig. 13 A, C**). Similarly, the proportion of apoptotic αTC1-6-cells was significantly higher than non-treated group following treatment with hIAPP but there was no noticeable difference between the proportion of apoptotic αTC1-6 cells following hIAPP treatment at either glucose concentration (**Fig 13 B, D**). However, when the two cell lines were compared, the proportion of apoptotic αTC1-6 cells was significantly lower as compared to INS-1 β-cells after treatment with hIAPP at both glucose concentrations (**Fig. 13**; $p < 0.05$).

Moreover, treatment of INS-1 β-cells with IL-1β significantly increased the proportion of apoptotic INS-1 β-cells after 24 hours compared to non-treated group and there was no difference in the proportion of apoptotic INS-1 β-cells between two glucose concentrations (**Fig 13 A, C**). Similarly, the number of apoptotic αTC1-6 cells was significantly higher following treatment with IL-1β than non-treated group and there was no difference between two glucose concentrations (**Fig 13 B, D**). However, the proportion of apoptotic αTC1-6 cells was significantly lower as compared to corresponding INS-1 β-cell group following treatment with IL-1β at both glucose concentrations (**Fig 13**; $p < 0.05$).

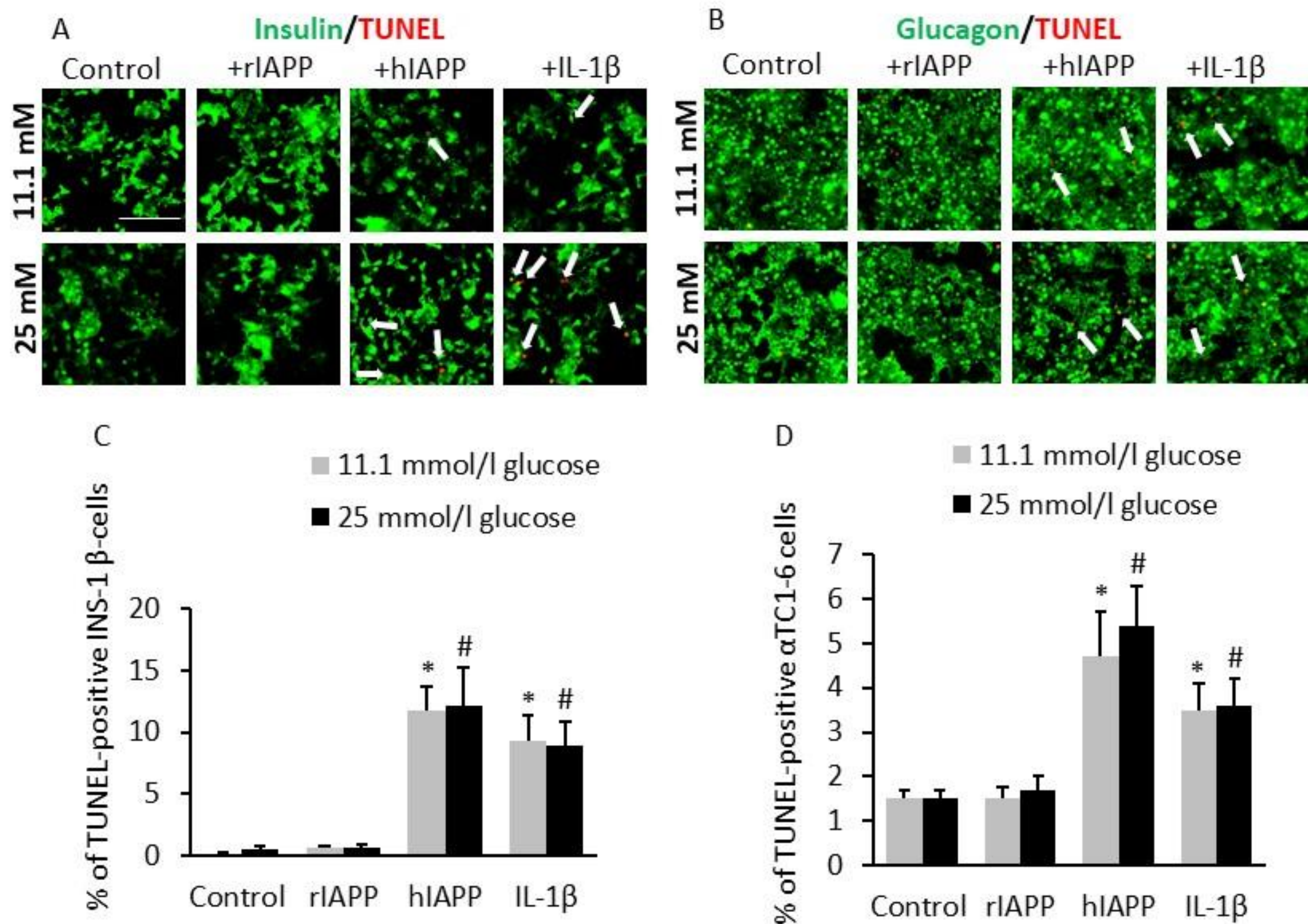


Figure 13: Treatment with hIAPP or IL-1β induces lower apoptosis in αTC1-6 cells than INS-1 βcells. INS-1 β-cells **(A)** and αTC1-6 cells **(B)** and without treatment (control) or following treatment with synthetic rIAPP (10 μM), hIAPP (10 μM) or IL-1β (1ng/ml) in either 11.1 mmol/l or 25 mmol/l glucose for 24 hours, were immunolabelled for glucagon (green) or insulin (green) and TUNEL (red); scale bar: 200 μM. White arrows denote TUNEL-positive areas. The proportion of TUNEL-positive (apoptotic) INS-1 β-cells **(C)** and αTC1-6 cells **(D)** after treatment with rIAPP, hIAPP and IL-1β in 11.1 mmol/l and 25 mmol/l glucose. Quantifications represent 4 micrographs per condition, 300-400 cells per microscopic field). Results are expressed as means ± SEM of three independent studies (n=3). *vs control (11.1 mmol/l glucose); #vs control (25 mmol/l glucose); *p*<0.05, two-way ANOVA).

CHAPTER 4: DISCUSSION

Amyloid deposits form in the pancreatic islets of patients with T2D as well as during islet culture and following transplantation into patients or animal models with T1D [1, 2]. Several studies from our and other research groups have demonstrated that hIAPP aggregates are toxic to islet β-cells and promote β-cell dysfunction and apoptosis [3, 4, 225]. In patients with T2D, amyloid formation is associated with progressive loss of β-cell mass and function [1, 3]. Also, it has been shown that amyloid formation in islets after transplantation into human patients or animal models with T1D is associated with loss of β-cells in the islet grafts [226], leading to recurrence of hyperglycemia [8, 9, 229]. However, the molecular mechanisms by which aggregation of endogenously produced hIAPP causes impaired β-cell function and apoptosis are still not clearly understood.

Increasing evidence from recent studies suggest that amyloid formation contributes to islet inflammation in T2D, mainly by mediating IL-1β signaling [10, 11]. Increased number of islet resident macrophages and elevated IL-1β levels have been reported in patients with T2D [13, 14, 54]. Also, studies from our research group have demonstrated that amyloid formation mediates IL-1β production in human and hIAPP-expressing transgenic mouse islets, which in turn leads to upregulation of the Fas cell death receptor and activation of the Fas-mediated apoptotic pathway in β-cells [11, 16, 17, 184]. Accordingly, blocking IL-1β signaling with IL-1RA treatment in diabetic mice transplanted with islets from hIAPP-expressing mice protected them from amyloid toxicity [187].

Importantly, islet α-cells have a better survival rate during diabetes. Studies have shown that α-cell mass remains unchanged or is increased whereas β-cell mass progressively declines during diabetes [98, 231]. For example, a recent study performed on human pancreatic tissues has reported that α-cell mass relative to β-cell mass was increased after the onset of T2D, which was associated with the length of chronic hyperglycemia [98]. The increased α-cell mass observed in both T1D and T2D is mainly due to increased α-cell proliferation (hyperplasia) and size (hypertrophy) [231], possibly mediated by elevated IL-6 levels [98]. Thus, patients with T1D and T2D have hyperglucagonemia in both basal and postprandial states associated with increased α-cell mass [19, 20].

An important finding from the previous studies in our lab is that both transformed and primary islet α-cells are more resistant than β-cells to the apoptotic effects of amyloid. The rate of apoptosis was much lower in transformed αTC1 cells treated with synthetic hIAPP than βTC-3 cells treated with the same concentrations of hIAPP [218]. Also, amyloid formation in both human and hIAPP-expressing mouse islets during *ex vivo* culture was associated with progressive β-cell loss, whereas α-cell mass remained almost the same, leading to decreased β/α cell ratio in amyloid forming islets [16, 17, 184, 232]. Finally, higher concentrations of IL-1β were required to induce apoptosis in αTC1-6 cells as compared with INS-1 β-cells [60].

The cellular mechanisms that protect α-cells from amyloid toxicity and other β-cell apoptotic factors such as elevated glucose and IL-1β are not well understood. It has been suggested that higher sensitivity of β-cells to the effects of IL-1β might be, at least partially, due to high abundance of its receptor, IL-1R1, on β-cell membrane [233]. The previous findings from our lab have shown that IL-1β/Fas/caspase-8 pathway plays an important role in amyloid-induced β-cell apoptosis in human islets and hIAPP-expressing transgenic mouse islets [49, 60, 184, 181]. In this MSc project, using transformed mouse αTC1-6 cells and rat INS-1 β-cells, we explored whether difference(s) in the expression of IL-1R1 and/or Fas receptor between α-cells and β-cells provides a potential cellular mechanism in α-cells that protects them from amyloid mediated IL-1β-induced apoptosis.

IAPP is specifically produced by islet β-cells. Therefore, β-cells are exposed to both intracellular and extracellular hIAPP aggregates, whereas α-cells do not produce IAPP and are exposed to extracellular hIAPP aggregates that are formed in the islets [113, 152, 166]. Therefore, in this project we focused on comparing the apoptotic effects of extracellular hIAPP aggregates on α-cells and β-cells. We used transformed rodent α-cells and β-cells and exposed both cell lines to extracellular recombinant hIAPP aggregates. Thus, in this experimental model, both cell lines were exposed to equal concentration of hIAPP and similar forms of extracellular hIAPP aggregates.

αTC1-6 cells and INS-1 β-cells were cultured in two glucose concentrations, 11.1 mmol/l which is the suggested optimal glucose concentration for INS-1 cells, and 25 mmol/l as elevated glucose concentration, to assess the potential synergetic effects of elevated glucose and amyloid-

or IL-1β-induced β-cell apoptosis. Rat IAPP, which is a non-fibrillogenic peptide due to the presence of three proline residues in its amino acid sequence and therefore is not toxic to β-cells, [113] was used as a control to detect any potential adverse effects of peptide treatment at the concentration used for hIAPP treatment in this study.

My studies with transformed αTC1-6 and INS-1 β-cells showed that IL-1R1 is consecutively expressed in both αTC1-6 cells and INS-1 β-cells but IL-1R1 immunoreactivity was lower in αTC1-6 cells than INS-1 β-cells as assessed by IL-1R1 mean fluorescence intensity. Moreover, there was no profound change in IL-1R1 immunoreactivity in αTC1-6 or INS-1 β-cells following 24 hours treatment with non-fibrillogenic rIAPP, fibrillogenic hIAPP or IL-1β in either 11.1 or 25 mmol/l glucose but hIAPP or IL-1β treated αTC1-6 cells had lower IL-1R1 immunoreactivity than corresponding INS-1 β-cell treated groups. These findings suggest that higher IL-1R1 immunoreactivity in INS-1 β-cells as compared to αTC1-6-cells may favor higher activation of the IL-1β signalling in INS-1 β-cells as compared to αTC1-6 cells. However, it should be noted that in these studies, we treated the cells with exogenous hIAPP or IL-1β for 24 hours and "n" of two independent studies were performed. Thus, future studies with longer time points and higher "n" are required to further validate these findings.

My studies further showed that neither αTC1-6 cells nor INS-1 β-cells expressed Fas at detectable levels under basal conditions. Treatment with hIAPP or IL-1β was associated with Fas upregulation after 8-12 hours in both αTC1-6 cells and INS-1 β-cells but the proportion of Fas-positive αTC1-6 cells was significantly lower following treatment with hIAPP or IL-1β as compared to corresponding INS-1 β-cell treated groups at both 11.1 and 25 mmol/l glucose concentrations. The lower number of Fas-positive αTC1-6 cells might be associated with the lower IL-1R1 immunoreactivity that we observed in αTC1-6 cells. Accordingly, higher Fas-positive INS-1 β-cells than αTC1-6 cells may promote higher binding of Fas ligand to its receptor on the β-cell membrane as compared to αTC1-6 cells, leading to higher activation of the Fas-mediated apoptotic signaling in INS-1 β-cells.

Finally, both αTC1-6 cells and INS-1 β-cells had very low number of apoptotic cells under basal conditions and following exposure to rIAPP, as assessed by quantification of TUNEL-positive cells. However, treatment with hIAPP or IL-1β significantly increased the proportion of apoptotic

cells in both αTC1-6 and INS-1 β-cells as compared to their corresponding non-treated groups. When αTC1-6 and INS-1 β-cells were compared, the rate of apoptosis was markedly lower in hIAPP- or IL-1β-treated αTC1-6 cells than INS-1 β-cells at either glucose concentration. Taken together, these findings suggest that the lower rate of apoptosis in hIAPP or IL-1β treated αTC1-6 cells than INS-1 β-cells is associated with the lower IL-1R1 and Fas expression in αTC1-6 cells as compared to INS-1 β-cells during culture at the same conditions.

It is worth noting that mouse αTC1-6 cells were generated by creating an adenoma in female mice expressing SV40 T oncogene with the rat preproglucagon promoter [234], whereas rat INS-1 β-cells were generated by a tumor induced by X-ray in male rats [235]. Therefore, potential impact of sex and species differences on IL-1R1 and Fas expression in these two cell lines should be considered. Additionally, there was a difference in passage number of αTC1-6 cells and INS-1 β-cells used in this study. Passage number for INS-1 β-cells (60-69) was higher than that of αTC1-6 cells (12-17) which may potentially affect their characteristics. It has been reported that increase in the passage number can negatively affect the cell heath and phenotype [236]. However, the impact of passage number on our studies is unlikely, because INS-1 β-cells have been reported to maintain a stable differentiated β-cell phenotype over 116 passages [237].

Finally, findings from these studies with transformed cell lines may not fully represent the conditions in primary islets. Human and hIAPP expressing mouse islets form both intracellular and extracellular amyloid in the presence of elevated glucose because endogenously produced hIAPP in primary β-cells can lead to both intracellular and extracellular hIAPP aggregation in β-cells [113, 179, 185, 211, 238]. Therefore, in primary islet β-cells, in addition to the Fas-mediated (extrinsic) apoptotic pathway initiated by extracellular hIAPP aggregates, the cytochrome c (intrinsic) pathway will also be activated by intracellular hIAPP aggregates during amyloid formation [238]. Also, in primary islets, elevated glucose promotes IAPP secretion from β-cells and thereby potentiates amyloid formation, whereas in transformed rodent cell line models that we used in this project, cells were exposed to recombinant hIAPP.

In summary, my studies show that amyloid formation and elevated IL-1β, two conditions associated with T2D, can promote apoptosis in both αTC1-6 and INS-1 β-cells *in vitro* through IL-1β/Fas apoptotic signalling pathway. Both IL-1R1 and Fas expression were higher in INS-1 β-cells

as compared to αTC1-6 cells treated with the same concentrations of hIAPP or IL-1β. The lower expression of IL-1R1 and Fas in αTC1-6 cells may provide a protective cellular mechanism that explains the better survival of αTC1-6 cells than INS-1 β-cells following exposure to hIAPP aggregates or elevated IL-1β.

Future directions

These studies were performed in transformed αTC1-6 and INS-1 β-cell lines *in vitro* which may not fully represent characteristics of primary islets cells. Therefore, studies with primary islets and animal models of amyloid formation are required to further validate these findings. Also, an important future study would be to assess the expression level of IL-1R2 that acts as a decoy receptor and supresses IL-1β signaling, at basal conditions and following exposure to amyloid and IL-1β in transformed αTC1-6 and INS-1 β-cells as well as following amyloid formation in human and hIAPP-expressing mouse islets.

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