EFFECTS OF ESTROGEN ON CARDIOMETABOLIC DISEASES

INVESTIGATING THE ROLE OF ESTROGENS ON THE MOLECULAR MECHANISMS MODULATING PANCREATIC BETA CELL HEALTH AND CARDIOMETABOLIC DISEASE

By MONICA DE PAOLI, MD

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Monica De Paoli, MD (Università degli Studi di Udine) SUPERVISOR: Dr Geoff H Werstuck

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LAY ABSTRACT

People who suffer from diabetes mellitus have a higher risk of developing heart attack and stroke compared to those who do not have diabetes. Moreover, the risk of heart attack and stroke is higher in men than in women. We still do not understand the underlying reasons for these differences. This thesis project has used unique mouse models that display many of the same sex differences in disease progression that we see in humans to study the pathways and mechanisms that promote diabetes development. Specifically, we examined the protective effects of estrogen towards the development of diabetes and cardiovascular disease and how this hormone affected specific cells and tissues. The results of these studies are important because they will provide more information regarding the effects of menopause and aging on chronic disease progression in women.

ABSTRACT

Sex-dependent differences in the prevalence of diabetes and cardiovascular diseases are well established. The objective of this project is to investigate the molecular mechanisms by which estrogen modulates chronic disease progression. Our lab, and others, have previously implicated endoplasmic reticulum (ER) stress in the development and progression of diabetes and cardiometabolic disease. We hypothesize that estrogens protect pancreatic beta cell health, and slow the progression of cardiometabolic disease, by modulating the unfolded protein response (UPR) in response to ER stress. Two distinct mouse models were used in these studies. The $ApoE^{-/-}Ins2^{+/Akita}$ mouse model of hyperglycemia-induced atherosclerosis, in which females are significantly protected from hyperglycemia and atherosclerosis relative to males, and the TALLYHO/JngJ mouse model, in which females are protected from chronic hyperglycemia relative to males. We found that ovariectomy of female $ApoE^{-/-}Ins2^{+/Akita}$ or TALLYHO/JngJ mice promoted chronic hyperglycemia. Supplementation with exogenous 17-beta estradiol significantly lowered blood glucose levels in ovariectomized $ApoE^{-/-}Ins2^{+/Akita}$ mice and reduced atherosclerotic lesion development in both male and ovariectomized female mice. Pancreatic islets from sham operated $ApoE^{-/-}Ins2^{+/Akita}$ female mice showed a significant increase in the expression of protective UPR factors and a decrease in pro-apoptotic factors, compared to males or ovariectomized females. To determine if alleviating ER stress could moderate hyperglycemia, male and ovariectomized female TALLYHO/JngJ mice were treated with the chemical chaperone 4-phenylbutryic acid (4-PBA). We showed that 4-PBA treatment significantly lowered fasting blood glucose levels and improved glucose tolerance. The results of this thesis suggest that estrogens play a protective role in the maintenance of beta cell health and blood glucose regulation by activating the adaptive UPR. This mechanism may explain the protection observed in premenopausal women and may lead to the development of targeted therapies to treat diabetes and cardiometabolic diseases.

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Finally, I want to dedicate this thesis to my niece, Allyson. May you always smile at life and grow up to become the confident and successful woman that lies within you.

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LIST OF ABBREVIATIONS AND SYMBOLS

4-PBA, 4-phenylbutyric acid

A1C, glycated hemoglobin

ACM, alpha cell mass

ANOVA, analysis of variance

ASK1, apoptosis signal-regulating kinase 1

ATF4, activating transcription factor 4

ATF6, activating transcription factor 6

ATP, adenosine triphosphate

BAK, Bcl-2-antagonist/killer 1

BAX, Bcl-2-associated x protein

BCM, beta cell mass

BIM, Bcl-2 like protein 11

CHD, coronary heart disease

CVD, cardiovascular disease

DAPI, 4′,6-diamidino-2-phenylindole

DKA, diabetic ketoacidosis

DM, diabetes mellitus

E1, estrone

E2, estradiol

E_3, estriol

EDEM, ERAD-enhancing alpha-mannosidase-like protein

eIF2α, translation initiation factor 2α

ELISA, enzyme-linked immunosorbent assay

ER, endoplasmic reticulum

ERAD, endoplasmic reticulum associated degradation

ERα, estrogen receptor alpha

ERβ, estrogen receptor beta

GADD153/CHOP, C/EBP homologous protein

GLUT2, glucose transporter 2

GPERs, G protein-coupled estrogen receptors

GRP78, glucose-regulated protein 78

GRP94, glucose-regulated protein 94

GTT, glucose tolerance test

HDL, high density lipoproteins

HHS, hyperosmolar hyperglycemic state

HRT, hormonal replacing therapy

ICAM-1, intracellular adhesion molecule 1

IFG, impaired fasting glucose

IgG, immunoglobulin G

IGT, impaired glucose tolerance

IL-10, interleukin 10

IL-6, interleukin 6

IP, intraperitoneal

IRE1α, inositol-requiring enzyme 1 alpha

JNK, Jun N-terminal kinase

KDEL, amino acid sequence Lysine-Aspartic acid-Glutamic acid-Leucine

MODY, maturity onset diabetes of the young

ovx, ovariectomized

PCR, polymerase chain reaction

PDI, protein disulfide isomerase

PERK, pancreatic endoplasmic reticulum kinase

qRT-PCR, real time quantitative polymerase chain reaction

rER, rough endoplasmic reticulum

SD, standard deviation

SEM, standard error of the mean

sER, smooth endoplasmic reticulum

SHBG, sex hormone-binding globulin

SMC, smooth muscle cells

STZ, streptozotocin

sXBP1, spliced x-box protein 1

tER, transitional endoplasmic reticulum

TH, TALLYHO/JngJ mice

TNFα, tumor necrosis factor alpha

TXNIP, thioredoxin-interacting protein

UPR, unfolded protein response

VCAM-1, vascular cell adhesion molecule 1

VLDL, very low density lipoproteins

WFS1, Wolframin ER Transmembrane Glycoprotein

XBP1, x-box protein 1

ZDF, Zucker diabetic fatty rat

DECLARATION OF ACADEMIC ACHIEVEMENT

This research project has been conducted between September 2015 and December 2021. The author started as a Master's student in the Chemical Biology graduate program, and then transferred in the PhD program in April 2017.

The current COVID-19 pandemic significantly impacted the progression of this research project. The delay was due to lab closures, mandates to operate at reduced capacity, and restrictions in conducting animal research (mainly related to the number of animals allowed to be enrolled for experimental procedures). During this time the author co-authored an additional review paper (Appendix 3). The Chemical Biology Graduate Program has granted an extension of 8 months to this project, corresponding to the approximate time lost during these unforeseen circumstances.

A detailed description of the author's original contributions, as well as that of other co-authors, is outlined at the beginning of each manuscript chapter (Chapters 2-4), under the paragraph "Contributions". Additionally, the author has conceived and approved the illustrations prepared by undergraduate students (mentored by the author) for the "Introduction" chapter. Figure 1 was prepared by Susanna Fang, Figures 2 and 4 were prepared by Zinal Patel, and Figure 3 has been prepared by Alexander Zakharia.

CHAPTER 1 – INTRODUCTION

Diabetes Mellitus definition, classification, diagnosis

Diabetes Mellitus (DM) is a metabolic disorder characterized by the presence of chronically elevated blood glucose levels (hyperglycemia) (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee & Cheng, 2013).

DM can be categorized as type 1 DM, type 2 DM, maturity onset diabetes of the young (MODY), and gestational DM. In type 1 DM insulin-producing pancreatic beta cells are progressively destroyed through an autoimmune process which leads to insulin deficit. Type 2 DM develops when a combination of genetic, metabolic, and environmental factors induce an altered response to insulin in the hepatic, skeletal, and/or adipose tissue. Generally, prior to developing overt type 2 DM, individuals show a dysregulation of glucose homeostasis in the form of impaired fasting glucose (IFG) or impaired glucose tolerance (IGT). MODY is a monogenic form of diabetes that exhibits autosomal dominant inheritance leading to the early onset of hyperglycemia and altered insulin secretion due to pancreatic beta cell dysfunction. This form of diabetes accounts of about 1-5% of people with DM (Hoffman et al., 2022; Urakami, 2019). Gestational DM is characterized by the development of insulin resistance as a result of metabolic changes during the later stages of pregnancy. This form of diabetes usually disappears after delivery, but women with gestational DM do have 30-60% risk to develop DM later in life (Johns et al., 2018). The shared feature of these various types of DM is hyperglycemia resulting from the inability of insulin, secreted by pancreatic beta cells, to effectively regulate blood glucose levels.

According to Diabetes Canada DM is characterized by the presence of any of the following diagnostic criteria:

- The presence of diabetes symptoms such as polyuria, polydipsia, weight loss, along with the finding of a random blood glucose level measurement of over 11.1 mM (200mg/dl)
- The presence of fasting blood glucose levels over 7mM (126mg/dl) when there is no caloric intake for at least 8 hours
- The presence of glycated hemoglobin (A1C) above 6.5% (in adults and not if type 1 DM is suspected)
- The detection of plasma glucose levels of over 11.1mM (200mg/dl) two hours after a 75g oral glucose tolerance test (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee & Cheng, 2013)

Normoglycemia is defined as fasting plasma glucose levels below 5.6mM (100mg/dl) or A1C below 5.6%. IFG is defined as fasting plasma levels between 6.1-6.9mM, whereas IGT presents with glucose levels between 7.8-11mM after an oral glucose challenge. Prediabetes is defined as IFG, IGT, or A1C between 6.0-6.4mM. People with prediabetes are considered at high risk of developing diabetes however, it may be

possible to restore normoglycemia by introducing lifestyle interventions such as modifying diet composition, increasing physical exercise and weight loss (Tuso, 2014).

Epidemiology of DM

DM is a growing health concern and recent statistics have shown that over 8% of the global adult population has DM, with the majority of these cases being type 2 DM (Tao et al., 2015). Estimates predict that the prevalence of diabetes will rise to over 10% of the global population by the year 2030 (Saeedi et al., 2019). DM this is not just a concern for the adult population as it has been observed that the incidence of DM, and particularly type 2 DM, is rapidly increasing in children and adolescents. Adolescent type 2 DM, shows a more aggressive phenotype, and the transition from prediabetes to DM is much faster compared to the adult population (Chen et al., 2012; Kao & Sabin, 2016; Temneanu et al., 2016). Moreover, people with DM tend to have a higher mortality rate than people without diabetes, and in recent years this disease has been the 8th leading cause of death among the worldwide population (Tao et al., 2015). In addition to being a global major health concern, the increasing prevalence of DM is also an economic concern, as treatment of this disease and its associated complications poses a significant burden on health care costs worldwide (Authors/Task Force Members et al., 2013; Rowley et al., 2017).

Acute and chronic complications of DM

DM is associated with the presence of complications that can be categorized as acute or chronic.

Acute complications develop rapidly and include diabetic ketoacidosis (DKA) and hyperosmolar hyperglycemic state (HHS). Both are considered medical emergencies and need immediate attention and intervention. DKA is the result of insulin deficiency (typically observed in type 1 DM) or insulin suppression due to high levels of counter insulin hormones, mainly glucagon, or catecholamines, cortisol and growth hormone (typically observed in type 2 DM). The reduced ratio between insulin and glucagon promotes the activation of gluconeogenesis, glycogenolysis and formation of ketone bodies. The high concentration of glucose results in loss of water, sodium, potassium, and chloride via the urinary tract, therefore inducing hypovolemia and electrolyte imbalance. The symptoms associated with DKA are nausea, vomiting, abdominal pain, tachycardia (elevated heartbeat rate), Kussmaul's breathing, acetone-odour breath, and a state of confusion (Umpierrez & Korytkowski, 2016).

HHS is an acute complication that can be seen more frequently in type 2 DM. Reduced levels of insulin associated with a reduced hydric intake and peripheral insulin resistance can promote HHS. These conditions are associated with an increased production of glucose by the liver through glycogenolysis and gluconeogenesis, and there is an impaired uptake of glucose by the skeletal muscle. Hyperglycemia induces a more severe hypovolemia than DKA and the state of confusion is more evident. Nausea, vomiting, abdominal pain and Kussmaul's breathing are absent in this manifestation. It is not fully understood why, in HHS, ketone bodies are minimal: some

studies suggest that this could be the result of insulin deficiency being less severe in HHS, compared to DKA. Other theories could be that the level of counter insulin hormones and free fatty acids are less prominent than in DKA so the liver may not produce as many ketone bodies, or that the ratio between insulin and glucagon does not favour ketogenesis (Adeyinka & Kondamudi, 2022; Canadian Diabetes Association Clinical Practice Guidelines Expert Committee & Cheng, 2013; Umpierrez & Korytkowski, 2016).

The chronic complications of DM develop over time and can be divided into vascular and non-vascular. Non-vascular complications of DM include an increased predisposition to infections, skin changes, gastroparesis and hearing loss (Powers, 2014). The vascular complications can be further distinguished as microvascular or macrovascular (Fowler, 2008; Stratton et al., 2000).

The microvascular complications of DM give rise to the conditions of diabetic retinopathy, diabetic nephropathy, and diabetic neuropathy.

Diabetic retinopathy can be subdivided into two types, non-proliferative and proliferative. The former typically manifests between 10 and 20 years after DM is diagnosed, and it is characterized by small aneurisms in the retina, flame hemorrhages and cotton wool spots. The manifestation can progress, modifying the vessel calibre and extending the previous alterations. This condition can lead to blindness. Proliferative retinopathy is the evolution of severe forms of non-proliferative retinopathy, and is characterized by excessive neovascularization, which can establish along the optical nerve or the macula. These new vessels are very fragile and can easily break, resulting in hemorrhages and fibrosis. The scar tissue formed can retract causing retinal detachment (Powers, 2014).

Diabetic nephropathy represents the major cause of end-stage renal disease, as well as one of the main causes of diabetes-related morbidity. People with diabetic nephropathy also typically present with diabetic retinopathy. The typical features of diabetic nephropathy are glomerular lesions called glomerulosclerosis, which irreversibly alter the structure of the glomeruli, a series of capillaries that form part of the nephron and are a key component of the filtering system of the kidney. Chronic hyperglycemia can damage the glomeruli altering their structure. These modifications impair the filtering functionality of the glomeruli. Initially, the remaining healthy glomeruli can compensate the filtering duties for the damaged ones, but eventually they may also sustain irreversible damage. Ultimately, this significant loss of function can lead to kidney failure (Qi et al., 2017).

Diabetic neuropathy can manifest itself through various pathways. One of these is polyneuropathy, a degeneration of peripheral nerves that can lead to numbness and tingling, and typically starts in the feet and legs. Diabetic neuropathy can also manifest with mononeuropathy, characterized by pain or motor weakness in the area innervated by a single nerve; or polyneuropathy if the area is innervated by two or more nerves. Finally, diabetic neuropathy can present as autonomic neuropathy which can lead to dysfunctions in the gastrointestinal system (altered gastric motility, gastroparesis), the cardiovascular system (with an increased heart rate at rest or orthostatic hypotension), and/or erectile dysfunction. Although the specific mechanisms underlying the

development of diabetic neuropathy are not yet fully understood, it is believed that these alterations can result from high levels of glucose either inducing damage to the nerves (demyelination), or through alterations to the vascular system that supports the nerves through the thickening of the arterioles and hyalinization (Powers, 2014).

The macrovascular complications of DM are characterized by the development of accelerated atherosclerosis of the aorta and large and medium calibre arteries. Atherosclerosis represents the main underlying cause of cardiovascular diseases (CVDs) which can clinically manifest as coronary heart disease, stroke, and peripheral vascular disease. These complications are the main cause of long-term sequelae or death in patients with diabetes and causing a significant burden on health care systems (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee & Cheng, 2013; Damaskos et al., 2020; Viigimaa et al., 2020).

It has been observed that modifications in lifestyle such as adopting a diet low in calories and fat, increasing physical exercise and improving blood glucose levels show a significant benefit for the microvascular complications and, if blood glucose level targets are achieved early, also for the macrovascular complications (Holman et al., 2008; Nathan et al., 2005).

Sex differences in DM and its associated CVDs

Accumulating evidence shows that sex differences in the prevalence of DM do exist, with premenopausal women appearing to be protected from the development of DM, compared to postmenopausal women or men (Gale & Gillespie, 2001; Meisinger et al., 2002; The Emerging Risk Factors Collaboration, 2010; Wändell & Carlsson, 2014). It has been observed that insulin resistance which is associated with the development of some forms of DM, significantly increases in postmenopausal women however, when treated with estrogen replacement therapy, the risk of developing metabolic disturbances is significantly reduced (J.-E. Kim et al., 2019; Pu et al., 2017). These findings suggest that there is a protective role of estrogens in the development of DM and metabolic disorders. This theory is supported by two large, randomized, double-blind, placebo-controlled clinical trials, the Women's Health Initiative and the Heart and Estrogen/Progesterin Replacement studies, which showed that women taking hormonal replacement therapy were less prone to developing DM than those receiving placebo (Margolis et al., 2004; Salpeter et al., 2006).

Sex differences also do exist in the presentation and outcome of diabetes-associated CVDs. Men with diabetes have a 3- to 5- fold increased risk of coronary heart disease (CHD) compared to premenopausal women (Winham et al., 2015). It has been observed that women tend to develop CHD far later in life than men, further supporting the potential protective role of estrogens (Anand et al., 2008). Additionally, evidence suggests that DM represents a more powerful risk factor for coronary heart disease in postmenopausal women, compared to men because it erases the biological female advantage in terms of global risk (Appelman et al., 2015). Various clinical studies support this observation as it has been shown that women with DM have a far higher increased risk of developing CHD and stroke compared to men with DM, even after

accounting for other major cardiovascular risk factors (Huxley et al., 2006; Peters et al., 2014a, 2014c; The Emerging Risk Factors Collaboration, 2010). Furthermore, women with DM tend to have a far greater risk of hospitalization and/or mortality due to diabetes-associated CVDs than men with DM, even as treatment of these diseases has become more equal between sexes (Peters et al., 2014a, 2014c; Roche & Wang, 2013).

Since the risk of developing DM and its associated cardiovascular complications tend to increase after menopause, these studies suggest that the presence of estrogen could play a protective role in cardiometabolic health.

Animal models of diabetes

To better understand the mechanisms underlying sex differences in cardiometabolic disease progression, rodent models can be used to uncover additional information regarding the pathogenesis of these metabolic disorders. Many rodent models of DM present with sexual dimorphism in terms of glucose homeostasis. Ovariectomy in female rodents is typically used to mimic estrogen deprivation that is observed in postmenopausal women (De Paoli & Werstuck, 2020). It is important to note that rodents normally do not exhibit chronic hyperglycemia, therefore this condition is typically induced by chemically inducing hyperglycemia or by inducing, or selecting for, genetic mutations/variants on one (monogenic) or more (polygenic) genes.

An example of a mouse model where chronic hyperglycemia is chemically induced is the streptozotocin (STZ)-induced rodent model. STZ is an antineoplastic drug that is selectively toxic to pancreatic beta cells and induces cell death by damaging cellular DNA. It has been observed that female rodents are more resistant to pancreatic beta cell damage than males, and higher doses and/or multiple injections of STZ are typically required to acquire a chronic hyperglycemic phenotype (Le May et al., 2006; Y. Li et al., 2018; Paik et al., 1982).

The Werstuck lab has developed a rodent model of hyperglycemia-induced atherosclerosis, the ApoE$^{-/-}$:Ins2$^{+/Akita}$ mouse. The deficiency of apolipoprotein E promotes atherosclerosis, and chronic hyperglycemia is induced by a point mutation in one allele of the Ins2 gene (monogenic induced hyperglycemia). This mutation (C96Y) results in the substitution of cystine 96 with tyrosine, thus interfering with proper insulin folding and maturation, leading to chronic hyperglycemia. It has been observed that male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice develop chronic hyperglycemia by three weeks of age, whereas female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice are only transiently hyperglycemic and normalize their blood glucose levels at about 5 weeks of age (chapter 2(Venegas-Pino et al., 2016)). An extensive characterization of this mouse model will be described in chapters 2 and 3 of this thesis.

An example of polygenic diabetes is represented by the TALLYHO/JngJ mouse model. These mice are a model of type 2 DM and obesity and show sexual dimorphism in terms of glucose regulation. Male TALLYHO/JngJ mice are chronically hyperglycemic, whereas females remain normoglycemic throughout their lifespan (J. H. Kim et al.,

2006). An extensive explanation of the derivation and characterization of the TALLYHO/JngJ mouse model is provided in chapter 4 of this thesis.

Endocrine pancreas and the regulation of blood glucose levels

The pancreas is a retroperitoneal organ oriented transversely and extending from the C loop of the duodenum to the hilum of the spleen. It can be divided into three parts: head, body and tail. The main pancreatic duct (Wirsung) drains into the duodenum at the Papilla of Vater where merges with the common bile duct creating the ampulla of Vater which is a common channel for pancreatic and biliary drainage. The accessory duct (Santorini) usually drains in a separate papilla aside from the Papilla of Vater.

The pancreas has a lobulated structure containing distinct exocrine and endocrine components. The exocrine pancreas represents the majority of the organ, and it is made up of acinar cells, which produce digestive enzymes that are conveyed to the duodenum stored in zymogen granules. The cells of the endocrine pancreas are usually clustered in the Islets of Langerhans. These represent the major center of blood glucose regulation.

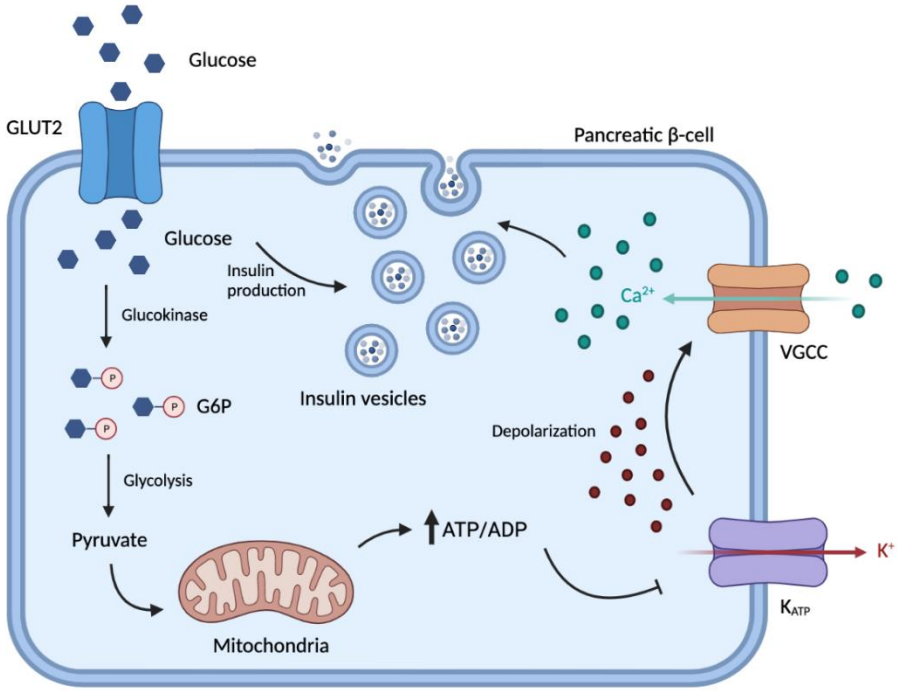
Within the islets of Langerhans at least four types of cells can be distinguished. Alpha cells are responsible for secreting glucagon, a hormone that stimulates an increase in blood glucose levels. Beta cells secrete insulin, the key hormone in reducing blood glucose levels. These cells usually represent the core of the islet, and their activity is mainly regulated by blood glucose levels. Gamma cells (also known as PP cells) are responsible for secretion of the pancreatic polypeptide, which can stimulate intestinal motility, as well as the secretion of gastric and intestinal enzymes. Delta cells secrete somatostatin, a hormone that can regulate the secretion of glucagon, insulin as well as other hormones (Skelin et al., 2010).

Mechanisms of insulin secretion

Insulin is synthesized in the endoplasmic reticulum (ER) starting from a precursor, preproinsulin, which contains two chains (A and B) linked together by the C-peptide. At the N-terminus there is a signal peptide that is responsible for translocating this protein from the ER-bound ribosomes into the ER lumen. Here, preproinsulin matures into proinsulin by the removal of the signal peptide and the formation of two disulfide bonds between the A and B chains. Proinsulin moves to the Golgi apparatus where it is stored in vesicles and is converted to insulin by the cleavage of the C-peptide and the formation of an additional disulfide bond in the A chain. Insulin is typically stored in intracellular granules in the form of hexamers containing zinc (Skelin et al., 2010).

Insulin release is directly regulated by blood glucose concentration. Glucose enters the beta cell through the glucose transporters 2 (GLUT2) where it is used by the mitochondria to produce adenosine triphosphate (ATP). When blood glucose levels increase, there is a corresponding increase in beta cell ATP production that induces the closure of the plasma membrane potassium channels K_{ATP}. This causes the depolarization of the cell membrane, which in turn induces the opening the voltage

gated $^{Ca2+}$ channels with a subsequent increase of the influx of extracellular Ca^{2+}. This stimulates the immediate release of insulin stored in the vesicles (Skelin et al., 2010). Should the secretory stimulus continue, the protracted depolarization promotes the de novo biosynthesis of insulin (Figure 1).



Introduction figure 1. Insulin secretion.

Glucose enters inside the beta cell through the GLUT2 transporter and is used by the mitochondrion to produce ATP. The increased ATP levels determine closure of plasma membrane potassium channels (K_{ATP}) which in turn result in depolarization. As a consequence, the voltage-gated-Ca^{2+} channels open and intracellular Ca^{2+} concentration increases. The subsequent electrical activity created induces movement of vesicles containing insulin to the plasma membrane where insulin is released. Image created with BioRender.com

The ER, ER stress and the unfolded protein response (UPR)

The ER is a subcellular organelle located in the cytosol of the eukaryotic cells. It is made up of a membrane-bound system of branching tubules and flattened sacs connected to the outer membrane of the nucleus and extending throughout the cytosol. The membranes surround a space which is separated from the cytoplasm called the ER lumen (Fagone & Jackowski, 2009). The ER can be subdivided into three areas, i) the rough ER (rER) which presents flattened saccules encapsulated by membranes that carry a high quantity of ribosomes, ii) the smooth ER (sER) which does not have ribosomes, and iii) the transitional ER (tER) which is partly rough and partly smooth. The rER functions in protein synthesis, translocation and folding; the sER is involved in the synthesis and storage of lipids; whereas the tER is involved in protein transportation towards the Golgi apparatus, calcium storage, drug handling, detoxification and lipid and steroid synthesis (Lavoie & Paiement, 2008).

Proteins synthesized in the ER are typically destined to be secreted out of the cell or to be localized to an intracellular membrane system. Pancreatic beta cells are capable of synthesizing and secreting large quantities of insulin and therefore they have a well-structured ER. Cells that do not secrete large quantities of proteins usually have a smaller less complex ER, with a reduced protein processing capacity (Oakes & Papa, 2015).

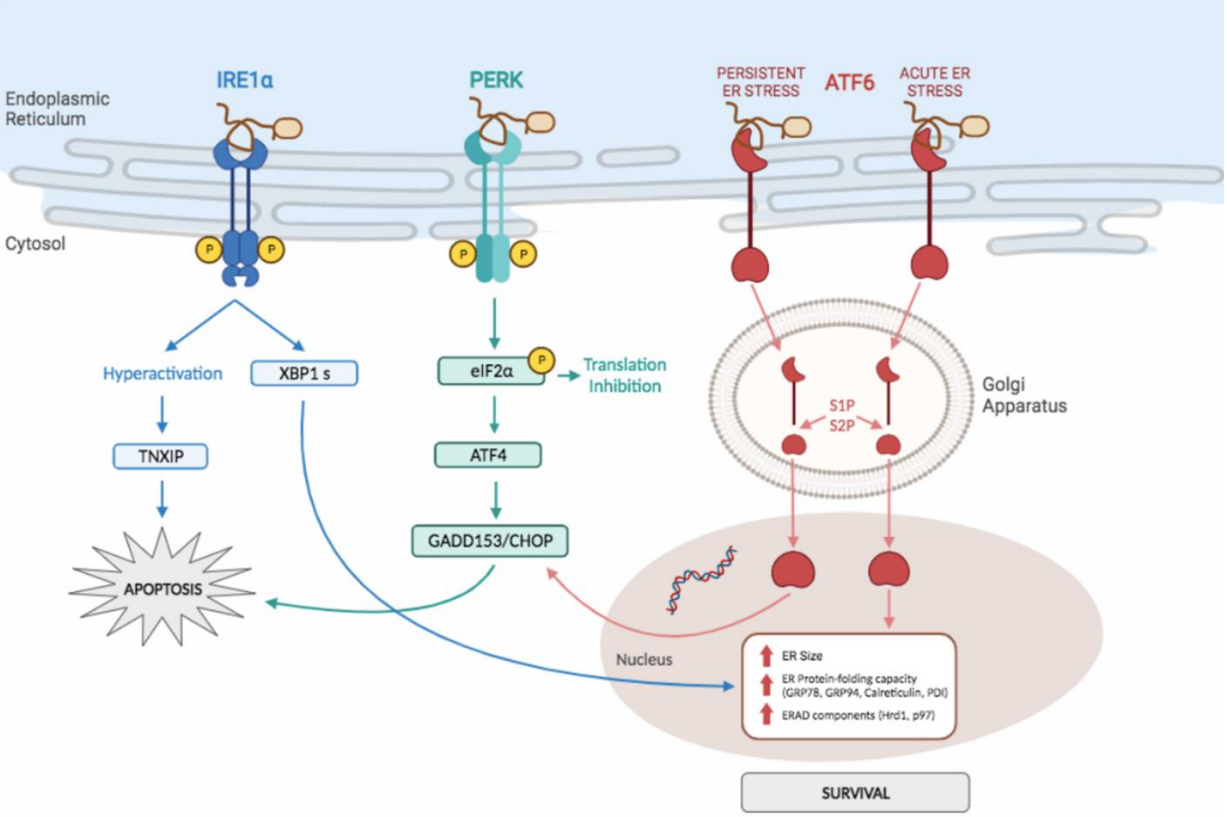
If the rate of the de novo protein synthesis exceeds folding capacity of the ER there can be an accumulation of misfolded proteins altering the homeostasis and disrupting the function of the ER. This condition is known as ER stress. Cells have developed a system to respond to this situation in order to restore homeostasis: the Unfolded Protein Response (UPR) (Koksal et al., 2021; Oakes & Papa, 2015; Tabas & Ron, 2011). The UPR is a signal transduction pathway that functions to restore ER homeostasis by increasing protein folding capacity (adaptive UPR) through the increased synthesis of molecular chaperones and by increasing the degradation of misfolded proteins through an increased expression of ER associated degradation (ERAD) components. If the adaptive UPR can limit ER stress and restore ER homeostasis, the cell can survive and continue to function. However, when ER homeostasis cannot be restored, the UPR induces the expression of proapoptotic molecules to induce cell death (apoptotic UPR) (Ron & Walter, 2007; Walter & Ron, 2011). The UPR is activated by three distinct ER transmembrane proteins: inositol-requiring enzyme 1α (IRE1α), pancreatic endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6) (Figure 2). Each of these signalling factors contains an ER luminal domain that can sense elevated concentrations of protein misfolding and each signalling pathway has a specific set of downstream targets (Ron & Walter, 2007; Tirasophon et al., 1998; S. Wang & Kaufman, 2012; X. Z. Wang et al., 1998).

Upon activation, IRE1α autophosphorylates thereby activating a RNAse domain. The IRE1α RNAse excises a 26-nucleotide intron from the mRNA encoding the X-box protein 1 (XBP1) transcription factor, which subsequently enhances the transcription of genes responsible for increasing ER protein folding capacity. If the stress levels are too high and homeostasis cannot be reached, IRE1α is hyperactivated by undergoing homo-oligomerization. Its RNAse activity then degrades mRNAs encoding an N-terminal signal sequence, which reduces protein-folding load and also reduces the expression of

proteins that repress apoptosis. IRE1α oligomerization also upregulates pro-inflammatory proteins such as thioredoxin-interacting protein (TXNIP), which in turn activate the caspase-1 pathway, as well as the expression of apoptosis signal-regulating kinase 1 (ASK1) and Jun N-terminal kinase (JNK) which activate Bcl2 like protein 11 (BIM). BIM inhibits anti-apoptotic molecules such as Bcl-2 and enhances the expression of pro-apoptotic molecules such as Bcl-2-associated x protein (BAX) and Bcl-2-antagonist/killer 1 (BAK), thereby promoting apoptosis through the mitochondrial pathway (Oakes & Papa, 2015).

Upon activation, PERK forms homodimers which activate a kinase domain. Activated PERK phosphorylates, and thereby inhibits, translation initiation factor 2α (eIF2α). This results in a reduction in general protein translation, which alleviates the protein load on the ER processing machinery. The reduced concentration of eIF2α promotes an upregulation of activating transcription factor 4 (ATF4), which in turn increases expression of C/EBP homologous protein (GADD153/CHOP). GADD153/CHOP promotes the transcription of XBP1 and ER resident chaperones. An extended block of the translation process induced by PERK, such as what happens when ER stress is protracted, is incompatible with cell survival. This leads to high levels of GADD153/CHOP which inhibit the expression of anti-apoptotic BCL-2 and upregulate pro-apoptotic molecules such as BIM, triggering the apoptotic process (Oakes & Papa, 2015; Song et al., 2017).

Activated ATF6 is translocated to the Golgi where it is cleaved by Site-1 and Site-2 protease. This causes the release of the ATF6(N) transcription factor, that along with XBP1s, functions to increase expression of proteins that enhance the ER protein-folding capacity. When homeostasis cannot be restored, ATF6 can indirectly activate the apoptotic mechanism by increasing the expression of XBP-1 and GADD153/CHOP (Song et al., 2017).



Introduction figure 2. The unfolded protein response to ER stress.

The accumulation of misfolded proteins in the ER activates three ER transmembrane signaling factors PERK, IRE1 and ATF6, which initiate the unfolded protein response (UPR). Initially the adaptive UPR acts to reestablish ER homeostasis by; i) decreasing protein flux into the ER (translation block), ii) enhancing the folding capacity of the ER by increasing the expression of molecular chaperones such as GRP78, Calreticulin and Protein Disulfide Isomerase (PDI) and WFS1, and iii) increasing the ER associated degradation of misfolded proteins (ERAD). Chronic ER stress results in apoptosis caused by hyperactivation of IRE1α, and/or the activation of GADD153/CHOP by PERK. Image created with BioRender.com

The role of ER stress in diabetes

Protracted ER stress and consequential cell injury have been implicated in the pathophysiology of many diseases including diabetes (Araki et al., 2003; Back & Kaufman, 2012; Cao et al., 2020; Cnop et al., 2017; S. Wang & Kaufman, 2012). Under conditions of chronically elevated glucose levels, pancreatic beta cells are required to secrete a significant amount of insulin. This increased demand of insulin production can overwhelm the ER of the pancreatic beta cells, beyond the capacity of the adaptive UPR. In this instance, when ER homeostasis cannot be restored, pro-apoptotic pathways are activated leading to beta cell loss (Araki et al., 2003; Back & Kaufman, 2012; Cao et al., 2020; Oyadomari et al., 2002).

The link between diabetes/hyperglycemia, ER stress and beta cell loss can be seen in the streptozotocin (STZ)-injected mouse model. In this experimental model hyperglycemia is induced by streptozotocin, a drug that is selectively toxic for pancreatic beta cells, and it has been observed that hyperglycemia in these mice is associated with increased levels of ER stress leading to pancreatic beta cell loss (Ahn et al., 2015; Werstuck et al., 2006). In humans the clinical signs of Wolfram Syndrome, an autosomal recessive disorder due to a mutation of the Wolframin ER Transmembrane Glycoprotein (WFS1) gene, are due to the presence of severe ER stress (Pallotta et al., 2019). WFS1 aids in protein folding and can stimulate the ERAD process and is also involved in regulating the ATF6 pathway. One of the disorders associated with this disease is the presence of DM, as these genes are highly expressed in pancreatic beta cells and are involved in maintaining ER homeostasis by regulating ATF6 activation. In people with Wolfram Syndrome ATF6 is hyperactivated leading to the expression of apoptotic UPR markers which eventually induce pancreatic beta cell loss (Stone et al., 2021). Another example is represented by the Wolcott-Rallison syndrome, another autosomal recessive type of diabetes characterized by mutations in the *EIF2AK3* gene which encodes PERK. These mutations cause a loss of function of the PERK kinase activity which can't properly regulate eIF2α. Therefore, when the demand of insulin is increased, the consequent ER stress cannot be mitigated leading to pancreatic beta cell loss (Fonseca et al., 2009; Stone et al., 2021).

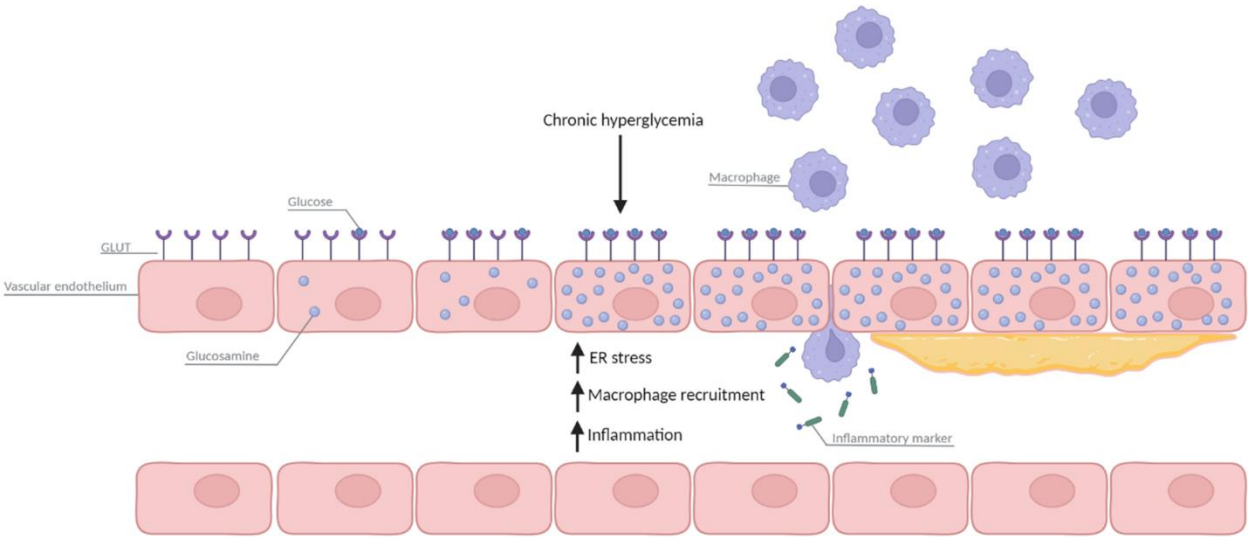
The PERK-knock out mouse is a mouse model that shows many features of Wolcott-Rallison syndrome, a disease whose main feature is the development of diabetes during neonatal or early stages of life. The lack of PERK results in an impaired UPR causing a rapid loss of beta cells by apoptosis (Gupta et al., 2010). These mice also develop other exocrine defects because of the premature death of secretory cells, however diabetes is the earliest consequence of this mutation, suggesting that pancreatic beta cells are quite sensitive to ER stress. Another rodent model is represented by mice with a deletion of Xbp1. These mice are not able to process proinsulin and therefore their insulin secretion is impaired (Oakes & Papa, 2015; Zhang et al., 2021). The $Wfs1^{-/-}$ mouse model resembles the features of Wolfram Syndrome. Deletion of WFS1 results in early loss of beta cells as a result of an ineffective response to ER stress (Fonseca et al., 2010; Takeda et al., 2001).

The loss of a significant number of pancreatic beta cells, whether it be due to aging or disease, will put an added burden on the remaining cells as they try to compensate for the deficiencies in insulin production. As chronic levels of hyperglycemia are

established, the remaining cells will experience protein overload and chronic ER stress (Oakes & Papa, 2015). These hypotheses can provide an explanation for the onset of the various kinds of DM. As an example, in type 1 DM the loss of pancreatic beta cells is due to an autoimmune attack. This would cause the remaining cells to compensate for the lost ones therefore protracting and aggravating ER stress. In type 2 DM, insulin resistance increases the demand for insulin production. In both cases the result is a progressive increase in insulin production and ER stress which could trigger the apoptotic pathway with consequent loss of beta cells leading to pancreatic beta cell failure and uncontrolled hyperglycemia.

The role of ER stress in atherogenesis

As explained earlier in this chapter, diabetes is associated with the accelerated development of atherosclerosis, which in turn leads to CVDs. However, the molecular mechanisms by which hyperglycemia promotes atherosclerosis initiation and progression are not clear. Studies on experimental rodent models suggest that ER stress might play a role (McAlpine et al., 2010). Apolipoprotein E is a component of very low density lipoproteins (VLDL) and high density lipoproteins (HDL), playing a major role in the clearance of these lipoproteins from the blood stream. ApoE deficiency in this rodent model is associated with a hyperlipidemic phenotype which leads to the development of atherosclerosis. It has been observed that hyperglycemia induced by STZ injections in the $ApoE^{-/-}$ mice significantly accelerates the development of atherosclerosis with plaques being significantly higher in volume than those of normoglycemic $ApoE^{-/-}$ mice (McAlpine et al., 2010). Furthermore, it was observed that the hyperglycemic $ApoE^{-/-}$ mice have high levels of glucosamine, a glucose metabolite and a potent ER stress inducer and proinflammatory agent. It has been observed that the accumulation of glucosamine in the vascular endothelial cells and infiltrating macrophages significantly promotes ER stress and the expression of UPR markers (Beriault & Werstuck, 2013; McAlpine et al., 2010). Based on this evidence it can be hypothesized that ER stress might play a role in the increased cardiovascular risk observed in people with DM. Inflammation could be further aggravated by dyslipidemia, which is typically present in people with diabetes, as well as by the accumulation of glucosamine in other components of the atherosclerotic process such as macrophages (Figure 3). Further research needs to be conducted to specifically delineate these mechanisms.



Introduction figure 3. Proposed mechanism that links hyperglycemia, ER stress and atherosclerosis initiation and progression.

Chronic hyperglycemia determines the accumulation of glucose's end product, glucosamine. Glucosamine can enter the vascular endothelial cells increasing levels of ER stress and activating a proinflammatory response that recruits macrophages, therefore initiating atherosclerotic plaque buildup. This can be aggravated by dyslipidemia, as well as the maintenance of the proinflammatory response due to chronic levels of glucosamine. Image created with BioRender.com

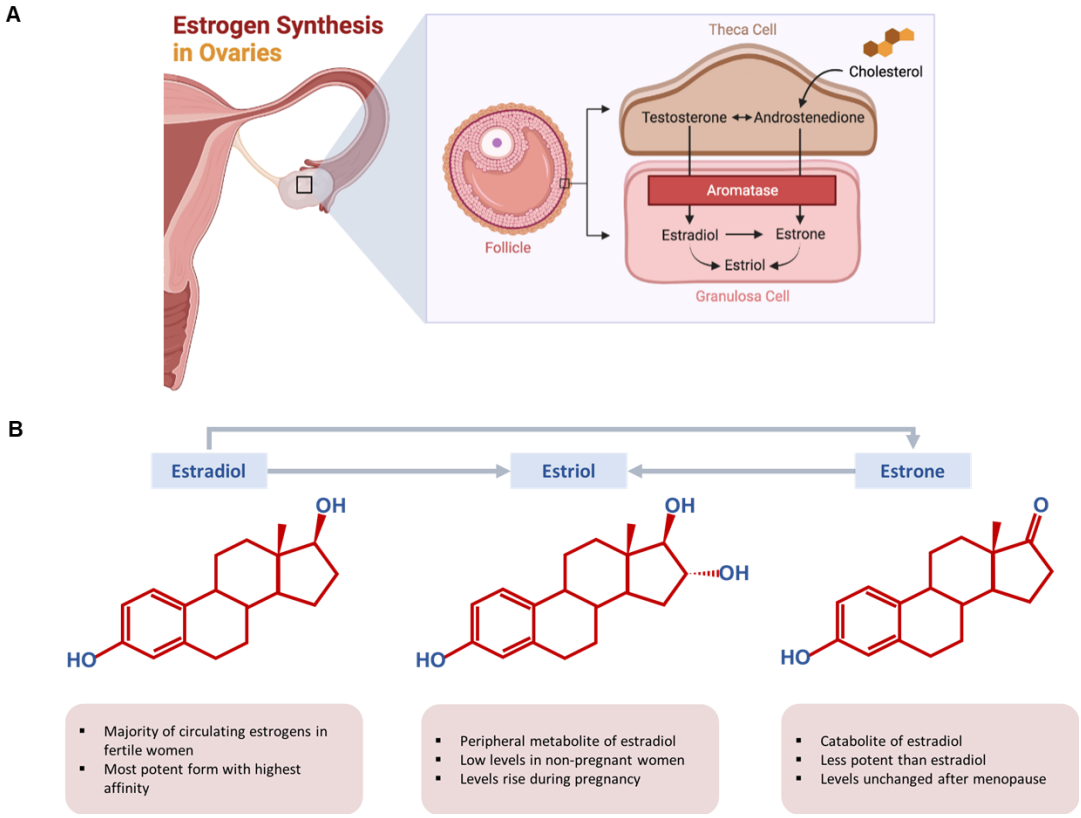
Estrogens

Estrogens are the primary sex hormones in the female body (Ruggiero & Likis, 2002). Estrogens are steroid hormones that are derived from cholesterol. They are mainly secreted by the ovaries, adrenal cortex, and can be peripherally converted from androstenedione (Figure 4A). The female body can secrete three main kinds of estrogen (Figure 4B): 17β-estradiol (estradiol or E2) which is produced by the ovaries and represents the majority of circulating estrogens in fertile women. E2 is the most potent form of estrogen because of the high affinity it has for estrogen receptors. Circulating concentrations fluctuate between 40-400pg/ml through the menstrual cycle but after menopause it drops to less than 20pg/ml (Ruggiero & Likis, 2002).

Estrone (E1) is less potent than estradiol. It is a catabolite of estradiol, and it is also produced from androstenedione which in turn derives from adipose tissue. In post-menopausal women its levels remain unchanged because the adrenal cortex maintains the production of androstenedione. Estriol (E_3) represents a peripheral metabolite of estradiol and is present in low concentrations in non-pregnant women. Its levels tend to rise during pregnancy as it is the main estrogen produced by the placenta (Ruggiero & Likis, 2002).

Estrogens are highly lipid-soluble and this allows them to easily diffuse through the plasma membrane and to enter the blood stream where they mainly circulate bound to sex hormone-binding globulins (SHBG) (R. E. White et al., 1995). Many factors influence the efficacy of estrogens. One of these factors is the relative potency of the various estrogen types and their affinity for estrogen receptors. Other factors are the amount of free hormone that can diffuse through the plasma membranes, the production of SHBG from the liver, and the agonist or antagonist action of estrogen receptors upon binding to the hormone (Ruggiero & Likis, 2002).

There are two main types of estrogen receptors that are present in both males and females: estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). ERα is typically expressed in the uterus, liver, breast and kidney whereas ERβ is found in the reproductive tissue as well as in the brain, bone, urinary tract and vascular system (Ruggiero & Likis, 2002). Both receptors are expressed in pancreatic beta cells and recent studies demonstrate their involvement as important regulators for these cells (Alonso-Magdalena et al., 2013). E2 modulates insulin release in an ERα-dependent manner and the activation of ERβ triggers the closure of ATP-sensitive K^+ channels inducing Ca^{2+} oscillations and consequential insulin release (Alonso-Magdalena et al., 2013). In addition to ERα and ERβ, which work in the nucleus by regulating gene expression, another subpopulation of estrogen receptors has been discovered, the G protein-coupled estrogen receptors (GPERs). GPERs are usually expressed in the reproductive system, brain, kidneys, vascular system, liver, adipose tissue and pancreatic beta cells (Prossnitz & Barton, 2011). GPERs are believed to function as fast-acting modulators of specific cell-signaling pathways (Barton, 2016). Evidence suggests that, in pancreatic islets, they are responsible for stimulating fast insulin secretion mediated by E2. Thus, the protective effects of estrogens on beta cell function may involve the activation of both ERα and GPER (Mauvais-Jarvis et al., 2017; Tiano et al., 2011).



Introduction figure 4. Estrogen synthesis and types of estrogen.

Estrogen is mainly produced in the follicles of the ovaries (**A**). Follicles are characterized by the presence of an oocyte surrounded by granulosa cells and an external layer of theca cells. Cholesterol enters the theca cells, and is converted into androgens (testosterone, androstenedione) which are precursors of estrogen. Subsequently, androgens enter the granulosa cells which contain aromatase, the central enzyme responsible for converting androgens into estrogens. Panel (**B**) summarizes the characteristics of the main types of estrogen. Image created with BioRender.com

Research hypothesis and objectives

Premenopausal women appear to be protected from the development of DM and its associated CVDs, compared to men or postmenopausal women (Appelman et al., 2015; Huebschmann et al., 2019; Meisinger et al., 2002; Raparelli et al., 2017). However, the mechanisms underlying this protection have yet to be elucidated. It is important to mention that the study of sex differences has been an understudied, and relatively neglected, area of biomedical research. In the past, most clinical studies have been conducted in male subjects, and the results of these observations have been applied to the general population which includes men, women, and gender diverse people. Basic scientists also often use male animal models, and the sex-derivation of cell lines is usually ignored. In recent years there has been a push by North American funding agencies to encourage researchers to account for sex as a biological variable in biomedical studies (Clayton & Collins, 2014; Mazure & Jones, 2015).

This research project aims to improve our understanding of the impact of biological sex in pancreatic beta cell health and cardiometabolic disease with a specific focus on the protective role of estrogen.

We hypothesize that estrogens protect pancreatic beta cell health and slow the progression of cardiometabolic disease by modulating the UPR in response to ER stress. Specifically, we hypothesize that estrogens can induce the expression of the adaptive UPR and/or repress the apoptotic UPR.

To effectively test this hypothesis this study addresses three main objectives:

- Objective 1: to characterize the role of estrogens in the maintenance of beta cell health and cardiometabolic disease in a sexually dimorphic mouse model system
- Objective 2: to determine how estrogens protect pancreatic beta cell health
- Objective 3: to confirm and extend our findings in an additional mouse model of type 2 DM and obesity

Following this introductive chapter, the thesis is structured as three main data chapters, which represent the three manuscripts that have emerged from this research project. These are followed by a concluding chapter that summarizes the significance of the results of this research. The first manuscript is a further characterization of the $ApoE^{-/-}$:$Ins2^{+/Akita}$ mouse model and focuses on outlining the protective role of estrogen in pancreatic beta cell health and atherosclerosis progression. The second manuscript continues the characterization of the $ApoE^{-/-}$:$Ins2^{+/Akita}$ mouse model with a specific focus on investigating a potential mechanism (UPR modulation) by which estrogens exert their protective role in beta cell health. The third manuscript extends our findings in an additional rodent model, the TALLYHO/JngJ mouse model, which shows sex differences in terms of the control of glucose homeostasis.

The reader should be advised that since these manuscripts have been submitted, or are being prepared, for publication there may be overlap in the introduction and methods sections.

CHAPTER 2 – INVESTIGATING THE PROTECTIVE EFFECTS OF ESTROGEN ON BETA CELL HEALTH AND THE PROGRESSION OF HYPERGLYCEMIA-INDUCED ATHEROSCLEROSIS

Monica De Paoli, Dempsey Wood, Mary Bohn, Arjun Pandey, Dana Borowitz, Daniel Venegas-Pino, Yuanyuan Shi, Geoff Werstuck

Contributions

Monica De Paoli, has conceived and designed the research project; analyzed and interpreted the results of the experiments; written, edited, and revised the manuscript as well as prepared the manuscript's figures. For the experimental part, the author has bred the experimental mice, performed surgeries, harvested organs and tissues, prepared samples for the analysis. Additionally, the author has measured fasting glucose levels (Figure 1), performed the insulin and glucagon immunofluorescent staining for insulin and glucagon in female mice (Figure 2A, 2C, 2E), performed and analysed ipGTT in experimental mice (Figure 3), performed and analysed atherosclerosis progression in male and female experimental mice through the Sudan IV staining in *en face* aortas and through Mason's trichrome analysis (Figures 4-7). The author also performed experiments, analysed, and created or helped creating all supplementary figures. The author has been directly involved in mentoring, supervising, reviewing, and approving all the work of the co-authors listed below.

Co-authors:

Dempsey Wood has performed immunohistochemical staining and analysis for alpha and beta cell mass at various time points in male and female experimental mice (Figure 2G-H, Supplementary Figure 5A).

Mary Bohn has performed immunofluorescent staining for glucagon and insulin in male mice (Figure 2B, 2D, 2F).

Arjun Pandey has performed immunohistochemical staining and analysis for alpha and beta cell mass in male and female mice supplemented or not with estrogen (Figure 2I, 2J, Supplementary Figure 5B).

Dana Borowitz has quantified and analysed metabolic parameters (Table 1).

Daniel Venegas-Pino, performed blood glucose measurements, harvested organs and tissues, prepared samples for analysis.

Yuanyuan Shi performed blood glucose measurements, harvested organs and tissues.

Supervisor:

Geoff Werstuck has conceived and designed research, wrote, edited, reviewed, and approved the final version of the manuscript.

All authors approved the final version of the manuscript.

Additionally, part of the introduction to this manuscript has been adapted from two previously published peer reviewed papers (Appendix 2, Appendix 3)

This manuscript has been submitted to the American Journal of Physiology-Endocrinology and Metabolism and it is currently in revision.

Abstract

Sex differences in the prevalence and development of diabetes and associated cardiometabolic complications are well established. The objective of this study was to analyze the effects of estrogen on the maintenance of beta cell health/function and atherosclerosis progression, using a mouse model of hyperglycemia-induced atherosclerosis, the ApoE$^{-/-}$:Ins2$^{+/Akita}$ mouse. ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice exhibit sexual dimorphism in the control of blood glucose levels. Male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice are chronically hyperglycemic due to a significant reduction in pancreatic beta cell mass. Female mice are only transiently hyperglycemic, maintain beta cell mass, and blood glucose levels normalize at 35±1 days of age. To determine the effects of estrogen on pancreatic beta cell health and function, ovariectomies and estrogen supplementation experiments were performed, and pancreatic health and atherosclerosis were assessed at various time points. Ovariectomized ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice developed chronic hyperglycemia with significantly reduced beta cell mass. To determine whether the observed effects on ovariectomized ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice were due to lack of estrogens, slow-releasing estradiol pellets were inserted subcutaneously. Ovariectomized ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice treated with exogenous estradiol showed normalized blood glucose levels, and maintained beta cell mass. Exogenous estradiol significantly reduced atherosclerosis in both ovariectomized female and male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice relative to controls. Together, these findings suggest that estradiol confers significant protection to pancreatic beta cell health and can directly and indirectly slow the progression of atherosclerosis.

Introduction

Diabetes mellitus (DM) is characterized by the inability of pancreatic beta cells to effectively regulate blood glucose levels, resulting in the development of chronic hyperglycemia (1). It is estimated that approximately 8.4% of adults worldwide had diabetes in 2017, and the prevalence is predicted to rise to 9.9% by 2045, posing a significant and growing burden on healthcare systems (2). Moreover, DM represents an independent risk factor for cardiovascular diseases (CVD), by significantly increasing the risk of developing atherosclerosis, which represents the main underlying cause of

CVD (3). People with DM have a two-fold increased risk of death due to CVD compared to those without diabetes (4).

Sex differences are known to modulate CVD presentation and outcome (5). These variations are thought to be the result of genetic, hormonal, and environmental factors. Pre-menopausal women present a slower progression towards atherosclerosis and this protection has been attributed to estrogens (6, 7). Differences in the prevalence of DM between sexes have also been identified as pre-menopausal women have a lower incidence of DM compared to men, with accumulating data shows significant sex differences in the regulation of glucose homeostasis and islet physiology and function (8–10). Clinical studies have shown that healthy women secrete more insulin after a meal compared to men (11). Similar results were attained in *ex vivo* experiments on human and mouse pancreatic islets (12, 13).

Many rodent models of DM exhibit similar, though exaggerated, sex dimorphism in glucose homeostasis to that observed in humans. Zucker diabetic fatty (ZDF) rats and TALLYHO/JngJ mice are characterized by the development of chronic hyperglycemia in males, whereas females remain normoglycemic (14, 15). Sex differences are also observed in the $Ins2^{+/Akita}$ mice and streptozotocin-injected (STZ) mouse models of impaired beta cell function (16, 17). Both male and female $Ins2^{+/Akita}$ mice develop a diabetic phenotype however, females present with less severe hyperglycemia (18, 19). Female STZ-injected mice are protected from STZ-induced beta cell toxicity, which can be reached only with increased dosing of the drug (20). Taken together, both human and animal studies suggest that estrogens play a protective role in the maintenance of glucose homeostasis as well as beta cell health.

To investigate the links between DM and CVD, we recently developed a mouse model of hyperglycemia-induced atherosclerosis, the $ApoE^{-/-}$:$Ins2^{+/Akita}$ mouse, by incorporating the Akita mutation into an $ApoE^{-/-}$ mouse. Unexpectedly, these mice present with an even more extreme sexual dimorphism than $Ins2^{+/Akita}$ mice with males developing chronic hyperglycemia. Female $ApoE^{-/-}$:$Ins2^{+/Akita}$ mice show transient hyperglycemia after which blood glucose levels normalize to levels similar to $ApoE^{-/-}$ controls (16). In this paper, we characterize the $ApoE^{-/-}$:$Ins2^{+/Akita}$ mouse model specifically examining the role of estrogens in beta cell health and function, and atherogenesis.

Research Design and Methods

Animal models

Experimental $ApoE^{-/-}$:$Ins2^{+/Akita}$ mice were created by crossing male $ApoE^{-/-}$:$Ins2^{+/Akita}$ mice with female $ApoE^{-/-}$:$Ins2^{+/+}$ mice. Fasting glucose levels were measured at four weeks of age. Genotypes were confirmed using PCR by methods previously described (see Supplementary Table 1A for primer sequences) (16). All experimental mice were fed a standard diet (2018 Teklad Global 18% Protein Rodent Diet, Harlan Teklad, Madison, WI, USA) *ad libitum* and had free access to water. A subset of female $ApoE^{-/-}$:$Ins2^{+/Akita}$ mice underwent ovariectomy at 4 weeks of age, and after two weeks of recovery, they were maintained on standard or switched to a western diet (Teklad

Adjusted Calories TD 97363; Harlan Teklad, Madison, WI, USA, see Supplementary Table 2 for diet composition) *ad libitum* with free access to water. The western diet contains 0.15% cholesterol and 21% anhydrous milk lipids, corresponding to 42% of calories derived from fat. A complete experimental paradigm is illustrated in Supplementary Figures 1-2. All animal procedures were pre-approved by the McMaster University Animal Research Ethics Board.

Ovariectomy

Ovariectomies were performed using methods previously described (16, 21). Four week old mice (n= 5-10 per experimental group) were anesthetized using isoflurane (5% induction, 2.5% maintenance of anaesthesia). The incision area (3 x 3 cm surrounding the iliac crest) was shaved and cleaned. A midline horizontal incision through the skin was performed. The ovary was localized, and another incision was made through the muscle layer to reach the abdominal cavity. The ovary was pulled out by gently removing the fat pat that surrounds it from the abdominal cavity. A double ligation of the uterine horn and vessels at about 0.7 cm and 1 cm distally from the ovary was performed and the ovary was removed. The remaining tissue was put back in the abdominal cavity, and the wound was sutured. The contralateral ovary was similarly removed. The skin wound was closed using a wound clipper. Sham operated animals (n=5-10 per experimental group) received the same incisions and isolation of ovaries however ovaries were not removed.

Blood/plasma analysis and Glucose Tolerance Test (GTT)

Blood glucose levels were measured using a glucometer (One Touch Verio Flex, LifeScan, Burnaby, BC, Canada). Samples that exceeded the limit of the glucometer (35 mM) were quantified using a colorimetric glucose assay (Infinity Glucose Hexokinase Reagent, Thermo Fisher, Middletown, VA, USA). Final values are expressed as an average of the two techniques. Prior to blood sampling, mice were fasted as follows: 1 hour fasting for 4 week old mice; 2 hour fasting for 5-6 week old mice; 4-6 hours fasting for >6 week old mice.

A glucose tolerance test was performed on a subset of mice (n=4-6 per experimental group) at experimental endpoint (18 weeks), prior to harvesting. Mice were fasted for 4-6 hours, and blood glucose levels were determined (time 0). A solution of glucose (2g/kg body weight of a 200mg/ml solution) was administered through an intraperitoneal (IP) injection. Blood samples were collected after 30, 60 and 120 minutes. Glucose levels were determined as described above.

Fasting plasma lipid levels were quantified using the colorimetric diagnostic kit for total cholesterol and triglyceride determination (Infinity Triglyceride, Infinity Cholesterol, Thermo Scientific, Middletown, VA).

Determination of sexual maturity in female mice

Sexual maturity in female $ApoE^{-/-}$:$Ins2^{+/Akita}$ and respective $ApoE^{-/-}$ controls (n= 12 per experimental group) was determined by monitoring age at vaginal opening, an external

indicator of puberty onset that represents an indirect marker of estrous cycle initiation, and it is caused by the rise in estradiol levels during peripubertal period. (22)

Effects of exogenous estradiol administration on glucose regulation and atherosclerosis progression

Female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice were sham operated or ovariectomized prior to the onset of puberty (4 weeks of age). A subset of four week old female ovariectomized and male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice received a subcutaneous estradiol pellet implant (0.1 mg, 90 days release, Innovative Research of America, Sarasota, FL, USA). The pellet continuously releases estradiol for 90 days at a dose of 0.1 mg/pellet, which allows the hormone to circulate at physiological concentrations (23, 24). Glucose levels were assessed at 4, 10, 15 and 18 weeks of age.

Tissue harvesting

Mice were anesthetized with isoflurane and serum and plasma samples obtained by cardiac puncture. Mice were euthanized by cervical dislocation and vasculature was rinsed with phosphate buffered saline. Heart, aorta, liver, fat pad, and pancreas were collected. All organs were fixed in 10% neutral-buffered formalin and stored at room temperature.

Pancreas analysis

Pancreata from male and female ApoE$^{-/-}$ and ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice were harvested at different time points (1, 5, 10, 15 and 25 weeks of age for females, 1, 15 and 25 weeks of age for males). Immunohistochemical staining for insulin or glucagon was performed on paraffin-embedded sections (6µm) from female ApoE$^{-/-}$ and ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice (n=5 for newborn groups, n=4 for all the other time points). The entire pancreas was analyzed at 180 µm intervals, and a total of six sections per mouse were examined. Areas staining positive for insulin or glucagon were quantified using ImageJ software (NIH, Bethesda, MD, USA; http://imagej.nih.gov/ij). Immunostaining was performed using monoclonal rat IgG insulin antibody (MAB1417, R&D Systems, Minneapolis, MN, USA), polyclonal rabbit glucagon antibody (NB100-91782, Novus Biologicals Canada, Oakville, ON, Canada), at a dilution of 1:100, 1:200, respectively. Immunostaining was detected using conjugated secondary antibodies goat anti-rat biotinylated IgG (BA 9400, Vector, Burlington, ON, Canada), goat anti-rabbit biotinylated IgG (BA 100, Vector, Burlington, ON, Canada), each diluted at 1:200.

Beta cell mass (BCM) was calculated using the following formula (25):

BCM= (average of insulin-positive stained area: total area of section) x pancreatic mass (mg)

Alpha cell mass (ACM) was calculated using the following formula (25):

ACM= (average of total glucagon-positive stained area/ total section area) x pancreatic mass (mg)

Glucagon and insulin content of pancreatic islets were determined by assessing immunofluorescence intensity using the previously described antibody against insulin (1:100 dilution) and polyclonal mouse glucagon antibody (CL8867AP-S, Burlington, ON, Canada) (1:200 dilution), and counterstained with DAPI (Invitrogen, Carlsbad, CA, USA) at a dilution of 1:5000. Immunostaining was detected using secondary antibodies Alexa Fluor 488 goat anti-rat IgG (A11006, Thermo Scientific, Middletown, VA, USA), Alexa Fluor 568 goat anti-mouse IgG (A11004, Thermo Scientific, Middletown, VA, USA) each at 1:200 dilution. Separate sections were stained with pre-immune IgG instead of the primary antibody, to control for non-specific staining. Six pancreatic sections per mouse were scanned and a total of n=15 islets per mouse were selected for the intensity of fluorescence analysis which was performed with ImageJ software. The intensity of fluorescence staining for each mouse was calculated as follows: (average intensity of fluorescence of the n=15 islets per mouse) - (average intensity of fluorescence of the n=15 islets of the negative control).

Islet isolation

Pancreatic islet isolation was carried out using established methods. (26, 27) Briefly, pancreata were excised from 4 or 8 week old mice and placed in a tube with collagenase (Collagenase type XI, C7657, Sigma Aldrich, Oakville, ON, Canada) for digestion. The tube was placed in a water bath (37°C) and hand shaken regularly for 20 minutes. Islets were purified on a histopaque gradient (Histopaque 1119, 11191 and Histopaque 1077, 10771, Sigma Aldrich, Oakville, ON, Canada). Islets were then washed with Hank's Balanced Salt Solution (HBSS) and handpicked under a microscope at 20X magnification. Each islet sample represents pooled islets from 4 pancreata (female mice) or 4-8 pancreata (male mice).

Gene expression analysis by qRT-PCR

Total RNA was isolated from pancreatic islets using TRIzol reagent (TRIzol®, 15596-018, Life Technologies, Burlington, ON, Canada). mRNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (4368813, Applied Biosystems, Foster City, California, USA). Real time PCR amplification was performed using SYBR green (SensiFAST™ SYBR Hi-ROX kit, Bioline, Wolston Warrington, UK) reagent. Transcript amplification was normalized to the reference gene beta actin. Primers sequences are listed in Supplementary Table 1B.

Atherosclerosis quantification

Hearts were paraffin-embedded and serial sections (5µm thick) of the aortic sinus and ascending aorta were collected on glass slides until lesions were no longer observed. Sections were stained with Masson's Trichrome (Sigma, St Louis, MO, USA). Lesion areas and volumes were measured using previously described methods. (28) Images were captured using a digital camera (Olympus DP71, Olympus Imaging, Center Valley, PA), mounted on a Leitz Laborlux S bright-field microscope (Leica Microsystems, Concord, ON) and analyzed using ImageJ.

En face aortas

Fixed aortas were cleaned of adjacent muscle and adventitial fat, longitudinally opened, and stained for lipid content with Sudan IV (Sigma, St Louis, MO, USA). Images of the whole aorta were taken using a L320 digital camera (Nikon, Mississauga, ON, Canada). The percentage of atherosclerotic area was assessed using ImageJ.

Statistical Analysis

Statistical analysis of multiple groups was assessed using t-test, one-way or two-way ANOVA, where appropriate. Data are presented as mean± Standard Error of the Mean (SEM) for >10 samples or mean± Standard Deviation (SD) for <10 samples. A value of $p<0.05$ was considered statistically significant.

Results

Sex-specific differences in the control of fasting blood glucose levels

Our lab previously reported sex-specific differences related to glucose homeostasis in the ApoE$^{-/-}$:Ins2$^{+/Akita}$ mouse model (16). In this study, we delineate the underlying mechanisms responsible for these differences. Male and female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice developed hyperglycemia by 4 weeks of age compared to age-matched, normoglycemic ApoE$^{-/-}$ controls (Figure 1A). Male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice remained chronically hyperglycemic for the entire time of the study (to 25 weeks of age). Female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice were transiently hyperglycemic and blood glucose levels normalized after 5 weeks of age to levels not significantly different than ApoE$^{-/-}$ controls (Figure 1A,B) (16). This corresponds to the time of sexual maturity in female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice and ApoE$^{-/-}$ controls (35 days ± 1 day of age), as determined by monitoring age at vaginal opening.

Effects of estrogen reduction and supplementation on glucose homeostasis

To determine if sex hormones are responsible for normalizing glucose levels in female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice, a subset of mice were ovariectomized. The ability of female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice to normalize blood glucose levels was lost in ovariectomized mice, which remained hyperglycemic to the end of the study (Figure 1B). To determine if this effect was driven by estrogen levels, slow release estradiol pellets were inserted subcutaneously into both male and female ovariectomized ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice (29, 30). Fasting blood glucose levels were measured at 4, 10, 15 and 18 weeks of age in all experimental mice (Figure 1C). As previously described, male and ovariectomized female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice developed chronic hyperglycemia whereas intact females were only transiently hyperglycemic with fasting glucose levels normalizing to those of the ApoE$^{-/-}$ controls. Supplementation with exogenous estradiol normalized glucose levels in female ovariectomized ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice and transiently improved fasting blood glucose levels in male mice, however this effect was lost by 15 weeks of age.

Evaluation of metabolic parameters

Body weight, lipid profiles, liver weight, pancreas weight and adipose tissue weight were evaluated in all experimental groups. Neither ovariectomy nor estradiol supplementation significantly affected any of the parameters measured across any of the female experimental groups (Table 1). Male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice supplemented with estradiol showed a significant increase in liver weight compared to age-matched male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice and ApoE$^{-/-}$ controls (Table 1). Male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice with or without estradiol supplementation showed reduced adipose tissue weight compared to ApoE$^{-/-}$ controls (Table 1B).

Expression of Estrogen Receptors

To determine if estrogens could directly act on the cells of the pancreatic islet, the expression of estrogen receptors ER alpha, ER beta, and GPER receptors (Supplementary Figures 3A-C) was confirmed in each experimental mouse strain using immunohistochemistry. Transcript levels of estrogen receptors from isolated pancreatic islets were quantified by qRT-PCR (Supplementary Figure 4). No significant difference in the expression of ER alpha, ER beta or GPER was observed in female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice and ApoE$^{-/-}$ controls at any time point (Supplementary Figure 4A) . Male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice showed a significant increase of ER alpha at eight weeks of age, compared to those at four weeks of age , and no significant differences in ER beta or GPER expression were observed (Supplementary Figure 4B).

Sex dimorphism in ApoE$^{-/-}$:Ins2$^{+/Akita}$ pancreatic islets

Insulin and glucagon content was determined by immunofluorescent staining of paraffin-embedded pancreatic sections from 25 week old male and female sham operated or ovariectomized ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice and respective ApoE$^{-/-}$ controls (Figure 2). Ovariectomy significantly reduced islet insulin content in female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice compared to age-matched sham operated ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice and ApoE$^{-/-}$ controls (Figure 2A,C). Insulin content was significantly reduced in male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice relative to age-matched ApoE$^{-/-}$ controls (Figure 2B,D). There was no significant difference in glucagon content in any experimental or control group (Figure 2E,F). Pancreatic islet architecture appeared disrupted in ovariectomized female and male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice, with alpha cells scattered throughout the central region of the islet section (Figure 2A,B).

Beta cell mass was similar in female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice and age-matched ApoE$^{-/-}$ controls at all experimental time points examined (Figure 2G, Supplementary Table 6A). Ovariectomized female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice showed significantly lower beta cell mass at 25 weeks of age compared to age-matched female ApoE$^{-/-}$:Ins2$^{+/Akita}$ and ApoE$^{-/-}$ controls. Male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice had significantly reduced beta cell mass at 15 and 25 weeks of age, compared to age-matched male ApoE$^{-/-}$ controls (Figure 2H, Supplementary Table 6B). There were no significant differences in alpha cell mass in any of the experimental groups or age-matched controls (Supplementary Figure 5A).

Supplementation with exogenous estradiol preserved beta cell mass in ovariectomized ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice to levels comparable to female ApoE$^{-/-}$:Ins2$^{+/Akita}$ sham operated mice and ApoE$^{-/-}$ controls (Figure 2I, Supplementary Table 7), but did not significantly affect beta cell mass in male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice (Figure 2J, Supplementary Table 7). There were no significant differences in alpha cell mass in any male or female experimental mouse groups (Supplementary Figure 5B).

To determine the effect of estradiol on the expression of genes encoding insulin and glucagon, Ins1, Ins2 and glucagon transcripts from isolated pancreatic islets of 4 and 8 weeks old male and female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice and ApoE$^{-/-}$ controls were quantified by qRT-PCR. Female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice did not show any significant differences in the expression of Ins1, Ins2 or glucagon transcripts across all experimental groups (Supplementary Figure 6A). Male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice at four and eight weeks of age, and eight week old ApoE$^{-/-}$ mice show reduced expression of Ins1, Ins2 and glucagon transcripts when compared to ApoE$^{-/-}$ controls at four weeks of age (Supplementary Figures 6B).

Glucose tolerance in the ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice

To assess pancreatic beta cell function a GTT was performed on a subset of mice at 18 weeks of age (Figure 3). Female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice showed a mild impairment in glucose tolerance, compared to normoglycemic ApoE$^{-/-}$ controls (Figure 3A,B). Ovariectomized, ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice showed an overt impairment of glucose tolerance. Estradiol supplementation significantly improved glucose tolerance in female ovariectomized and male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice (Figures 3C,D), compared to non-treated counterparts, with male's effect being milder than in females.

Effects of exogenous estradiol supplementation on atherosclerosis progression in the ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice

The ApoE$^{-/-}$:Ins2$^{+/Akita}$ mouse is a model of hyperglycemia-induced atherosclerosis. (16) To determine the effect of the exogenous administration of estradiol on atherosclerosis progression we quantified atherosclerotic lesion development in the *en face* aorta (Figures 4, 5), and aortic sinus (Figures 6,7) of these experimental mice.

Exogenous administration of estradiol significantly reduced lipid accumulation in the aortic arch, thoracic and abdominal aorta of ovariectomized ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice to levels similar to those of sham operated ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice and ApoE$^{-/-}$ controls (Figure 4). Male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice supplemented with estradiol had significantly reduced lipid accumulation in the aortic arch, abdominal and thoracic aorta to levels similar to the ApoE$^{-/-}$ controls (Figure 5).

In mice the aortic sinus is an area of high susceptibility to developing atherosclerosis (31). We quantified atherosclerotic lesion area/volume in the aortic sinus and observed that administration of exogenous estradiol significantly reduced atherosclerosis in the ovariectomized female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice to levels similar to those of the ApoE$^{-/-}$ controls (Figure 6). Interestingly, the same effect was observed in male ApoE$^{-/-}$:Ins2$^{+/Akita}$

mice, where supplementation with estradiol significantly reduced the amount of atherosclerosis to levels comparable of those of the ApoE$^{-/-}$ controls (Figure 7). Consistent with these findings, supplementation of estradiol in female ovariectomized ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice and male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice significantly reduced the necrotic core area/volume within the atherosclerotic plaques (Figures 6D, 7D).

Discussion

Accumulating evidence suggests that female sex hormones play a protective role in beta cell health and function, as premenopausal women are less prone to develop diabetes compared to men or women after menopause (32). Preclinical research aimed at increasing our understanding of the mechanisms that confer this protection use rodent models of diabetes showing sexual dimorphism in blood glucose regulation (9, 15, 16, 18, 33, 34). It is known that mice carrying the Ins2$^{+/Akita}$ mutation do show sex-differences in blood glucose regulation, with females having less severe hyperglycemia than males (19). We previously reported that the ApoE$^{-/-}$:Ins2$^{+/Akita}$ mouse model shows more pronounced sex-differences in glucose homeostasis (16). While male mice ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice are chronically, and severely, hyperglycemic, female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice are only transiently hyperglycemic, and their blood glucose levels start to normalize at five weeks of age which is when these mice reach sexual maturity, characterized by a significant increase in the levels of circulating estrogens. We verified that ApoE-deficiency does modulate blood glucose metabolism by examining glucose levels in female heterozygous ApoE$^{-/+}$Ins2$^{+/Akita}$. Fasting blood glucose levels in these mice are significantly elevated compared to female ApoE$^{-/-}$:Ins2$^{+/Akita}$ but significantly lower than age matched male ApoE$^{-/-}$:Ins2$^{+/Akita}$ (Supplementary Figure 7). The mechanism by which the presence or absence of ApoE modulates blood glucose levels in female mice is not yet known.

Female sex hormones appear to have a major influence on glucose levels because ovariectomized ApoE$^{-/-}$:Ins2$^{+/Akita}$ females remain chronically hyperglycemic and administration of exogenous estradiol significantly improved fasting blood glucose levels , beta cell mass and glucose tolerance to levels similar to the ApoE$^{-/-}$:Ins2$^{+/Akita}$ sham females and ApoE$^{-/-}$controls. Transient improvements were seen in male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice , suggesting that estrogen only partially rescues this effect, or that higher doses of estrogen might be needed to attain good glycemic control.

In this study we show that estrogens have a direct and specific effect on beta cell health. These results are in accordance with various studies conducted in different rodent models that show sexual dimorphism in terms of beta cell health and function, with male rodents typically showing hyperglycemia and altered beta cell health, compared to female counterparts (35). For instance, Wistar obese male rats develop hyperglycemia while females remain normoglycemic when fed either a normal diet or a hyperglycemic diet (36). Furthermore, female Sprague-Dawley rats fed a western-type high fat diet maintain glucose tolerance compared to male counterparts (37). In our

experiments we show that ovariectomy significantly reduced beta cell mass and islet insulin content to levels comparable with those of the male counterparts. Estrogen replacement was able to preserve beta cell mass and glucose homeostasis. These findings are consistent with current guidelines on hormone replacement therapy in menopausal women, which show a beneficial effect in terms of glucose homeostasis in both women with and without diabetes mellitus (38).

Diabetes is associated with an increased risk of developing atherosclerosis, which represents the main underlying cause of cardiovascular diseases (1). Accumulating data shows that sex differences in prevalence and severity of diabetes and its associated cardiovascular risk do exist, with pre-menopausal women being protected, compared to men or post-menopausal women. After menopause, women developing diabetes have a higher mortality risk due to diabetes-associated cardiovascular diseases compared to women without diabetes and men (39, 40). Our previously published data showed that ovariectomized female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice developed advanced atherosclerotic lesions compared to intact females (16). Furthermore, in previous studies we have shown that when fed a western diet, which represents an additional stressor contributing to the development of atherosclerosis, male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice exacerbated atherosclerosis progression, significantly increasing mortality due to myocardial infarction between 20 and 25 weeks of age (41). Conversely, female ApoE$^{-/-}$:Ins2$^{+/Akita}$ counterparts did not show any variation in life span (41). In this study, we observed a significant increase in mortality in ovariectomized ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice fed a western-type high fat diet, compared to sham operated female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice (Supplementary Figure 8). These findings suggest that female sex hormones may protect against cardiovascular events.

Exogenous administration of estradiol in ovariectomized female and male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice significantly reduced lipid accumulation in the *en face* aortas and the volume of atherosclerotic plaques to a degree similar to that of the respective ApoE$^{-/-}$ controls. Furthermore, the necrotic core volume within the plaques was significantly reduced in both male and female ovariectomized ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice supplemented with estradiol compared to the untreated counterparts, suggesting that supplementation with exogenous estradiol reduces overall atherosclerosis progression and prevents the formation of advanced lesions.

Human studies and animal models show that estrogens play a protective role in glucose homeostasis and that the loss of estrogen leads to the development of hyperglycemia (42). Hyperglycemia is also linked to the development and progression of atherosclerosis. One hypothesis is that elevated blood glucose can contribute to the production of pro-inflammatory molecules that ultimately induce vascular damage and initiate and/or progress the atherosclerotic processes (43). Estrogens may indirectly protect against atherogenesis by normalizing blood glucose levels and reducing the potential inflammatory stimuli on the vasculature, as suggested by our findings in female ovariectomized ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice supplemented with estradiol. However, male

ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice supplemented with estradiol presented with a significant reduction in atherosclerosis progression, even if their blood glucose normalization was transient. This could be explained by the fact that along with the effects on glucose homeostasis, estrogens may also have a direct effect on vascular physiology specifically by protecting from vascular injury as well as by promoting anti-inflammatory responses (44, 45). Although the specific mechanisms have yet to be elucidated, the direct protective effect of estrogen could be exerted in various ways. Estrogen could act by inhibiting fat accumulation in the vascular tissue (46). Estrogen may also modulate the expression of proinflammatory and anti-inflammatory cytokines and/or the interactions between immune cells and other cells involved in the atherosclerotic inflammatory response (47). Finally, estrogen may exert vascular protection by increasing nitric oxide bioactivity in the vascular endothelium, which in turn can increase vasodilation and can also reduce the adhesiveness of endothelial cells to white blood cells therefore having an anti-inflammatory activity (48, 49).

Taken together, these data show a protective role of estrogens on beta cell health and function, glucose homeostasis and hyperglycemia-associated atherosclerosis progression. Further studies are required to evaluate the downstream mechanisms by which estrogens are able to exert their protective effect(s).

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Tables

Mouse genotype/ intervention	Body weight (g)	Plasma Cholesterol (mM)	Plasma Triglycerides (mM)	Pancreas weight (g)	Liver weight (g)	Adipose weight (g)
Female ApoE$^{-/-}$ sham	23.1 ± 2.38	5.86 ± 0.99	0.45 ± 0.14	0.115 ± 0.02	0.84 ±0.09	0.12 ± 0.05
Female ApoE$^{-/-}$:Ins2$^{+/Akita}$ sham	22.2 ± 1.86	5.42 ± 1.25	0.47 ± 0.06	0.124 ± 0.01	0.95 ± 0.19	0.15 ± 0.06
Female ApoE$^{-/-}$:Ins2$^{+/Akita}$ ovx	22.7 ± 1.95	6.00 ± 1.35	0.73 ± 0.18	0.124 ± 0.02	0.96 ± 0.12	0.17 ± 0.07
Female ApoE$^{-/-}$:Ins2$^{+/Akita}$ ovx+E2	21.8 ± 0.96	4.24 ± 1.73	0.44 ± 0.07	0.136 ± 0.02	0.97 ± 0.38	0.08 ± 0.05
Male ApoE$^{-/-}$ sham	28.5 ± 0.84	7.23 ± 0.82	0.50 ± 0.06	0.125 ± 0.03	1.11 ± 0.11	0.29 ± 0.06
Male ApoE$^{-/-}$:Ins2$^{+/Akita}$ sham	26.3 ± 1.88	7.73 ± 0.47	0.75 ± 0.30	0.131 ± 0.02	1.22 ± 0.08	0.06 ± 0.03 ***
Male ApoE$^{-/-}$:Ins2$^{+/Akita}$ +E2	28.3 ± 0.74	5.54 ± 1.16	0.61 ± 0.20	0.161 ± 0.03*	1.47 ± 0.17 **, ***	0.16 ± 0.19 *

Table 1. Metabolic parameters in 18 week old male and female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice and age-matched controls (ApoE$^{-/-}$) supplemented with 17β-Estradiol (E2).

Average values are presented with the Standard Deviation (SD). n=5-12/group. Liver weight: ** p<0.01 male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice +E2 vs male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice, *** p<0.001 male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice +E2 vs male ApoE$^{-/-}$ mice. Adipose tissue weight:

*** $p<0.001$ male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice vs male ApoE$^{-/-}$ mice, * $p<0.05$ male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice +E2 vs male ApoE$^{-/-}$ mice

Figure captions

Figure 1. Fasting blood glucose levels. (A) Fasting blood glucose levels in male and female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice and age-matched controls (ApoE$^{-/-}$). n=4-10 per group. 4-25 weeks **** p<0.0001 male ApoE$^{-/-}$:Ins2$^{+/Akita}$ vs age-matched male and female ApoE$^{-/-}$; 6-25 weeks ****p<0.0001 male ApoE$^{-/-}$:Ins2$^{+/Akita}$ vs age-matched female ApoE$^{-/-}$:Ins2$^{+/Akita}$. (**B)** Fasting blood glucose levels in female sham operated and ovariectomized (ovx) ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice and respective age-matched controls. n=4-10 per group. 6 weeks: *** p<0.001 female sham operated ApoE$^{-/-}$:Ins2$^{+/Akita}$ vs age-matched female ovx ApoE$^{-/-}$:Ins2$^{+/Akita}$, 9 weeks: ** p<0.01 female sham operated ApoE$^{-/-}$:Ins2$^{+/Akita}$ vs age-matched female ovx ApoE$^{-/-}$:Ins2$^{+/Akita}$, 10 weeks: ****p<0.0001 female sham operated ApoE$^{-/-}$:Ins2$^{+/Akita}$ vs age-matched female ovx ApoE$^{-/-}$:Ins2$^{+/Akita}$. (**C)** Effect of in vivo administration of 17β-Estradiol (E2) on fasting blood glucose levels in male and female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice and age-matched controls. n=8-14 per group. **p<0.01 male ApoE$^{-/-}$:Ins2$^{+/Akita}$ vs age-matched male ApoE$^{-/-}$:Ins2$^{+/Akita}$ +E2. ^p<0.05 and ^^^^p<0.0001 female ovx ApoE$^{-/-}$:Ins2$^{+/Akita}$ vs age-matched female ovx ApoE$^{-/-}$:Ins2$^{+/Akita}$ +E2. Error bars represent standard error of the mean (SEM). A comprehensive statistical analysis can be found in Supplementary Tables 3-5. Statistical test: two way-ANOVA.

Figure 2. Insulin and glucagon content and beta cell mass in pancreas sections of male and female ApoE$^{-/-}$ and ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice. Pancreatic sections from **(A)** male and **(B)** female ApoE$^{-/-}$ and ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice were immunostained with antibodies against insulin (green fluorescence) and glucagon (red fluorescence). Magnification=x20. Scale bars equal 100 µm. Quantification of **(C)** insulin and **(D)** glucagon staining intensity of female sham operated or ovariectomized (ovx) ApoE$^{-/-}$ and ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice. n=60 pooled islets from n=4 mice/group (corresponding to n=15 islets per experimental mouse). Error bars represent Standard Error of the Mean (SEM). ****p<0.0001 female ovx ApoE$^{-/-}$:Ins2$^{+/Akita}$ vs female sham operated ApoE$^{-/-}$, ****p<0.0001 female ovx ApoE$^{-/-}$:Ins2$^{+/Akita}$ vs female ovx ApoE$^{-/-}$, **p<0.01 female ovx ApoE$^{-/-}$:Ins2$^{+/Akita}$ vs female sham operated ApoE$^{-/-}$:Ins2$^{+/Akita}$. Quantification of **(E)** insulin and **(F)** glucagon staining intensity of male ApoE$^{-/-}$ and ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice. n=60 pooled islets from n=4 mice/group. Error bars represent Standard Error of the Mean (SEM). ****p<0.0001 male ApoE$^{-/-}$ vs male ApoE$^{-/-}$:Ins2$^{+/Akita}$. Beta cell mass quantification of **(G)** female and male **(H)** ApoE$^{-/-}$ and ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice. n=4-5 per group. Bars show mean and standard deviation (SD). **** p<0.01 female sham operated ApoE$^{-/-}$ vs age-matched ovx ApoE$^{-/-}$:Ins2$^{+/Akita}$, p<0.01 female sham operated ApoE$^{-/-}$:Ins2$^{+/Akita}$ vs age-matched ovx ApoE$^{-/-}$:Ins2$^{+/Akita}$. **** p<0.0001 male ApoE$^{-/-}$ vs age-matched ApoE$^{-/-}$:Ins2$^{+/Akita}$. Beta cell mass quantification of female **(I)** and male **(J)** ApoE$^{-/-}$ and ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice supplemented with 17β-estradiol. * p< 0.05, **p<0.01, ***p<0.001. n=5/group. Error bars represent Standard Deviation (SD). Statistical test: one way-ANOVA.

Figure 3. Effects of in vivo administration of 17β-Estradiol (E2) on glucose tolerance. GTT and relative Area Under the Curve (AUC) analysis in female **(A, B)** and male **(C, D)** ApoE-/-:Ins2+/Akita mice and age-matched controls supplemented with estradiol. Error bars represent Standard Deviation (SD). **** p<0.0001, * p<0.05. Experimental groups:female ApoE$^{-/-}$(n=5), female ApoE$^{-/-}$:Ins2$^{+/Akita}$ (n=4), female ApoE$^{-/-}$:Ins2$^{+/Akita}$ ovx (n=7), female ApoE$^{-/-}$:Ins2$^{+/Akita}$ ovx +E2 (n=5), male ApoE$^{-/-}$(n=4), male ApoE$^{-/-}$:Ins2$^{+/Akita}$ (n=6), male ApoE$^{-/-}$:Ins2$^{+/Akita}$ +E2 (n=4). Statistical test: one way-ANOVA.

Figure 4. Effects of in vivo administration of 17β-Estradiol (E2) on aortic atherosclerosis in female mice. (A) En face aortas from female ApoE$^{-/-}$ and ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice were stained with Sudan IV. Subgroups were ovariectomized and supplemented with 17β-Estradiol as indicated. Surface area contain plaque in the **(B)** aortic arch and thoracic aorta and **(C)** abdominal aorta containing plaque were quantified. n=4-8/group. Error bars represent Standard Deviation (SD). ***p<0.001. **p<0.02, * p<0.05. Statistical test: one way-ANOVA.

Figure 5. Effects of in vivo administration of 17β-Estradiol (E2) on aortic atherosclerosis in male mice. A) En face aortas from male ApoE$^{-/-}$ and ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice were stained with Sudan IV. A subgroup was supplemented with 17β-estradiol as indicated. Surface area contain plaque in the **(B)** aortic arch and thoracic aorta and **(C)** abdominal aorta containing plaque were quantified. n=4-8/group. Error bars represent Standard Deviation (SD). ***p<0.001. **p<0.02, * p<0.05. Statistical test: one way-ANOVA.

Figure 6. Effects of in vivo administration of 17β-Estradiol (E2) on atherosclerosis at the aortic sinus in female mice. (A) Representative images of cross sections of aortic sinus from female ApoE-/- and ApoE-/-:Ins2+/Akita mice ovariectomized and supplemented with 17β-Estradiol, as indicated. Magnification = x10. Scale bars equal 500µm. **(B+C)** Quantification of atherosclerosis in the aortic sinus and ascending aorta. **(D)** Necrotic core area at the aortic sinus. * p<0.05, **p<0.01, ***p<0.001. n=4-6/group. Error bars represent Standard Deviation (SD). Statistical test: one way-ANOVA.

Figure 7. Effects of in vivo administration of 17β-Estradiol (E2) on atherosclerosis at the aortic sinus in male mice. (A) Representative images of cross sections of aortic sinus from male ApoE-/- and ApoE-/-:Ins2+/Akita mice supplemented with 17β-Estradiol, as indicated. Magnification = x10. Scale bars equal 500µm. **(B+C)** Quantification of atherosclerosis in the aortic sinus and ascending aorta. **(D)** Necrotic core area at the aortic sinus. ****p<0.0001, ***p<0.001, * p<0.05. n=5-7/group. Error bars represent Standard Deviation (SD). Statistical test: one way-ANOVA.

Supplementary Table 1. (A) Primers sequences for genotype analysis. **(B)** Primers sequences for qRT-PCR analysis.

Supplementary Table 2. Composition of western diet Teklad Adjusted Calories TD 97363, customized diet from TD 88137.

Supplementary Table 3. (A) Detailed statistical analysis of data in figure 1A, fasting blood glucose levels in male and female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice and age-matched controls. NS, not significant. **(B) Detailed statistical analysis of figure data in 1B, fasting blood glucose levels in female sham operated or ovariectomized (ovx) ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice and age-matched controls.** NS, not significant.

Supplementary Table 4. Detailed statistical analysis of data in figure 1C, effects of in vivo administration of 17β-Estradiol (E2) in female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice. NS, not significant.

Supplementary Table 5. Detailed statistical analysis of data in figure 1C, effects of in vivo administration of 17β-Estradiol (E2) in male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice. NS, not significant. Comparisons between groups not reported in the table are not significant.

Supplementary Table 6. (A) Female average beta cell mass adjusted for body weight for each experimental group. Beta cell mass for female ApoE$^{-/-}$:Ins2$^{+/Akita}$ ovx was calculated at endpoint (25 weeks). **(B)** Male average beta cell mass adjusted for body weight for each experimental group. Beta cell mass for Female ApoE$^{-/-}$:Ins2$^{+/Akita}$ ovx was calculated at endpoint (25 weeks).

Supplementary Table 7. Average beta cell mass adjusted for body weight for each experimental group treated or not with exogenous estradiol.

Supplementary figure 1. Experimental paradigm for mice fed standard diet with free access to water.

Supplementary figure 2. Experimental paradigm for mice fed western diet with free access to water.

Supplementary Figure 3. Immunohistochemistry staining in paraffin-embedded pancreas sections for 25 week old female sham operated, ovariectomized (ovx) and male ApoE$^{-/-}$ and ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice. Sections were stained against (**A**) ER alpha, (**B**) ER beta and (**C**) GPER with a 1:100, 1:50 and 1:200 dilution respectively.

Supplementary Figure 4. (A) Expression of ER alpha, ER beta and GPER in pancreactic islets isolated from female ApoE$^{-/-}$ and ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice at 4 and 8 weeks of age. Each dot represents pooled islets from n=3-5 mice. n=3-4 per group. Bars show mean and standard deviation (SD). **(B) Expression of ER alpha, ER beta and GPER in pancreatic islets isolated from male ApoE$^{-/-}$ and ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice at 4 and 8 weeks of age.** Each dot represents pooled islets from n=3-5 mice. n=3-4 per group. Bars show mean and standard deviation (SD). * $p<0.05$.

Supplementary Figure 5. (A) Quantification of alpha cell mass in pancreas sections from (right) male and (left) female ApoE$^{-/-}$ and ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice. n=4-5 per group. Bars show mean and standard deviation (SD). **(B) Effects of in vivo administration of 17β-Estradiol on alpha cell mass of female (left) and male (right)**

ApoE-/-:Ins2+/Akita mice. Alpha cell mass quantification in pancreas sections from (**left**) female and (**right**) male ApoE-/- and ApoE-/-:Ins2+/Akita mice supplemented with estradiol as indicated. n=5/group. Bars represent Standard Deviation (SD).

Supplementary Figure 6. (A) Expression of Ins1, Ins2 and glucagon in pancreatic islets isolated from female ApoE$^{-/-}$ and ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice at 4 and 8 weeks of age. Each dot represents pooled islets from n=3-5 mice. n=3-4 per group. Bars show mean and standard deviation (SD). **(B) Expression of Ins1, Ins1 and glucagon in pancreatic islets isolated from male ApoE$^{-/-}$ and ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice at 4 and 8 weeks of age.** Each dot represents pooled islets from n=3-5 mice. n=3-4 per group. Bars show mean and standard deviation (SD). * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

Supplementary Figure 7. Fasting blood glucose levels in male and female ApoE$^{+/-}$:Ins2$^{+/Akita}$ mice and age-matched ApoE$^{+/-}$ controls. ****p<0.0001 25 weeks old male male ApoE$^{+/-}$:Ins2$^{+/Akita}$ vs age-matched male ApoE$^{+/-}$; ***p<0.001 4 weeks old male ApoE$^{+/-}$:Ins2$^{+/Akita}$ vs age-matched male ApoE$^{+/-}$; ***p<0.001 4 weeks old female ApoE$^{+/-}$:Ins2$^{+/Akita}$ vs age-matched female ApoE$^{+/-}$, **p<0.01 25 weeks old male female ApoE$^{+/-}$:Ins2$^{+/Akita}$ vs age-matched female ApoE$^{+/-}$ n=6-9 per group.

Supplementary Figure 8. Effects of western diet (high fat diet) versus regular chow diet on overall survival in ovariectomized ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice. n=8-10/group.

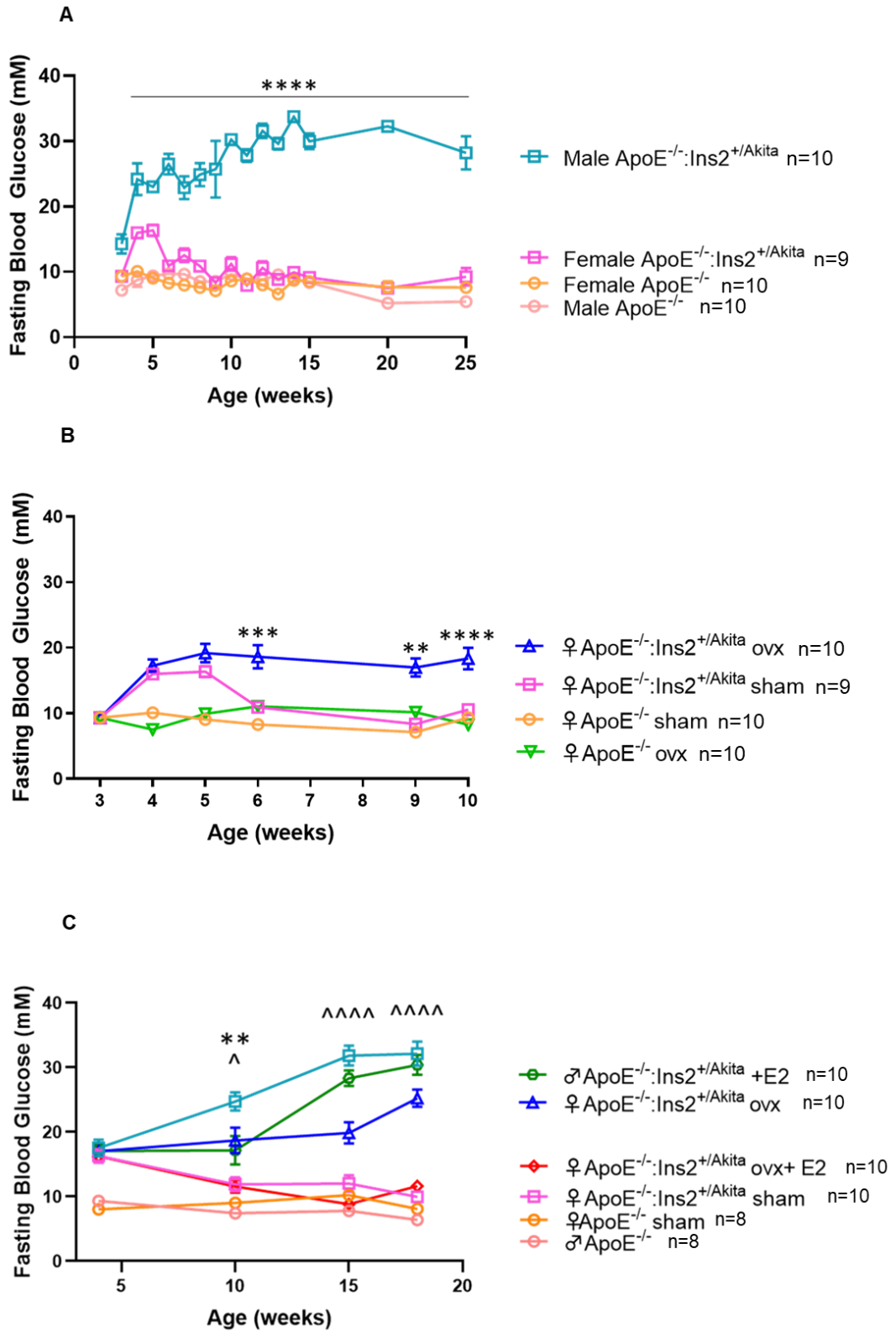


Figure 1. Fasting blood glucose levels.

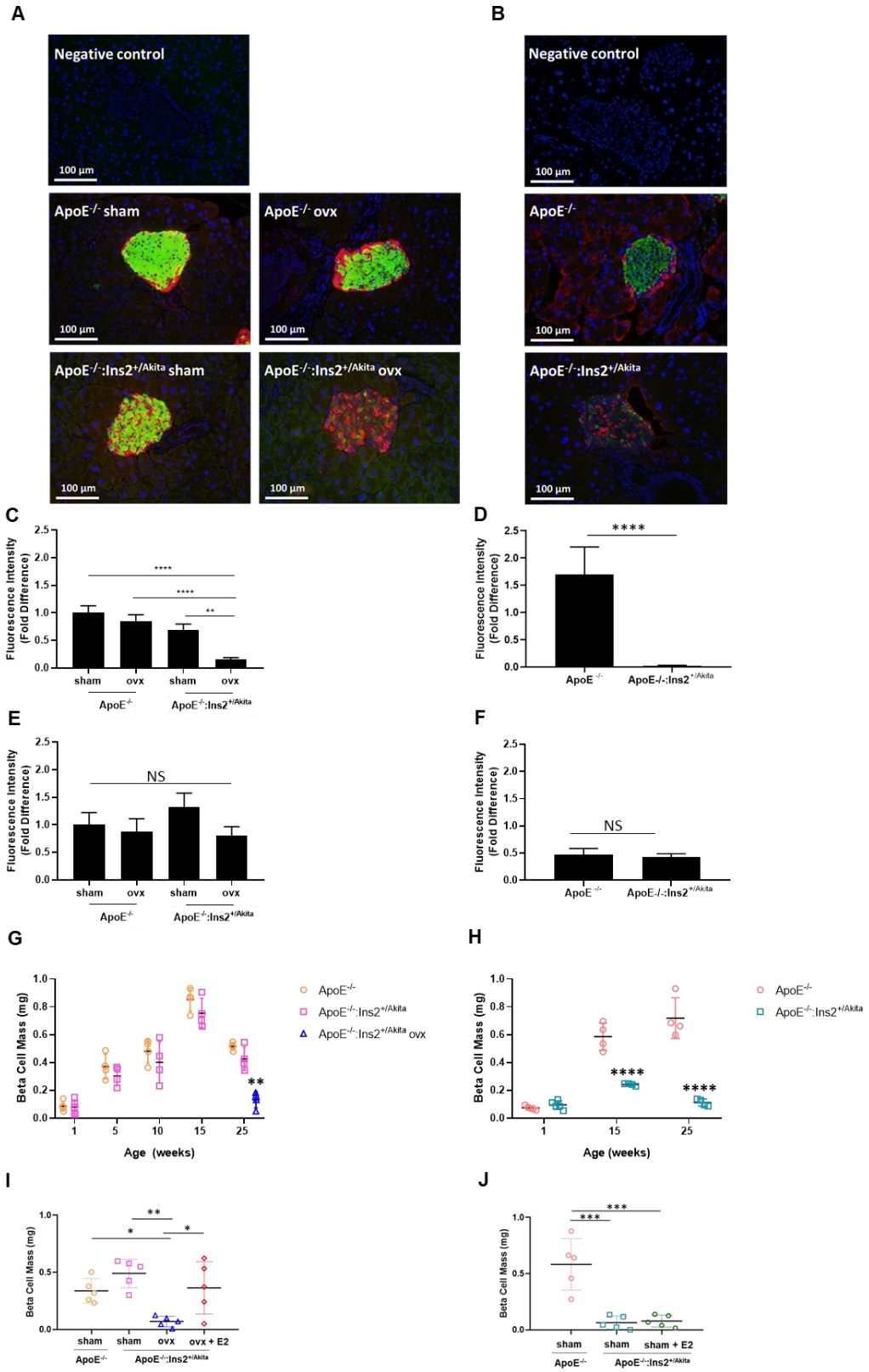


Figure 2. Insulin and glucagon content and beta cell mass in pancreas sections of male and female ApoE$^{-/-}$ and ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice.

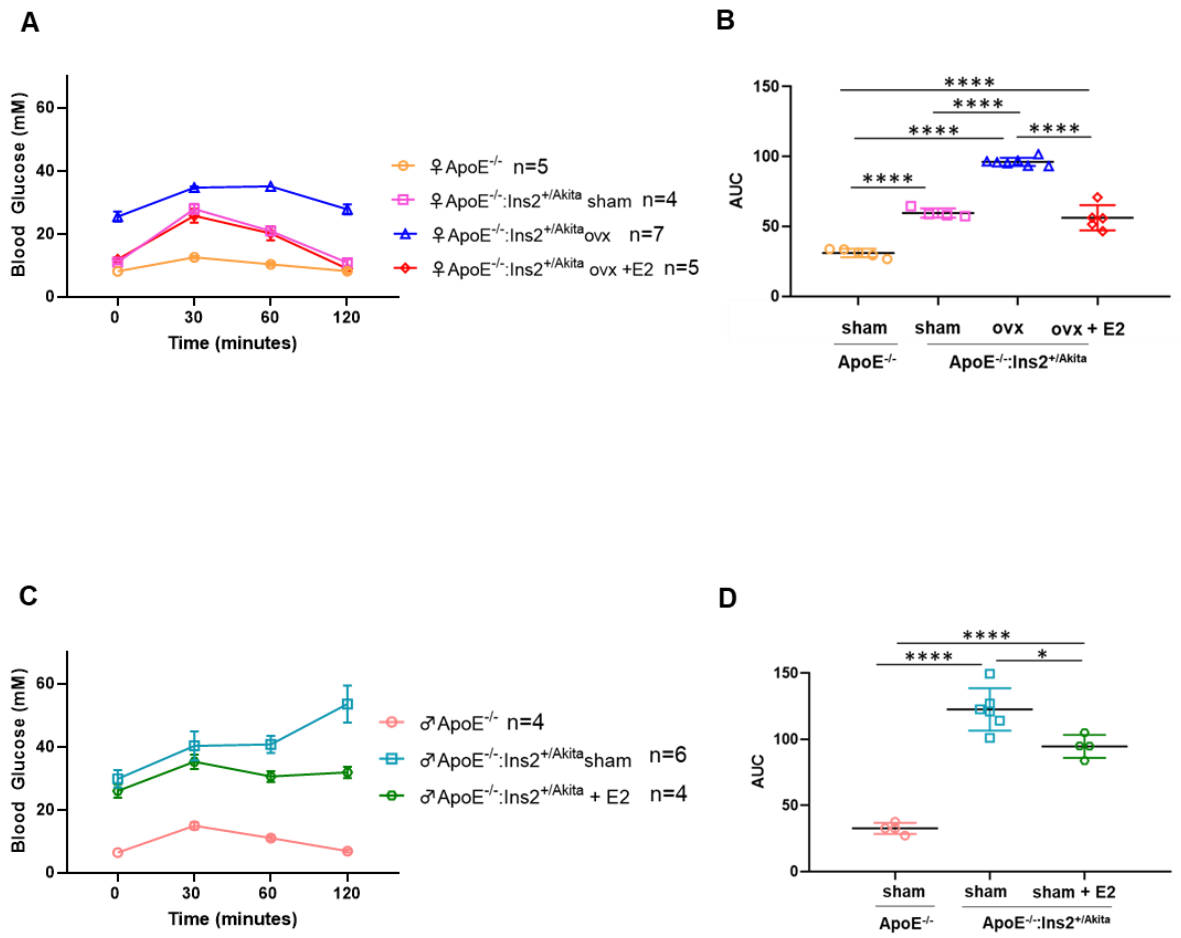


Figure 3. Effects of in vivo administration of 17β-Estradiol (E2) on glucose tolerance.

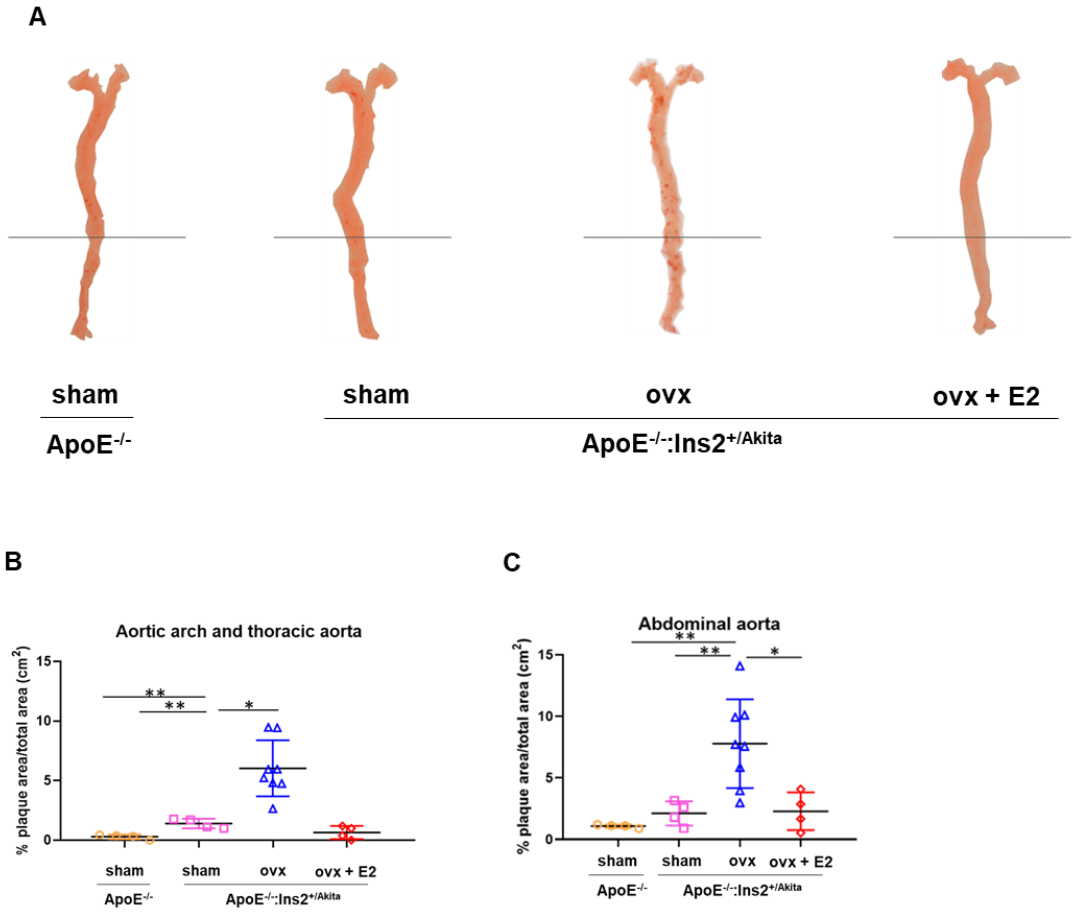


Figure 4. Effects of in vivo administration of 17β-Estradiol (E2) on aortic atherosclerosis in female mice.

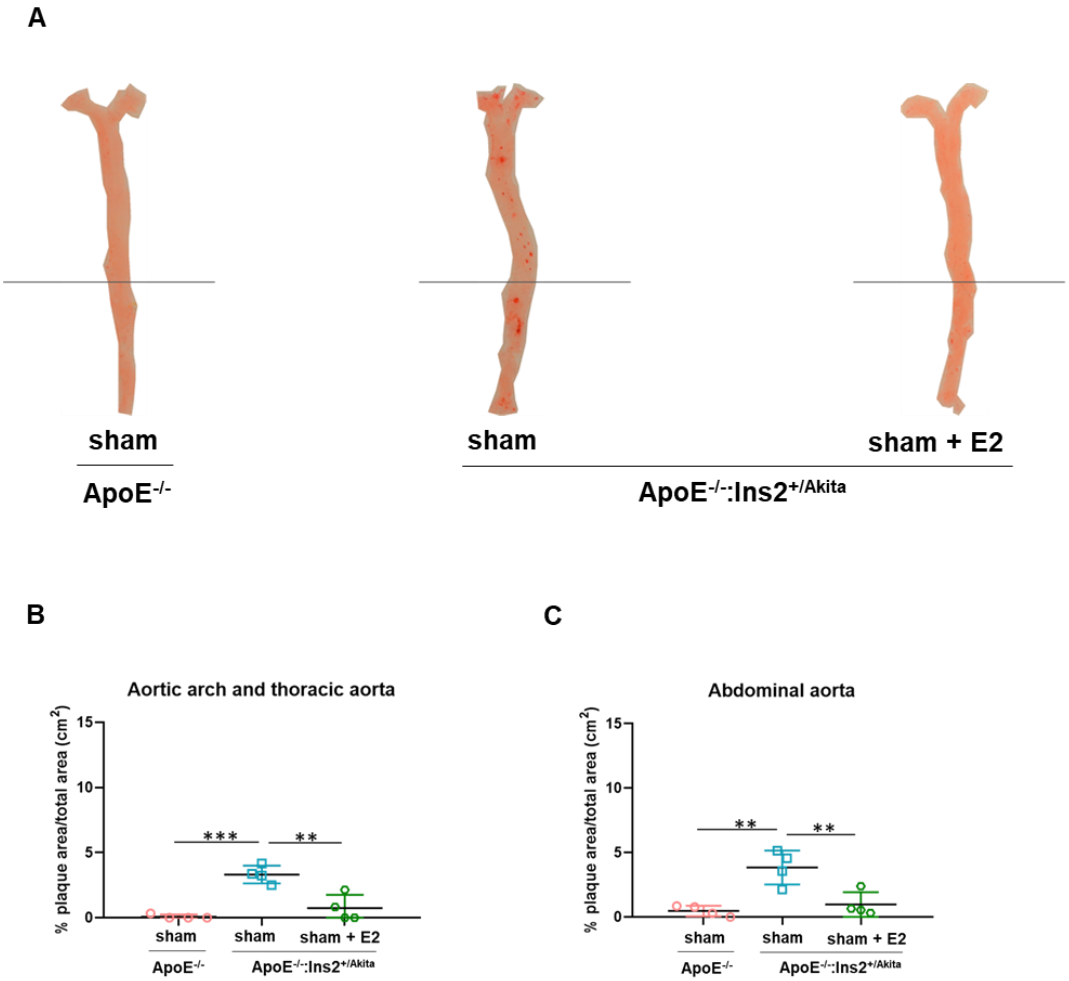


Figure 5. Effects of in vivo administration of 17β-Estradiol (E2) on aortic atherosclerosis in male mice.

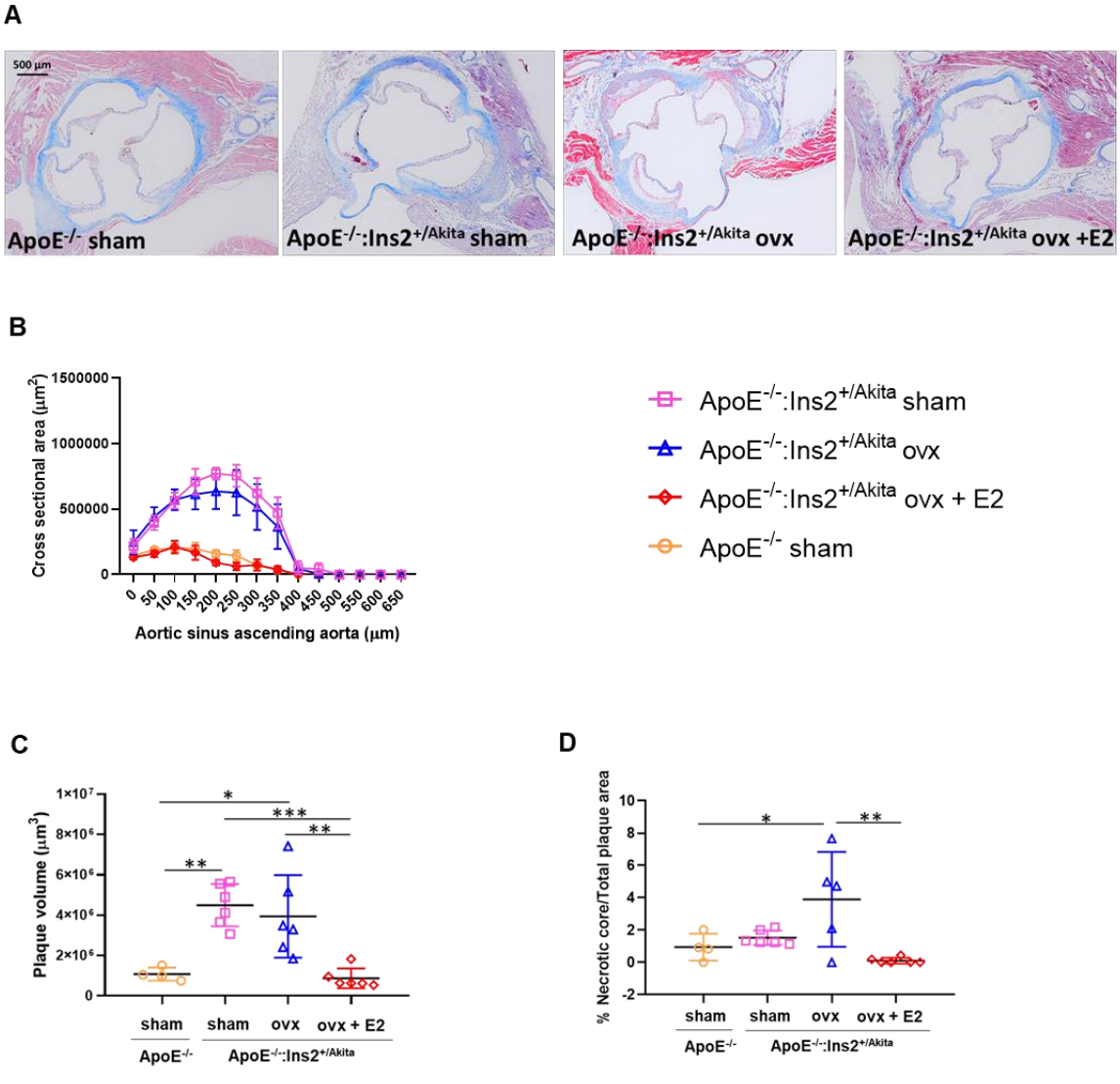


Figure 6. Effects of in vivo administration of 17β-Estradiol (E2) on atherosclerosis at the aortic sinus in female mice.

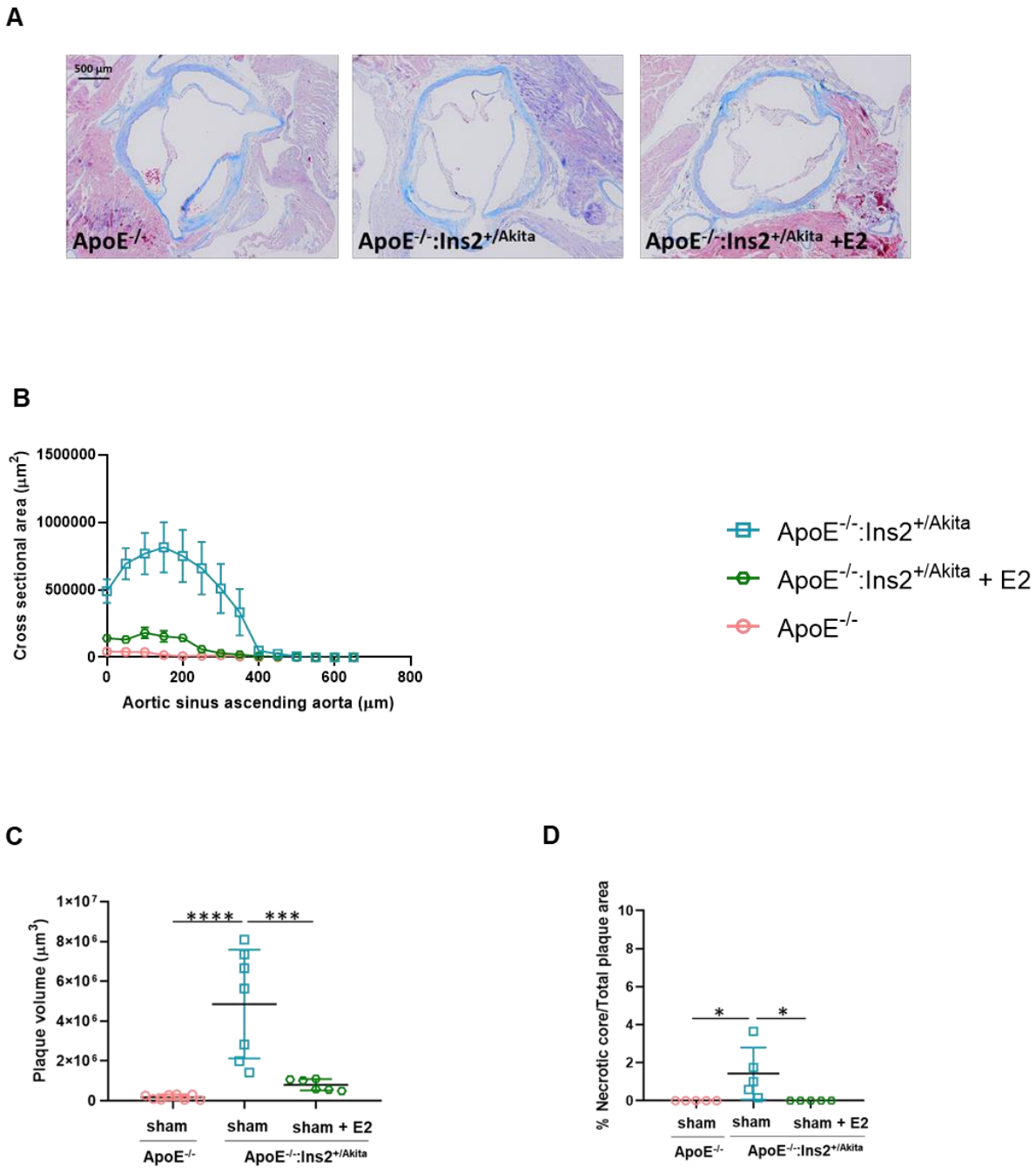


Figure 7. Effects of in vivo administration of 17β-Estradiol (E2) on atherosclerosis at the aortic sinus in male mice.

Supplementary material

Gene of interest	Forward	Reverse
Mouse Ins2	5'-TGCTGATGCCCTGGCCTGCT-3'	5'-TGGTCCCACATATGCACATG-3'
Mouse ApoE$^{+/+}$	5'-GCCGCCCCGACTGCATCT-3'	5'-TGTGACTTGGGAGCTCTGCAGC-3'
Mouse ApoE$^{-/-}$	5'-GCCGCCCCGACTGCATCT-3'	5'-GCCTAGCCGAGGGAGAGCCG-3'

Supplementary Table 1A. Primers sequences for genotype analysis.

Gene of interest	Forward	Reverse
Mouse Ins1	5'-GAAGCGTGGCATTGTGGAT-3'	5'-TGGGCCTTAGTTGCAGTAGTTCT-3'
Mouse Ins2	5'-AGCCTTAAGTGATCCGCTACAA-3'	5'-CATGTTGAAACAATAACCTGGAAGA-3'
Mouse ERα	5′-ACCATTGACAAGAACCGGAG-3′	5′-CCTGAAGCACCCATTTCATT-3′
Mouse ERβ	5′-TGTGTGTGAAGGCCATGATT-3′	5′-TCTTCGAAATCACCCAGACC-3′
Mouse GPER1	5'-TCATTTCTGCCATGCACCCA-3'	5'-GTGGACAGGGTGTCTGATGT-3'
Mouse GAPDH	5'-ACCACAGTCCATGCCATCAC-3'	5'-CACCACCCTGTTGCTGTAGCC-3'

Supplementary Table 1B. Primers sequences for qRT-PCR analysis.

Component	**g/kg**
Casein	195.0
DL-Methionine	3.0
Sucrose	341.46
Corn Starch	50.0
Maltodextrin	100.0
Anhydrous Milkfat	210.0
Cholesterol	1.5
Cellulose	50.0
Mineral Mix, Teklad, AIN-76 (170915)	35.0
Calcium Carbonate $CaCO_3$	4.0
Vitamin mix, Teklad (40060)	10.0
Ethoxyquin (antioxidant)	0.04

Supplementary Table 2. Composition of western diet Teklad Adjusted Calories TD 97363, customized diet from TD 88137.

Comparison	4 weeks	5 weeks	6-25 weeks
female ApoE$^{-/-}$ vs age-matched female ApoE$^{-/-}$:Ins2$^{+/Akita}$	NS	p<0.001	NS
female ApoE$^{-/-}$ vs age-matched male ApoE$^{-/-}$:Ins2$^{+/Akita}$	p<0.0001	p<0.0001	p<0.0001
male ApoE$^{-/-}$ vs age-matched female ApoE$^{-/-}$:Ins2$^{+/Akita}$	p<0.05	p<0.001	NS
male ApoE$^{-/-}$ vs age-matched male ApoE$^{-/-}$:Ins2$^{+/Akita}$	p<0.0001	p<0.0001	p<0.0001
female ApoE$^{-/-}$:Ins2$^{+/Akita}$ vs age-matched male ApoE$^{-/-}$:Ins2$^{+/Akita}$	p<0.0001	NS	p<0.0001
female ApoE$^{-/-}$ vs age-matched male ApoE$^{-/-}$	NS	NS	NS

Supplementary Table 3A. Detailed statistical analysis of data in figure 1A, fasting blood glucose levels in male and female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice and age-matched controls.

Comparison	4 weeks	5 weeks	6 weeks	9 weeks	10 weeks
female sham operated ApoE$^{-/-}$ vs female sham operated ApoE$^{-/-}$:Ins2$^{+/Akita}$	p<0.01	p<0.0001	NS	NS	NS
female ovx ApoE$^{-/-}$ vs female sham operated ApoE$^{-/-}$:Ins2$^{+/Akita}$	p<0.0001	p<0.0001	NS	NS	NS
female sham operated ApoE$^{-/-}$ vs female ovx ApoE$^{-/-}$:Ins2$^{+/Akita}$	p<0.001	p<0.0001	p<0.0001	p<0.0001	p<0.0001
female ovx ApoE$^{-/-}$ vs female ovx ApoE$^{-/-}$:Ins2$^{+/Akita}$	p<0.0001	p<0.0001	p<0.0001	p<0.01	p<0.0001
female sham operated ApoE$^{-/-}$:Ins2$^{+/Akita}$ vs female ovx ApoE$^{-/-}$:Ins2$^{+/Akita}$	NS	NS	p<0.001	p<0.0001	p<0.01
female sham operated ApoE$^{-/-}$ vs female ovx ApoE$^{-/-}$	NS	NS	NS	NS	NS

Supplementary Table 3B. Detailed statistical analysis of figure data in 1B, fasting blood glucose levels in female sham operated or ovariectomized (ovx) ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice and age-matched controls.

Comparison	4 weeks	10 weeks	15 weeks	18 weeks
female ApoE$^{-/-}$ vs female sham operated ApoE$^{-/-}$:Ins2$^{+/Akita}$	p<0.001	NS	NS	NS
female ApoE$^{-/-}$ vs female ovx ApoE$^{-/-}$:Ins2$^{+/Akita}$	p<0.0001	p<0.001	p<0.0001	p<0.0001
female ApoE$^{-/-}$ vs female ovx ApoE$^{-/-}$:Ins2$^{+/Akita}$ +E2	p<0.001	NS	NS	NS
female ApoE$^{-/-}$ vs male ApoE$^{-/-}$:Ins2$^{+/Akita}$	p<0.0001	p<0.0001	p<0.0001	p<0.0001
female ApoE$^{-/-}$ vs male ApoE$^{-/-}$:Ins2$^{+/Akita}$ +E2	p<0.01	p<0.01	p<0.0001	p<0.0001
female sham operated ApoE$^{-/-}$:Ins2$^{+/Akita}$ vs female ovx ApoE$^{-/-}$:Ins2$^{+/Akita}$	NS	p<0.01	p<0.01	p<0.0001
female sham operated ApoE$^{-/-}$:Ins2$^{+/Akita}$ vs female ovx ApoE$^{-/-}$:Ins2$^{+/Akita}$ +E2	NS	NS	NS	NS
female ovx ApoE$^{-/-}$:Ins2$^{+/Akita}$ vs female ovx ApoE$^{-/-}$:Ins2$^{+/Akita}$ +E2	NS	p<0.05	p<0.0001	p<0.0001
female ovx ApoE$^{-/-}$:Ins2$^{+/Akita}$ vs male ApoE$^{-/-}$:Ins2$^{+/Akita}$ +E2	NS	p<0.0001	p<0.0001	NS
Female ovx ApoE$^{-/-}$:Ins2$^{+/Akita}$ +E2 vs male ApoE$^{-/-}$:Ins2$^{+/Akita}$ +E2	NS	NS	p<0.0001	p<0.0001
female ApoE$^{-/-}$ vs male ApoE$^{-/-}$	NS	NS	NS	NS

Supplementary Table 4. Detailed statistical analysis of data in figure 1C, effects of in vivo administration of 17β-Estradiol (E2) in female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice.

Comparison	4 weeks	10 weeks	15 weeks	18 weeks
male ApoE$^{-/-}$ vs male ApoE$^{-/-}$:Ins2$^{+/Akita}$	p<0.001	p<0.0001	p<0.0001	p<0.0001
male ApoE$^{-/-}$ vs female sham operated ApoE$^{-/-}$:Ins2$^{+/Akita}$	p<0.05	NS	NS	NS
male ApoE$^{-/-}$ vs female ovx ApoE$^{-/-}$:Ins2$^{+/Akita}$	p<0.01	p<0.0001	p<0.0001	p<0.0001
male ApoE$^{-/-}$ vs female ovx ApoE$^{-/-}$:Ins2$^{+/Akita}$ +E2	p<0.05	p<0.0001	NS	NS
male ApoE$^{-/-}$ vs male ApoE$^{-/-}$:Ins2$^{+/Akita}$ +E2	p<0.01	p<0.0001	p<0.0001	p<0.0001
male ApoE$^{-/-}$:Ins2$^{+/Akita}$ vs female sham operated ApoE$^{-/-}$:Ins2$^{+/Akita}$	NS	p<0.0001	p<0.0001	p<0.0001
male ApoE$^{-/-}$:Ins2$^{+/Akita}$ +E2 vs female sham operated ApoE$^{-/-}$:Ins2$^{+/Akita}$	NS	NS	p<0.0001	p<0.0001
male ApoE$^{-/-}$:Ins2$^{+/Akita}$ vs female ovx ApoE$^{-/-}$:Ins2$^{+/Akita}$	NS	NS	p<0.0001	p<0.0001
male ApoE$^{-/-}$:Ins2$^{+/Akita}$ vs female ovx ApoE$^{-/-}$:Ins2$^{+/Akita}$ +E2	NS	p<0.0001	p<0.0001	p<0.0001
male ApoE$^{-/-}$:Ins2$^{+/Akita}$ +E2 vs male ApoE$^{-/-}$:Ins2$^{+/Akita}$	NS	p<0.01	NS	NS

Supplementary Table 5. Detailed statistical analysis of data in figure 1C, effects of in vivo administration of 17β-Estradiol (E2) in male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice.

Female ApoE$^{-/-}$	Female ApoE$^{-/-}$:Ins2$^{+/Akita}$ sham	Female ApoE$^{-/-}$:Ins2$^{+/Akita}$ ovx	Age (weeks)
0.08	0.07		1
0.37	0.30		5
0.48	0.40		10
0.85	0.75		15
0.52	0.43	0.13	25

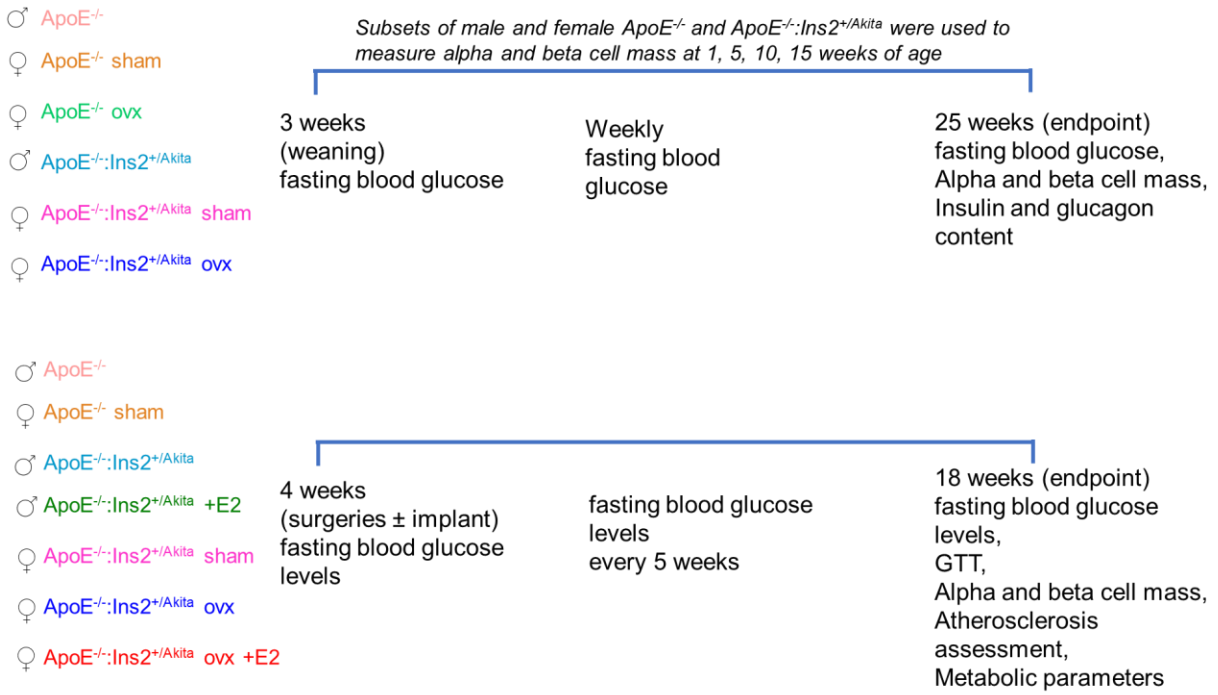
Supplementary Table 6A. Female average beta cell mass (expressed in mg) adjusted for body weight.

Male ApoE$^{-/-}$	Male ApoE$^{-/-}$:Ins2$^{+/Akita}$	Age (weeks)
0.07	0.09	1
0.59	0.24	15
0.72	0.11	25

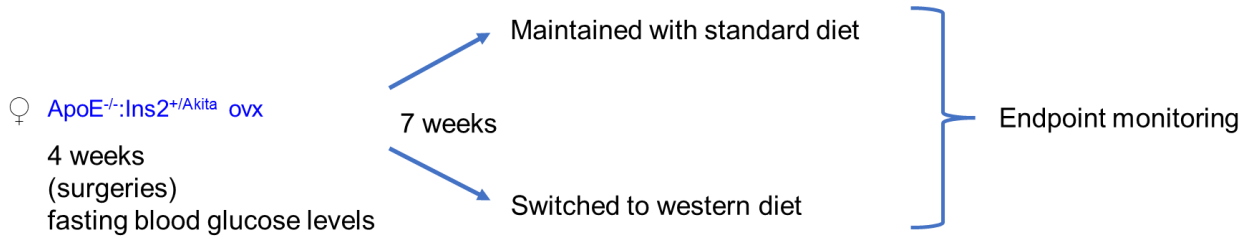
Supplementary Table 6B. Male average beta cell mass (expressed in mg) adjusted for body weight.

Experimental group	Average beta cell mass adjusted for body weight
Female ApoE$^{-/-}$	0.29
Female ApoE$^{-/-}$:Ins2$^{+/Akita}$ sham	0.49
Female ApoE$^{-/-}$:Ins2$^{+/Akita}$ ovx	0.06
Female ApoE$^{-/-}$:Ins2$^{+/Akita}$ ovx +E2	0.31
Male ApoE$^{-/-}$	0.51
Male ApoE$^{-/-}$:Ins2$^{+/Akita}$	0.05
Female ApoE$^{-/-}$:Ins2$^{+/Akita}$ +E2	0.07

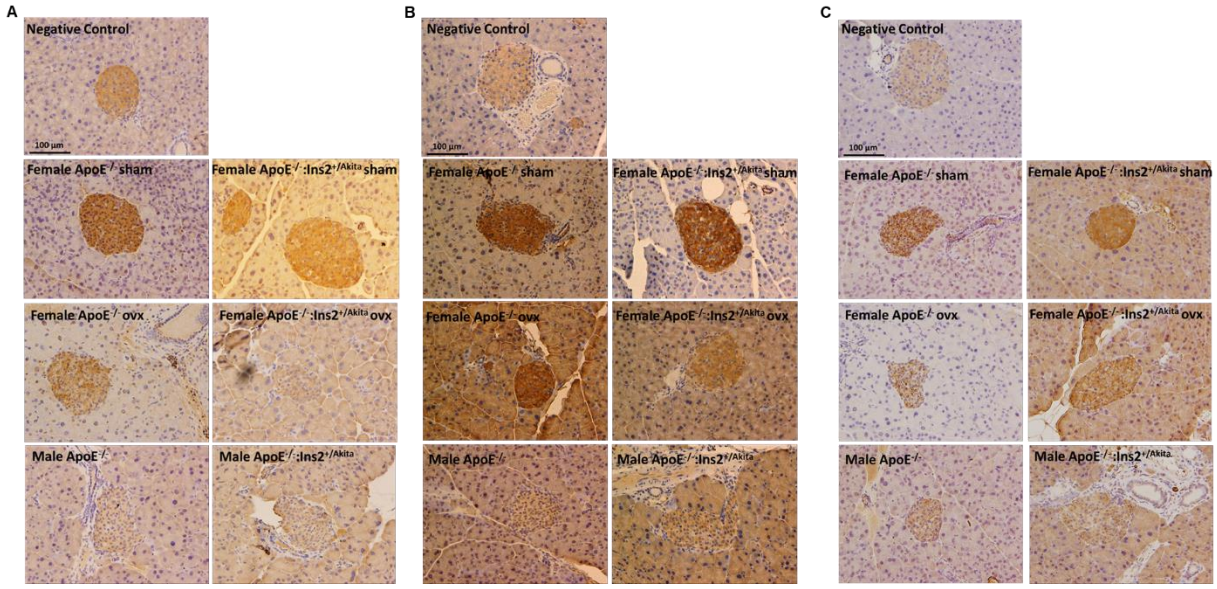
Supplementary Table 7. Average beta cell mass (expressed in mg) adjusted for body weight for each experimental group treated or not with exogenous estradiol



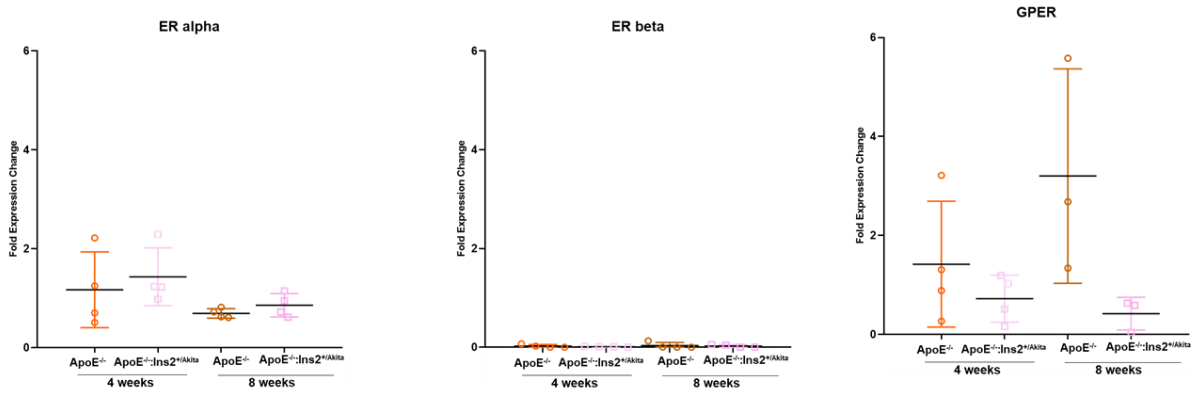
Supplementary figure 1. Experimental paradigm for mice fed standard diet with free access to water.



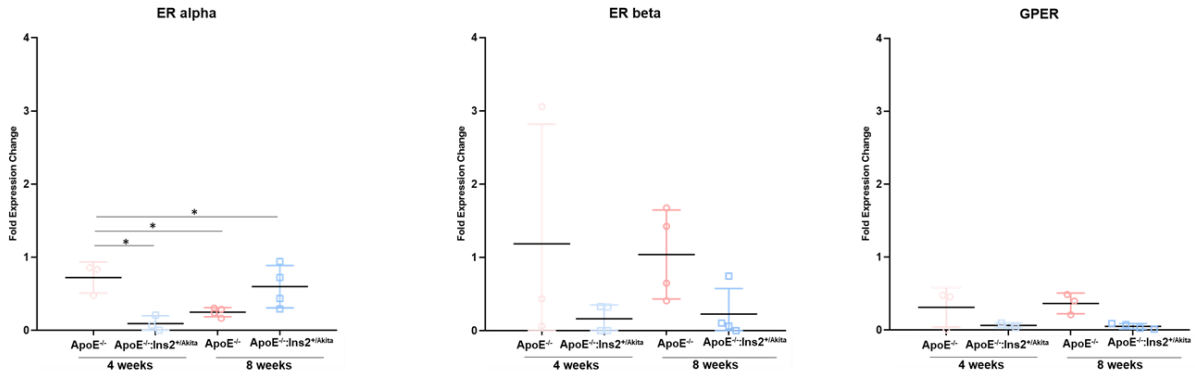
Supplementary figure 2. Experimental paradigm for mice fed western diet with free access to water.



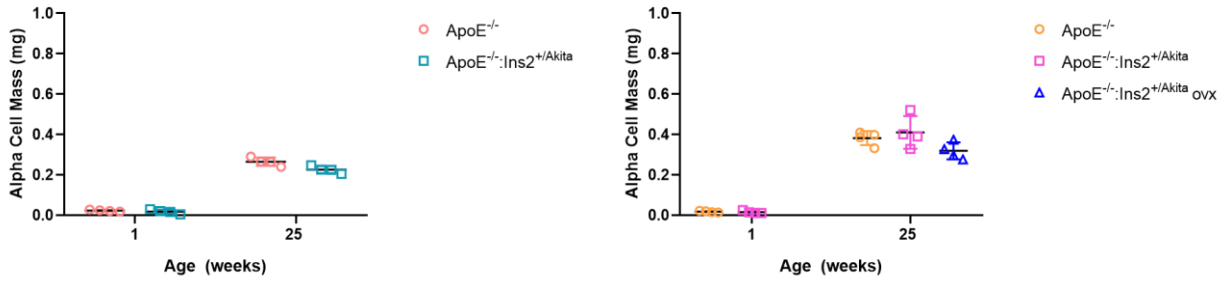
Supplementary Figure 3. Immunohistochemistry staining in paraffin-embedded pancreas sections for 25 week old female sham operated, ovariectomized (ovx) and male ApoE$^{-/-}$ and ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice.



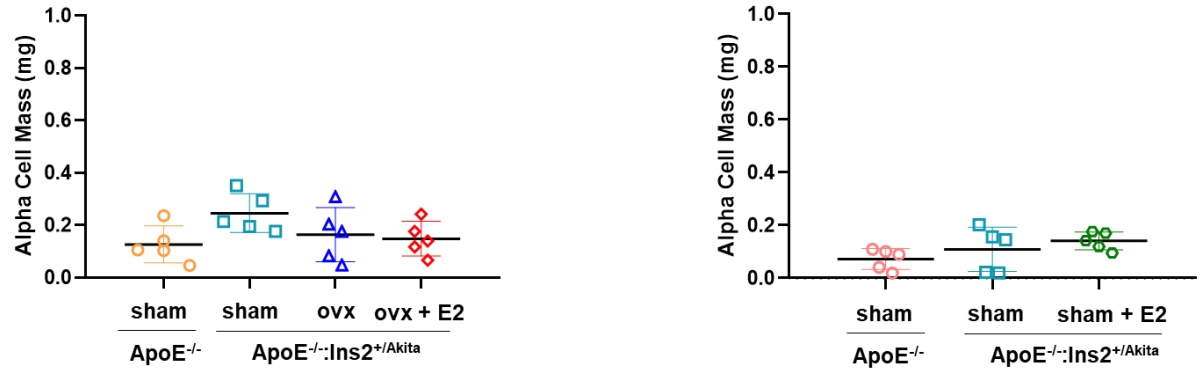
Supplementary Figure 4A. Expression of ER alpha, ER beta and GPER in pancreatic islets isolated from female ApoE$^{-/-}$ and ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice at 4 and 8 weeks of age.



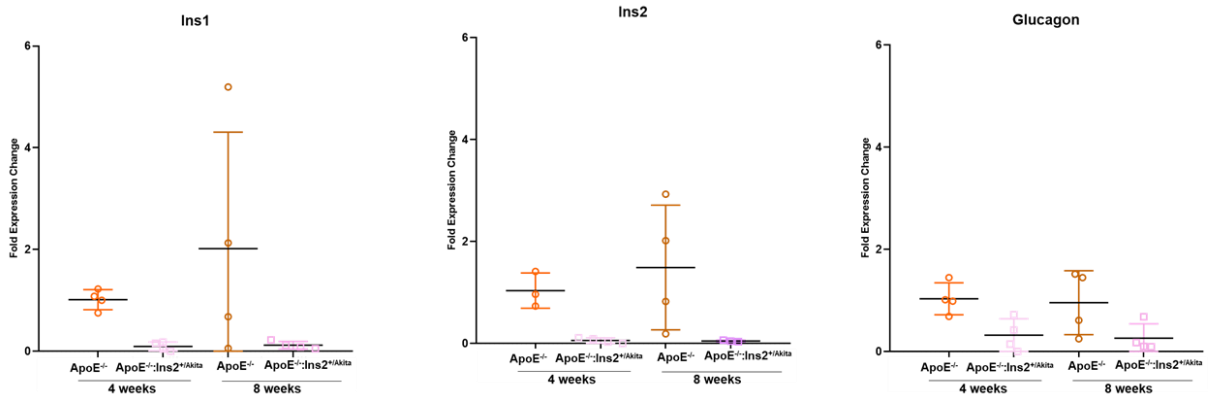
Supplementary Figure 4B. Expression of ER alpha, ER beta and GPER in pancreatic islets isolated from male ApoE$^{-/-}$ and ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice at 4 and 8 weeks of age.



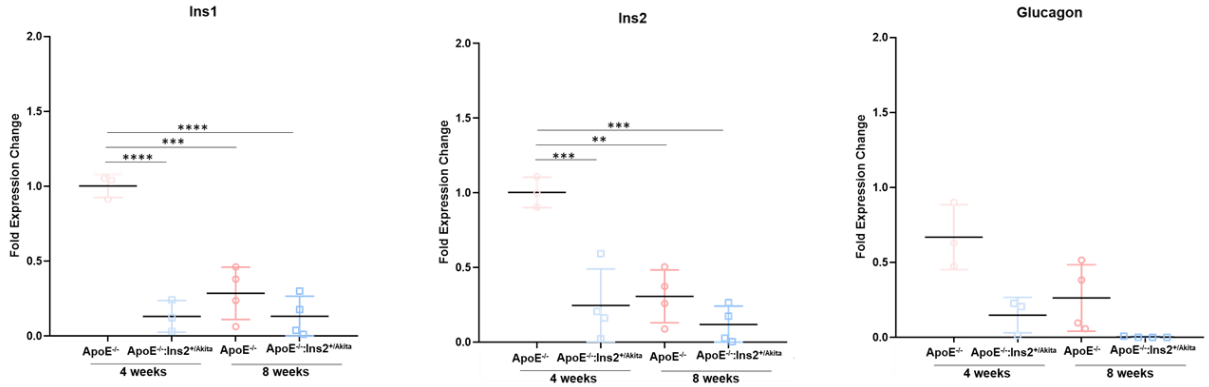
Supplementary Figure 5A. Quantification of alpha cell mass in pancreas sections from (right) male and (left) female ApoE$^{-/-}$ and ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice.



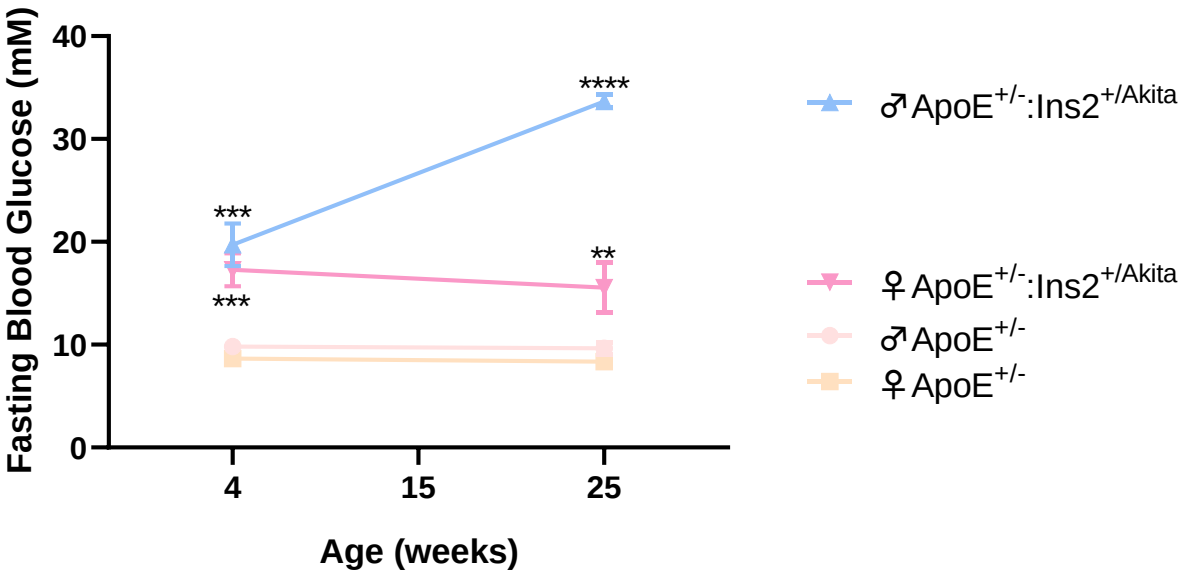
Supplementary Figure 5B. Effects of in vivo administration of 17β-Estradiol on alpha cell mass of female (left) and male (right) ApoE-/-:Ins2+/Akita mice



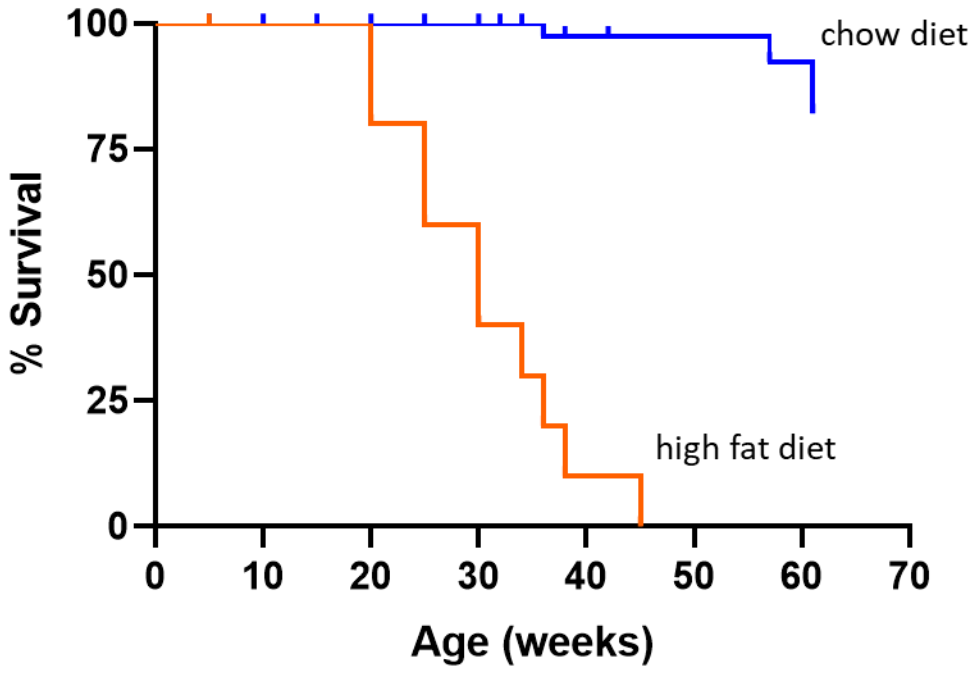
Supplementary Figure 6A. Expression of Ins1, Ins2 and glucagon in pancreatic islets isolated from female ApoE$^{-/-}$ and ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice at 4 and 8 weeks of age.



Supplementary Figure 6B. Expression of Ins1, Ins1 and glucagon in pancreatic islets isolated from male ApoE$^{-/-}$ and ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice at 4 and 8 weeks of age.



Supplementary Figure 7. Fasting blood glucose levels in male and female ApoE$^{+/-}$:Ins2$^{+/Akita}$ mice and age-matched ApoE$^{+/-}$ controls.



Supplementary Figure 8. Effects of western diet (high fat diet) versus regular chow diet on overall survival in ovariectomized ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice.

CHAPTER 3 – INVESTIGATING THE ROLE OF ESTROGEN IN THE REGULATION OF THE UNFOLDED PROTEIN RESPONSE (UPR) IN PANCREATIC BETA CELLS

Monica De Paoli, Mary Bohn, Deep Shah, Alexander Zakharia, Zil Patel, Zinal Patel, Geoff Werstuck

Contributions

The author, Monica De Paoli, has conceived and designed the research project; analyzed and interpreted the results of the experiments; written, edited, and revised the manuscript as well as prepared the manuscript's figures. For the experimental part, the author has bred the experimental mice, performed surgeries, harvested organs and tissues, prepared samples for the analysis. Additionally, the author has measured fasting glucose levels (Figure 1), performed and analysed qRT-PCR for the UPR markers in isolated pancreatic islets (Figure 2), performed immunofluorescent staining for GRP78/GRP94 in female mice (Supplementary Figure 1) performed all statistical analysis of the immunofluorescent and immunohistochemical analysis on UPR markers (Figure 3, Figure 4, Supplementary Figure 1-2). The author has been directly involved in mentoring, supervising, reviewing, and approving all the work of the co-authors listed below.

Co-authors:

Mary Bohn has performed immunofluorescent staining and quantified GRP78/GRP94 in male mice (Figure 2C).

Deep Shah has performed immunofluorescent and immunohistochemical staining and quantified for GRP78/GRP94, PDI, ATF4, and GADD153/CHOP in male and female mice supplemented with estrogen or not (Figure 2A, 2C, 2E, 2G; Figure 3A, 3C, 3E, 3G). Additionally, Deep has performed immunohistochemical staining for GADD153/CHOP in female mice (Supplementary Figure 2A).

Alexander Zakharia performed immunohistochemical staining and analysed the levels of GADD153/CHOP in male mice (Supplementary Figure 2C, 2D)

Zil Patel and Zinal Patel quantified the levels of GADD153/CHOP in female mice (Supplementary Figure 2B).

Supervisor:

Geoff Werstuck has conceived and designed research, wrote, edited, reviewed, and approved the final version of the manuscript.

All authors approved the final version of the manuscript.

Additionally, part of the introduction to this manuscript has been adapted from two previously published peer reviewed papers (Appendix 2, Appendix 3)

Abstract

Diabetes mellitus is a metabolic disorder characterized by the inability of insulin, secreted by pancreatic beta cells, to effectively regulate blood glucose levels. Sex differences in the presentation and outcome of diabetes do exist, and premenopausal women have a reduced risk of developing diabetes, relative to men, or women after menopause. Accumulating evidence shows a potential protective role for estrogens in the maintenance of pancreatic beta cell health, however the mechanisms underlying this protection are still unknown. To elucidate the potential mechanisms by which estrogens might exert this protection we used a mouse model of hyperglycemia-induced atherosclerosis, the ApoE$^{-/-}$:Ins2$^{+/Akita}$ mouse, which exhibits sexual dimorphism in glucose regulation. Male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice are chronically hyperglycemic whereas female mice are only transiently hyperglycemic. However, when female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice are ovariectomized, they retain a chronic hyperglycemic phenotype. Supplementation with exogenous estrogen can normalize blood glucose levels in ovariectomized females. In this study we propose that estrogens might protect pancreatic beta cells by modulating the unfolded protein response (UPR) in response to endoplasmic reticulum (ER) stress. We observed that ovariectomized female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice show significantly increased expression of apoptotic UPR markers in a pattern similar to what observed in male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice. Sham operated female and ovariectomized female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice supplemented with exogenous estrogen show a significant increase in the expression of adaptive UPR markers, compared to non-supplemented ovariectomized female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice. These findings suggest that estrogen may protect pancreatic beta cells from dysfunction by enhancing the adaptive UPR activation in response to pancreatic ER stress.

Introduction

Diabetes mellitus is a metabolic disorder where pancreatic beta cells are unable to secrete sufficient insulin to adequately regulate blood glucose levels. This condition is associated with a variety of vascular disorders including an increased risk of developing atherosclerosis, the underlying cause of cardiovascular diseases (1). The prevalence and incidence of diabetes mellitus has been increasing worldwide due to a more sedentary lifestyle and an increased prevalence of obesity, making it a significant burden on health care and health care costs (2). Sex differences in the prevalence and development of diabetes mellitus and its associated cardiovascular complications are known to exist. Pre-menopausal women are significantly less likely to develop these conditions relative to men or women after menopause (3,4). Accumulating data suggest that estrogens exert a protective effect in pancreatic beta cell health and function however, the mechanisms underlying this protection are still unknown (5,6).

Chronic hyperglycemia promotes insulin biosynthesis in pancreatic beta cells. Secreted proteins, like insulin, are co-translationally translocated and folded in the endoplasmic

reticulum (ER). If increased protein demand overloads the ER, and the folding capacity of the ER is exceeded, unfolded or misfolded proteins can accumulate causing a condition known as ER stress (7). Cells have developed mechanisms to restore ER homeostasis through the activation of the adaptive unfolded protein response (UPR) (8). The adaptive UPR relieves ER stress by increasing protein folding capacity by inducing the synthesis of molecular chaperones and/or increasing the ER associated degradation (ERAD) of terminally misfolded proteins (8). When there is a protracted condition of ER stress and homeostasis cannot be restored, the apoptotic UPR is activated, increasing the synthesis of pro-apoptotic factors (8). Secretory cells, such as pancreatic beta cells, have a heavy workload and are therefore susceptible to ER stress. Several studies have shown an association between diabetes/hyperglycemia and pancreatic beta cell ER stress and apoptosis (7,9–11).

In this study, we investigate the effect of estrogen on the beta cell UPR using a mouse model of hyperglycemia-induced atherosclerosis, the ApoE$^{-/-}$:Ins2$^{+/Akita}$ mouse, which shows sexual dimorphism in terms of glucose regulation and atherosclerosis progression (12). Male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice are chronically hyperglycemic, whereas female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice are transiently hyperglycemic, with blood glucose levels normalizing by five weeks of age, which represents the time when they reach sexual maturity. Ovariectomized female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice are chronically hyperglycemic however, supplementation of exogenous estradiol restores glucose homeostasis (see chapter 2).

We hypothesize that estrogen protects pancreatic beta cell health and function by modulating the UPR specifically by enhancing the adaptive UPR and/or repressing the apoptotic UPR.

Materials and Methods

Animal models

Male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice were crossed with female ApoE$^{-/-}$:Ins2$^{+/+}$ mice to create the experimental ApoE$^{-/-}$:Ins2$^{+/Akita}$ mouse model. Genotypes were confirmed using PCR by methods previously described (see chapter 2). All experimental mice received a standard diet (2018 Teklad Global 18% Protein Rodent Diet, Harlan Teklad, Madison, WI, USA) *ad libitum* with free access to water. Subsets of female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice underwent ovariectomy at 4 weeks of age with the experimental endpoint at 25 weeks of age. Additional subsets of female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice were sham operated or ovariectomized at four weeks of age, and received a subcutaneous estradiol pellet implant (0.1 mg, 90 days release, Innovative Research of America, Sarasota, FL, USA). A subset of male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice also received the estradiol pellet implant at four weeks of age. This pellet continuously releases estradiol at a dose of 0.1 mg/pellet for 90 days allowing the hormone to circulate at a physiological range (13,14). The experimental endpoint for mice receiving the estradiol pellet and their respective

controls was 18 weeks of age. All animal procedures were pre-approved by the McMaster University Animal Research Ethics Board.

Ovariectomy

Ovariectomies were performed using methods previously described (12,15). Mice at four weeks of age (n= 5-10 per experimental group) were anesthetized using isoflurane (5% induction, 2.5% maintenance of anaesthesia). A 3 x 3 cm incision area surrounding the iliac crest was shaved and cleaned, and a midline horizontal incision through the skin was performed. The ovary was identified, and an incision was made through the muscle layer to reach the abdominal cavity. The ovary was pulled out by gently removing the surrounding fat pad from the abdominal cavity. The uterine horn and vessels were double ligated (0.7 cm and 1 cm distally from the ovary), and the ovary was excised. The remaining tissue was put back in the abdominal cavity, and the incision was sutured. The contralateral ovary was removed in a similar fashion. The skin wound was closed using a wound clipper. Sham operated animals (n=5-10 per experimental group) received the same incisions and isolation of ovaries was performed however, ovaries were not removed.

Tissue harvesting

Mice were anesthetized with isoflurane and euthanized by cervical dislocation. Vasculature was rinsed with phosphate buffered saline. Heart, aorta, liver, fat pad, and pancreas were collected. All organs were fixed in 10% neutral-buffered formalin and stored at room temperature.

Analysis of the pancreas

Pancreata from male and female ApoE$^{-/-}$ and ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice were harvested at 18 or 25 weeks of age. Immunohistochemical or immunofluorescent staining for the following UPR markers was performed using the indicated antibodies at the reported dilutions: GRP78/GRP94 (monoclonal mouse KDEL antibody, ADI-SPA-827-J, Enzo/Cedarlane, Burlington, ON, Canada, 1:250 dilution), GADD153/CHOP (monoclonal mouse GADD153 (B3) sc-7531, Santa Cruz Biotechnology, Dallas, TX, USA, 1:50 dilution), PDI (monoclonal mouse antibody, ADI-SPA-891-F, Enzo/Cedarlane, Burlington, ON, Canada, 1:200 dilution), ATF4 (polyclonal rabbit antibody, 10835-1-AP, Thermo Fischer, Mississauga, ON, Canada, 1:200 dilution). Secondary antibodies used were goat anti-mouse biotinylated IgM (BA 2020, Vector, Burlington, ON, Canada), Alexa Fluor 488 goat anti-mouse IgG (A11001, Thermo Scientific, Middletown, VA, USA), Alexa Fluor 488 goat anti-rabbit IgG (A11008, Thermo Scientific, Middletown, VA, USA), at a dilution of 1:200 each. All immunofluorescent staining experiments were counterstained with DAPI (Invitrogen, Carlsbad, CA, USA, 1:5000 dilution). Separate sections were stained with pre-immune IgG instead of the primary antibody, to control for non-specific staining. Threshold levels were selected to correct for non-islet fluorescence.

Staining was performed on paraffin-embedded sections of pancreas (6µm thick) from male and female ApoE$^{-/-}$ and ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice (n=5-8 per experimental group). The

entire pancreas was analyzed at 180 µm intervals for a comprehensive overview of the islets within the organ. A total of six sections per mouse were used to perform immunohistochemical and immunofluorescent analysis, and a total of n=12-15 islets per mouse were selected for the intensity of fluorescence analysis. Immunofluorescence intensity was visualized, and images were captured using a Leica STELLARIS 5 confocal microscope. Analysis was performed using ImageJ software (NIH, Bethesda, MD, USA; http://imagej.nih.gov/ij). The intensity of fluorescence staining for each mouse was calculated as follows:

Intensity of fluorescence = (average intensity of fluorescence of the pancreatic islets per mouse) - (average intensity of fluorescence of the pancreatic islets of the negative control)

Immunohistochemical analysis was captured using a Leitz LABORLUX S microscope connected to a DP71 Olympus camera. Quantification of GADD153/CHOP was performed by counting the brown-stained nuclei versus the total number of nuclei (n= 12-20 islets per mouse; n= 4-8 mice per experimental group).

Islet isolation

Pancreatic islet isolation was carried out using established methods (16,17). Pancreata were excised from 4 or 8 week old mice and placed in collagenase (Collagenase type XI, C7657, Sigma Aldrich, Oakville, ON, Canada) at 37°C for digestion, and hand shaken regularly for 20 minutes. Islets were isolated on a histopaque gradient (Histopaque 1119, 11191 and Histopaque 1077, 10771, Sigma Aldrich, Oakville, ON, Canada), and washed with Hank's Balanced Salt Solution (HBSS). Each islet sample represents pooled islets from 4-8 pancreata.

Gene expression analysis by qRT-PCR

Total RNA was isolated from pancreatic islets using TRIzol reagent (TRIzol®, 15596-018, Life Technologies, Burlington, ON, Canada). mRNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (4368813, Applied Biosystems, Foster City, California, USA). Real time PCR amplification was performed using SYBR green (SensiFAST™ SYBR Hi-ROX kit, Bioline, Wolston Warrington, UK) reagent. Transcript amplification was normalized to the reference gene beta actin. Primers sequences are listed in Table 1.

Statistical Analysis

Statistical analysis of multiple groups was assessed using t-test, one-way or two-way ANOVA, where appropriate. Data are presented as mean± Standard Error of the Mean (SEM). Analyses were performed using ImageJ. A value of $p<0.05$ was considered statistically significant.

Results

We previously reported sex differences in the control of glucose homeostasis in the ApoE$^{-/-}$:Ins2$^{+/Akita}$ mouse model (see chapter 2), with male mice developing chronic hyperglycemia, and female mice showing normalized glucose levels by five weeks of age corresponding to the age when they reach sexual maturity (Figure 1). To determine if these changes in glucose regulation correspond to modifications in the activation of the UPR, pancreatic islets were isolated from four and eight week old male and female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice and ApoE$^{-/-}$ controls. Four weeks of age corresponds to a time where both male and female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice are hyperglycemic, and eight weeks of age corresponds to a time when male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice are hyperglycemic whereas females have normalized glucose levels. Total RNA was isolated from the islets and the expression of specific UPR genes were quantified. We observed that 8 week old female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice presented with a significant increase in the expression of markers of the adaptive UPR compared to four week old female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice and age matched ApoE$^{-/-}$ controls. (Figure 2A). These include GRP78, an ER resident chaperone involved in protein folding (18), and ERAD-enhancing alpha-mannosidase-like protein (EDEM), which is involved in the endoplasmic reticulum associated degradation (ERAD) (19). Furthermore, the protein disulfide isomerase (PDI) isoforms PDIa3 and PDIa6 are also significantly induced in 8 week old female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice relative to 4 week old female mice. No significant activation of the adaptive UPR is observed in male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice (Figure 2B). Markers of the apoptotic UPR, ATF4 and GADD153/CHOP, are not induced in female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice, but are significantly increased in male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice at 4 and 8 weeks of age, respectively (Figure 2C,D).

To determine the effects of estrogen and estrogen supplementation on UPR activation, male and female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice were implanted with a slow release estrogen pellet at four weeks of age and pancreata were harvested at eighteen weeks of age. We observed that estrogen supplementation normalizes blood glucose levels in ovariectomized female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice, whereas this treatment only transiently improves glucose homeostasis in male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice (see chapter 2). The expression of the adaptive UPR markers GRP78/GRP94 in female sham operated and ovariectomized female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice supplemented with estradiol is significantly increased compared to female ovariectomized ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice (Figure 3A,B). No significant differences are observed across male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice experimental groups in the expression of this adaptive UPR marker (Figure 3C,D). PDI expression was significantly induced in the female ovariectomized ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice, compared to the other female experimental groups (Figure 3E,F), whereas no significant differences were observed in the expression of this adaptive UPR marker across the male experimental groups (Figure 3G,H).

The apoptotic UPR marker ATF4 was significantly increased in the ovariectomized female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice, but supplementation of estrogen in female ovariectomized ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice significantly downregulates the expression of this

marker to levels comparable to the sham operated ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice and the ApoE$^{-/-}$ controls (Figure 4A,B). No significant differences in ATF4 expression were observed in male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice in the presence or absence of supplemented estrogen, relative to the ApoE$^{-/-}$ controls (Figure 4C,D). Similar to what is observed with ATF4, the expression of GADD153/CHOP is significantly induced in the ovariectomized female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice (Figure 4E,F). Ovariectomized female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice supplemented with estrogen do not show this increase in the expression of this apoptotic UPR marker. GADD153/CHOP was significantly expressed in male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice relative to the ApoE$^{-/-}$ controls. Supplementation with estrogen reduced GADD153/CHOP to levels of ApoE$^{-/-}$ controls (Figure 4G,H).

Discussion

Sex differences in the development of diabetes do exist, and premenopausal women appear to be protected from diabetes compared to men and women after menopause. This suggests that estrogens, and perhaps other sex hormones, play a protective role (20). Furthermore, at least part of this protective effect appears to be linked to estrogen's ability to alleviate ER stress, a condition that can be found in pancreatic beta cells under conditions of protracted hyperglycemia, such as diabetes (21). We hypothesize that estrogen protects pancreatic beta cell health and function by enhancing the adaptive UPR and/or repressing the apoptotic UPR. To test this hypothesis we used a mouse model of hyperglycemia-induced atherosclerosis, the ApoE$^{-/-}$:Ins2$^{+/Akita}$ mouse, which shows sexual dimorphism in terms of glucose regulation and homeostasis (Figure 1) (12).

This mouse model is notable because of the observation that male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice are chronically hyperglycemic, whereas females are only transiently hyperglycemic. Blood glucose levels in female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice normalize by five weeks of age, which represents the time when these mice become sexually mature, and estrogen levels increase (Figure 1). We quantified the expression of UPR genes in isolated pancreatic islets from male and female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice and ApoE$^{-/-}$ controls at four and eight weeks of age, as these ages bracket the observed normalization of blood glucose levels (and the attainment of sexual maturity) observed in female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice. We observed that the normalization of blood glucose levels in female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice is associated with activation of the adaptive UPR. Specifically, there is an increase in expression of ER resident chaperone GRP78 involved in protein folding, EDEM which is part of ERAD, and certain PDI isoforms which are involved in the formation of disulfide bonds, a crucial step for the proper folding and processing of insulin (Figure 2). Conversely, in male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice, the adaptive UPR is not significantly induced, but there is a significant increase in apoptotic UPR markers ATF4 and GADD153/CHOP, compared to age-matched ApoE$^{-/-}$ controls. These findings are consistent with the fact that the male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice show a significant loss of beta cell mass and are chronically hyperglycemic (Figure 1)

(see chapter 2). As these results show a potential role of estrogen in inducing the adaptive UPR, we sought to evaluate the expression of the adaptive markers GRP78/GRP94 and apoptotic marker GADD153/CHOP in conditions of estrogen depletion. Our previous studies showed that ovariectomy, a surgical procedure that significantly reduces the levels of circulating estrogens, promotes a chronic hyperglycemic phenotype in female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice (see chapter 2). Consistent with results of gene expression analysis of the isolated pancreatic islets, GRP78/94 proteins are significantly induced in female sham-operated ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice, but not in the age-matched ovariectomized ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice (Supplementary Figure 1A). Ovariectomy in female ApoE$^{-/-}$ controls, which are normoglycemic, does not significantly affect this adaptive UPR marker. Male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice also show significantly induced expression of GRP78/GRP94, compared to age-matched controls (Supplementary Figure 1). This can be expected since the Akita mutation is characterized by a point mutation in one allele of the Ins2 gene (C96Y) that leads to insulin misfolding (Figure 4B) (12). However, male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice also show a significant increase in the expression of GADD153/CHOP (Supplementary Figure 2), and similar results can be seen in the ovariectomized female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice, but not in ovariectomized female ApoE$^{-/-}$ controls.

Estrogen supplementation rescued the normoglycemic phenotype in ovariectomized female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice but not in males (see chapter 2). We analysed the expression of the adaptive UPR marker GRP78/GRP94 and we observed that estrogen supplementation significantly induces the expression of GRP78/GRP94 in ovariectomized female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice, but not in male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice (Figure 2). Furthermore, it was observed that ovariectomized female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice showed increased expression of the apoptotic UPR markers ATF4 and GADD153/CHOP, but supplementation of estrogen downregulated this expression (Figure 3). These results are consistent with estrogen modulating the UPR to increase insulin folding capacity, by increasing the expression of molecular chaperones GRP78/GRP94 and downregulating the expression of proapoptotic molecules such as ATF4 and GADD153/CHOP. We also observed that PDI is significantly induced in the ovariectomized female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice, but not in the sham operated or ovariectomized female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice supplemented with estrogen (Figure 2). This could be explained by the fact that PDI is directly involved in aiding with the formation of disulfide bonds, which are important components of insulin processing and maturation. Ovariectomized female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice are hyperglycemic, therefore their demand of insulin is significantly increased, compared to that of normoglycemic female sham operated, or female ovariectomized ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice supplemented with estrogen. Hence, ovariectomized female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice require increased expression of PDI. This result also shows that the expression of PDI is differentially regulated, compared to other UPR markers. Further studies are needed to verify this hypothesis.

Overall, our findings suggest that, under conditions that stimulate ER stress, such as chronic hyperglycemia, estrogen exerts a protective function by modulating the UPR, by enhancing the adaptive UPR and repressing the apoptotic UPR. The results from our animal studies are consistent with various in vitro studies showing that estrogen has a similar modulation of the adaptive and apoptotic UPR in different cell types. A study in a human gastric adenocarcinoma cell line treated with an ER stress inducer, tunicamycin, showed that those treated with estrogen significantly reduced ER stress-induced apoptosis (22). A similar result was also observed in another study using a mouse-derived osteoblast cell line, where ER stress was induced using another ER stress inducer, thapsigargin (23). In this case estrogen was able to reduce ER stress by increasing the expression of the protein chaperone GRP78, as well as repressing apoptosis by inhibiting the caspase cascade. Finally, another study on pancreatic cell lines (INS1) showed that estrogens were able to reduce the levels of ER stress in the presence of high glucose levels, protecting them from cell death (21).

Taken together, these results could provide a possible explanation of why pre-menopausal women have a lower risk of developing diabetes than men or post-menopausal women (3,21–24). Further research will be needed to determine the specific molecular mechanisms by which estrogen can modulate the adaptive UPR.

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Tables

Target	Forward	Reverse
Beta actin	5'-GGCACCACACCTTCTACAATG -3'	5'-GGGGTGTTGAAGGTCTCAAAC -3'
GRP78	5′ -CATGGTTCTCACTAAAATGAAAG G -3′	5′-GCTGGTACAGTAACAACTG -3′
EDEM	5'-CTACCTGCGAAGAGGCCG -3'	5'-GTTCATGAGCTGCCCACTGA -3'
PDIa1	5'- CAAGATCAAGCCCCACCTGAT -3'	5'-AGTTCCCCCCAACCAGTACTT -3'
PDIa3	5'- GATGGAATTGTCAGCCACTTG -3'	5'-GGTGTGTGCAAATCGGTAGTT -3'
PDIa4	5'- AGCTCCTTGGCAGCTTTCTC -3'	5'-TGCAGACATTATTTTGGTGGA -3'
PDIa6	5'- CTAGCAGTCAGCGGTCTGTAT -3'	5'-CACAGGCCGTCACTCTGAAT -3'
ATF4	5′-ATGGCCGGCTATGGATGAT -3′	5′-CGAAGTCAAACTCTTTCAGATC -CATT -3′
GADD153 / CHOP	5'TATCTCATCCCCAGGAAACG-3'	5'-CTGCTCCTTCTCCTTCATGC-3'

Table 1. Mouse primer sequences for qRT-PCR analysis

Figure captions

Figure 1. Fasting blood glucose levels in female and age-matched male ApoE$^{-/-}$ and ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice between 3 and 8 weeks of age. n=4-7 mice per experimental group. ****p<0.0001 4 week old male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice vs age matched male and female ApoE$^{-/-}$ controls; ****p<0.0001 4 week old female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice vs age matched male and female ApoE$^{-/-}$ controls; ****p<0.0001 5 week old male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice vs age matched male and female ApoE$^{-/-}$ controls; ****p<0.0001 5 week old male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice vs age matched female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice; ****p<0.0001 8 week old male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice vs age matched male and female ApoE$^{-/-}$ controls; ****p<0.0001 8 week old male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice vs age matched female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice. Bars represent standard error of the mean (SEM).

Figure 2. Expression of adaptive and apoptotic UPR markers in isolated pancreatic islets. Transcripts of isolated pancreatic islets were analysed for the expression of adaptive UPR markers GRP78, EDEM, PDIa1, PDIa3, PDIa4, PDIa6 in **(A)** female and **(B)** male ApoE$^{-/-}$ and ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice at 4 and 8 weeks of age. Pooled islets from n=4-8 mice. n=4 per group. *p<0.05, NS, not significant. Bars represent standard error of the mean (SEM). Expression of apoptotic UPR markers ATF4 and CHOP in transcripts from isolated pancreatic islets of female **(C)** and male **(D)** ApoE$^{-/-}$ and ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice at 4 and 8 weeks of age. Pooled islets from n=4-8 mice. n=4 per group. *p<0.05, NS, not significant. Bars represent standard error of the mean (SEM).

Figure 3. Expression of adaptive UPR markers in pancreatic slide sections. Immunofluorescence staining was performed to measure the expression of adaptive UPR markers GRP78/GRP94 in pancreatic islet sections of female **(A, B)** sham-operated, female ovariectomized, female ovariectomized supplemented with estrogen (E2) ApoE$^{-/-}$:Ins2$^{+/Akita}$ and female ApoE$^{-/-}$ mice; and male **(C, D)** male ApoE$^{-/-}$:Ins2$^{+/Akita}$ supplemented with estrogen **(E2)** or not. n=4-5 per group. *p<0.05, **p<0.01, NS, not significant. Bars represent standard error of the mean (SEM). Immunostaining was performed to evaluate the expression of adaptive UPR marker PDI in pancreatic islet sections of female **(E, F)** sham-operated, female ovariectomized, female ovariectomized supplemented with estrogen (E2) ApoE$^{-/-}$:Ins2$^{+/Akita}$ and female ApoE$^{-/-}$ mice; and male **(G, H)** male ApoE$^{-/-}$:Ins2$^{+/Akita}$ supplemented with estrogen (E2) or not. n=4-5 per group. *p<0.05, **p<0.01, NS, not significant. Bars represent standard error of the mean (SEM).

Figure 4. Expression of apoptotic UPR markers in pancreatic slide sections. Immunostaining was performed to measure the expression of apoptotic UPR marker ATF4 in pancreatic islet sections of female **(A, B)** sham-operated, female

ovariectomized, female ovariectomized supplemented with estrogen (E2) ApoE$^{-/-}$:Ins2$^{+/Akita}$ and female ApoE$^{-/-}$ mice; and male **(C, D)** male ApoE$^{-/-}$:Ins2$^{+/Akita}$ supplemented with estrogen (E2) or not. n=4-5 per group. *p<0.05, NS, not significant. Bars represent standard error of the mean (SEM). Immunohistochemistry staining was performed to quantify the expression of apoptotic UPR marker GADD153/CHOP in pancreatic islet sections of female **(E, F)** sham-operated, female ovariectomized, female ovariectomized supplemented with estrogen (E2) ApoE$^{-/-}$:Ins2$^{+/Akita}$ and female ApoE$^{-/-}$ mice; and male **(G, H)** male ApoE$^{-/-}$:Ins2$^{+/Akita}$ supplemented with estrogen (E2) or not. Data is expressed as percentage of brown-stained nuclei versus total nuclei. n=4-5 per group. *p<0.05. Bars represent standard error of the mean (SEM).

Supplementary Figure 1. Expression of adaptive UPR markers GRP78/GRP94 in pancreatic islet sections of 25 week old female sham-operated, female ovariectomized, and male ApoE$^{-/-}$ and ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice at 25 weeks of age. Immunofluorescent staining was performed on pancreatic section slides to evaluate the effects of ovariectomy in the expression of adaptive UPR markers GRP78/GRP94 in **(A,B)** female ApoE$^{-/-}$ and ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice versus age-matched ApoE$^{-/-}$ controls. Additionally, the expression of GRP78/GRP94 was also evaluated in **(C,D)** male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice versus age-matched ApoE$^{-/-}$ controls. n=4 per group. *p<0.05, **p<0.01. Bars represent standard error of the mean (SEM).

Supplementary Figure 2. Expression of apoptotic UPR marker GADD153/CHOP in pancreatic islet sections of female sham operated, female ovariectomized, and male (C, D) ApoE$^{-/-}$ and ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice at 25 weeks of age. Immunohistochemistry staining was performed on pancreatic section slides to evaluate the effects of ovariectomy in the expression of apoptotic UPR marker GADD153/CHOP in **(A,B)** female ApoE$^{-/-}$ and ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice versus age-matched ApoE$^{-/-}$ controls. Additionally, the expression of GADD153/CHOP was also evaluated in **(C,D)** male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice versus age-matched ApoE$^{-/-}$ controls. Quantification is expressed as percentage of brown-stained nuclei versus total nuclei. n=4-5 per group. *p<0.05, **p<0.01, ***p<0.001. Bars represent standard error of the mean (SEM).

Figures

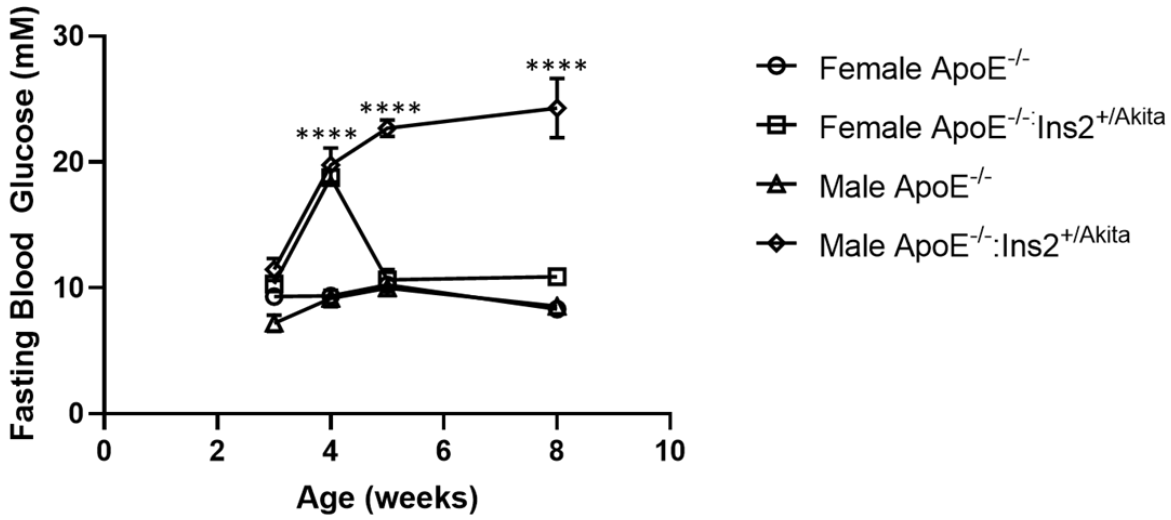


Figure 1. Fasting blood glucose levels in female and age-matched male ApoE$^{-/-}$ and ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice between 3 and 8 weeks of age.

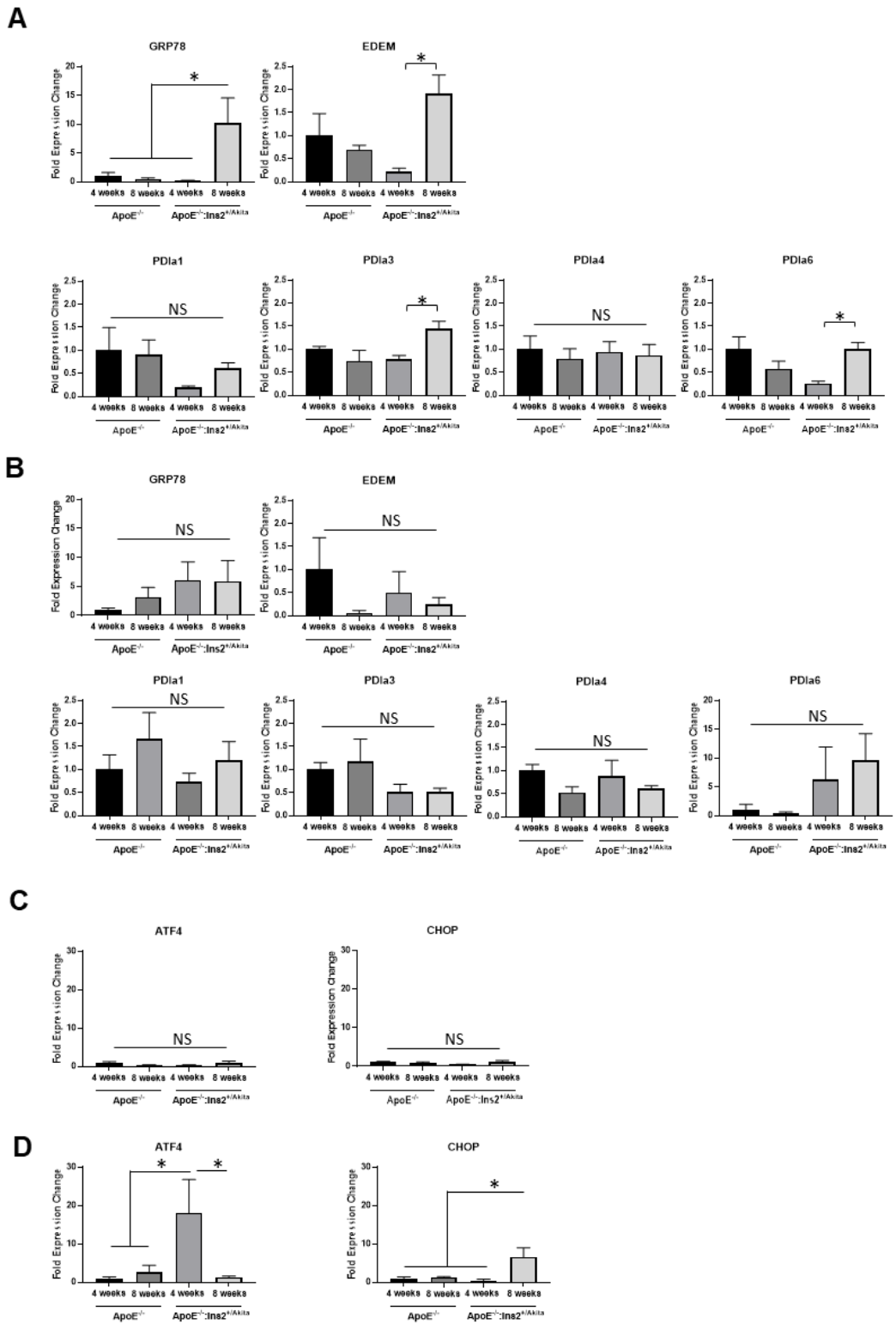


Figure 2. Expression of adaptive and apoptotic UPR markers in isolated pancreatic islets.

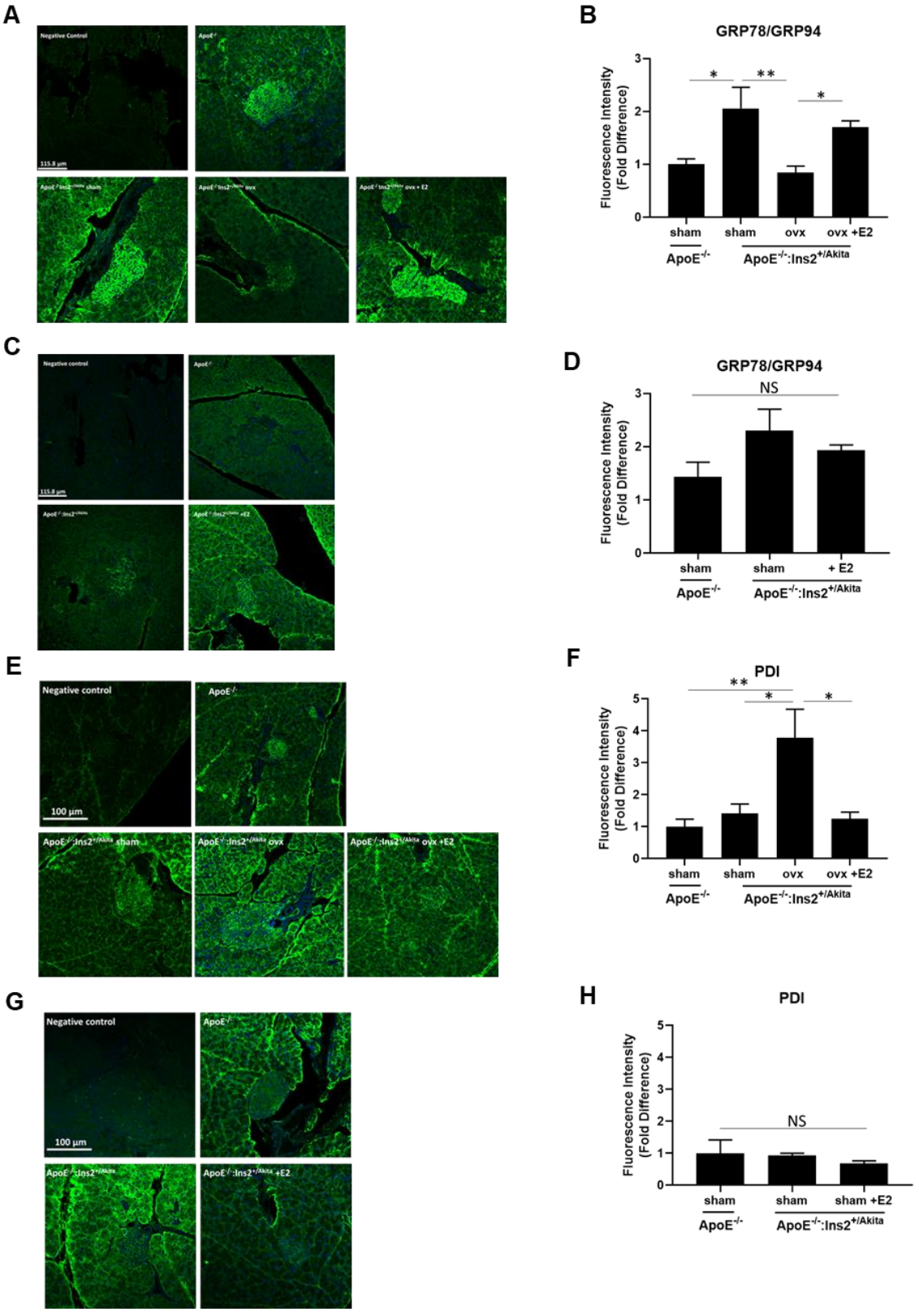


Figure 3. Expression of adaptive UPR markers in pancreatic slide sections.

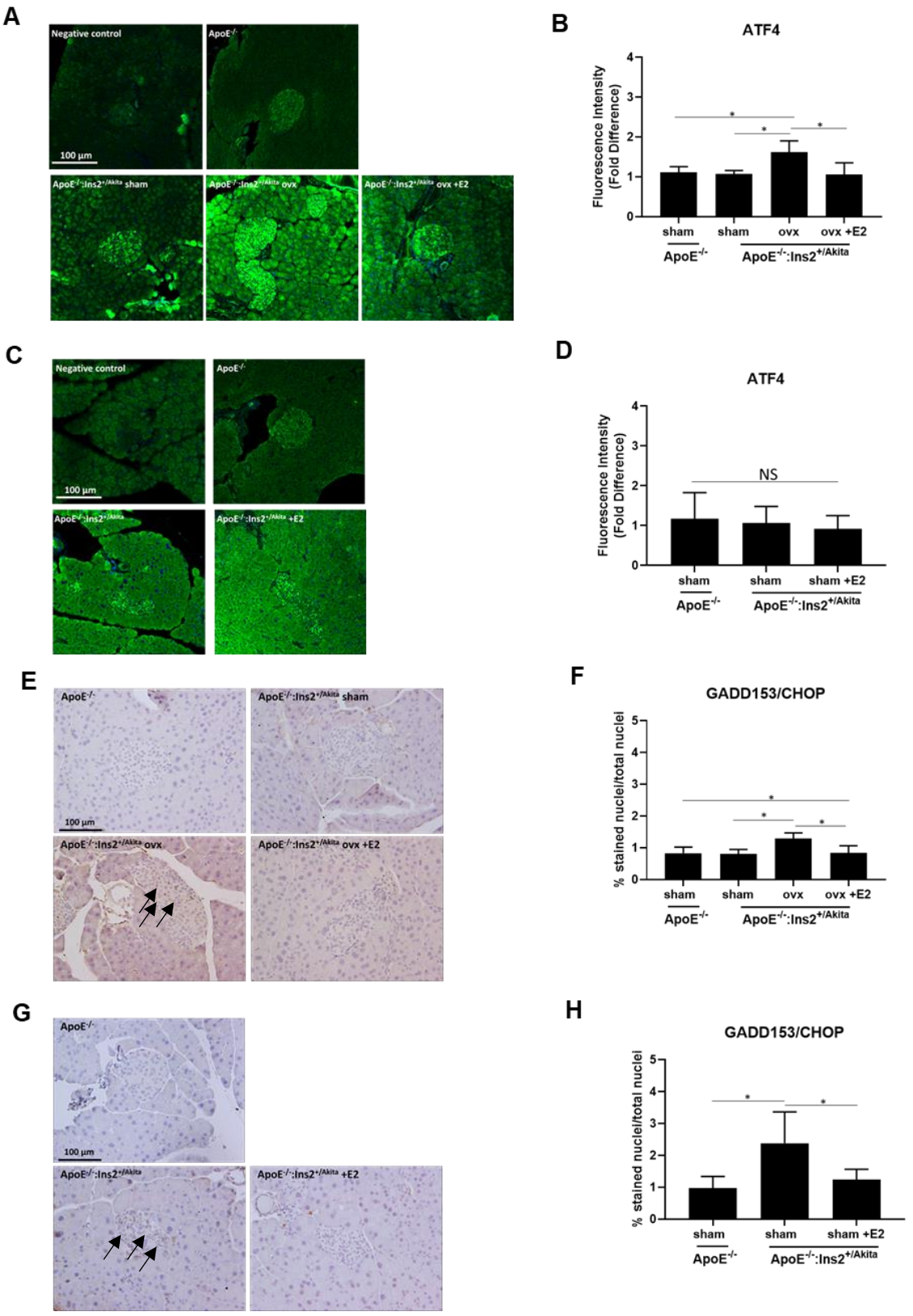
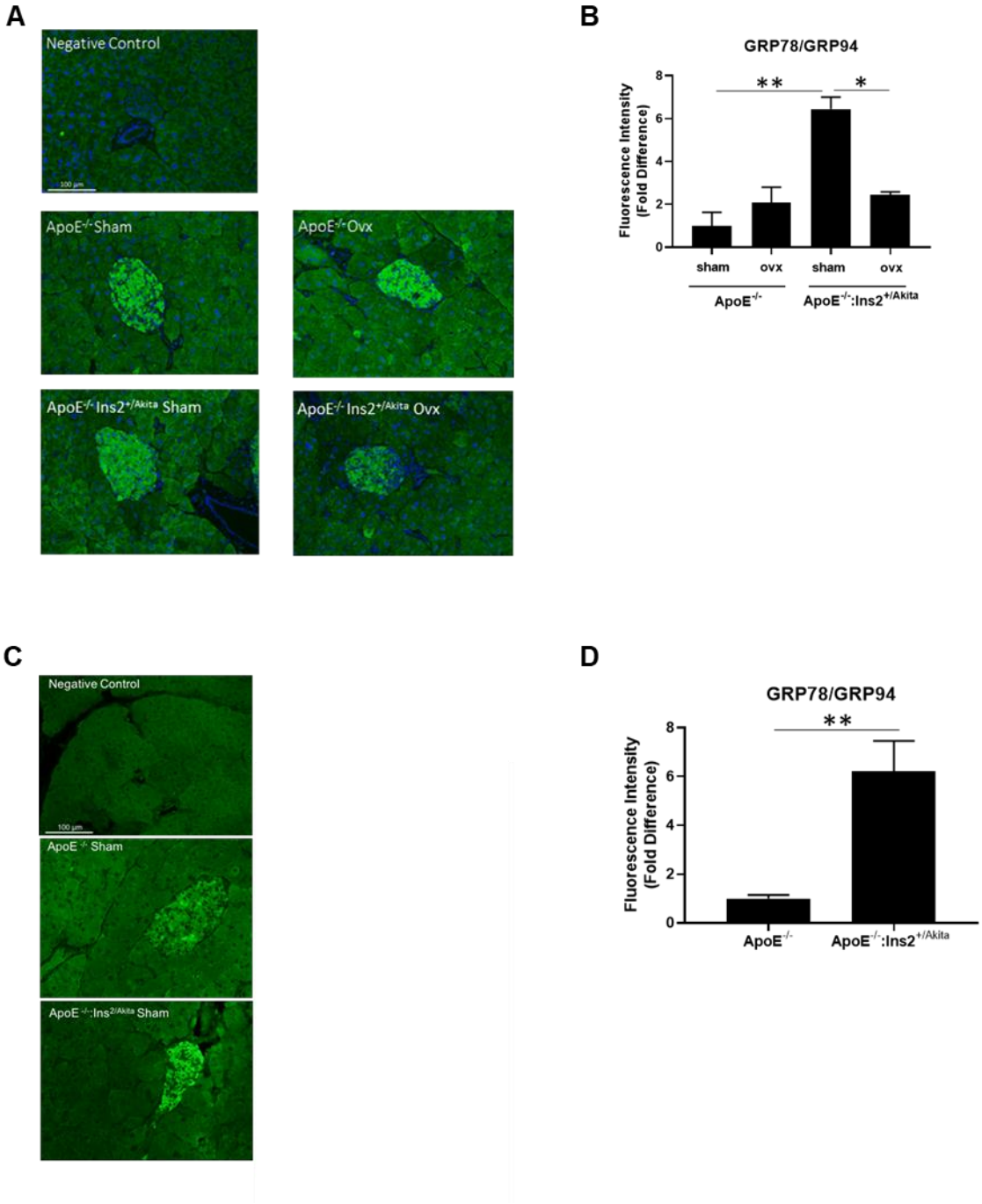
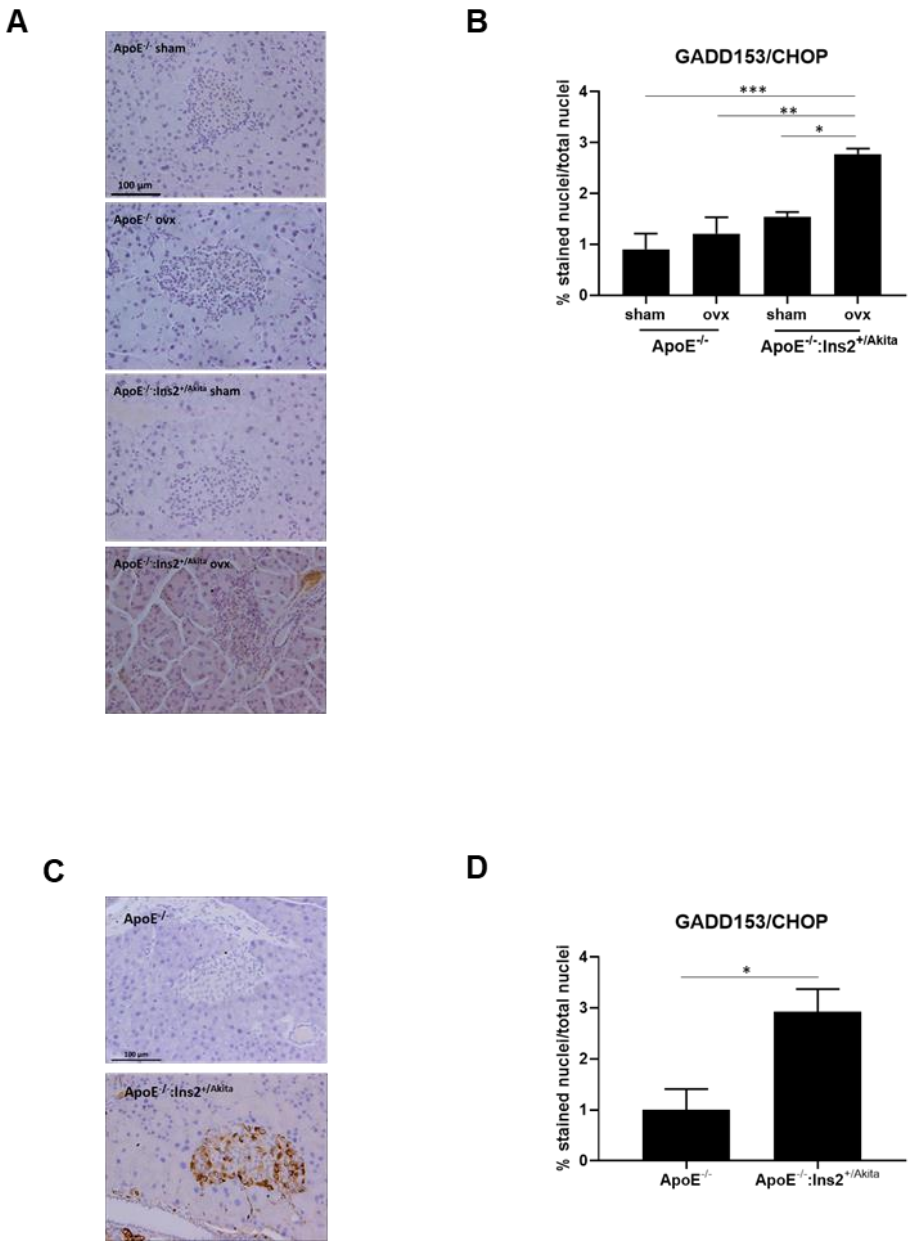


Figure 4. Expression of apoptotic UPR markers in pancreatic slide sections.

Supplementary material



Supplementary Figure 1. Expression of adaptive UPR markers GRP78/GRP94 in pancreatic islet sections of 25 week old female sham-operated, female ovariectomized, and male ApoE$^{-/-}$ and ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice at 25 weeks of age.



Supplementary Figure 2. Expression of apoptotic UPR marker GADD153/CHOP in pancreatic islet sections of female sham operated, female ovariectomized, and male ApoE$^{-/-}$ and ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice at 25 weeks of age.

CHAPTER 4 – SEX DIFFERENCES AND THE ROLE OF ER STRESS IN THE DEVELOPMENT OF HYPERGLYCEMIA IN THE TALLYHO/JngJ MOUSE, A MOUSE MODEL OF POLYGENIC OBESITY AND TYPE 2 DIABETES

Monica De Paoli, Zinal Patel, Susanna Fang, Geoff Werstuck

Contributions

The author, Monica De Paoli, has conceived and designed the research project; analyzed and interpreted the results of the experiments; written, edited, and revised the manuscript as well as prepared the manuscript's figures. For the experimental part, the author has bred the experimental mice, performed surgeries, harvested organs and tissues, prepared samples for the analysis. Additionally, the author has measured fasting glucose levels (Figure 1A, Figure 3A), performed and analysed ipGTT in experimental mice (Figure 2B, 2C; Figure 3B-E), tested and analysed the metabolic parameters for body weight, pancreas weight, liver weight, adipose tissue weight (Tables 1-2). Additionally, the author has performed all statistical analysis of the immunofluorescent and immunohistochemical analysis on alpha and beta cell mass quantification, insulin and glucagon content, and all UPR markers (Figures 4-6). The author has been directly involved in mentoring, supervising, reviewing, and approving all the work of the co-authors listed below.

Co-authors:

Susanna Fang has performed immunohistochemical analysis and quantification for alpha and beta cell mass quantification, immunofluorescent analysis and quantification for insulin and glucagon content (Figure 4), and quantification of serum insulin levels (Tables 1-2).

Zil Patel has performed immunofluorescent and immunohistochemical staining and quantified for GRP78/GRP94, PDI, ATF4, and GADD153/CHOP in male and female mice (Figures 5-6)

Supervisor:

Geoff Werstuck has conceived and designed research, wrote, edited, reviewed, and approved the final version of the manuscript.

All authors approved the final version of the manuscript.

Additionally, part of the introduction to this manuscript has been adapted from two previously published peer reviewed papers (Appendix 2, Appendix 3).

Abstract

Insulin is the central regulator of blood glucose levels. When pancreatic beta cells are not capable of secreting sufficient amounts of insulin to effectively regulate blood glucose levels, chronic hyperglycemia is established. Diabetes mellitus is a heterologous group of metabolic disorders that are clinically defined by the presence of chronic hyperglycemia. The global incidence of diabetes mellitus is increasing, causing a heavy burden on health care management and costs. It is now clear that there are sex differences in the incidence and prevalence of diabetes mellitus, with premenopausal women being protected from developing this disease, compared to men or postmenopausal women. The mechanisms underlying these differences have yet to be elucidated however, the use of experimental animal models can greatly help to gain a better understanding of these mechanisms. In this study we characterized a mouse model of polygenic type 2 diabetes, the TALLYHO/JngJ mice, that shows sexual dimorphism in blood glucose regulation, with male mice developing chronic hyperglycemia by five weeks of age, and female mice remaining normoglycemic. We first evaluated the effect of estrogen deprivation in female TALLYHO/JngJ mice by performing ovariectomies. Ovariectomized mice become chronically hyperglycemic, similar to males. We also evaluated the role of endoplasmic reticulum (ER) stress and the activation of the unfolded protein response (UPR) in the development of hyperglycemia. We observed that the expression of markers of the unfolded protein response (UPR) are significantly upregulated in hyperglycemic male and ovariectomized female TALLYHO/JngJ mice. Treatment with a chemical chaperone 4-phenylbutyric acid (4-PBA), that is known to reduce ER stress, significantly improves blood glucose levels in male and ovariectomized female TALLYHO/JngJ mice. Together, these findings support a protective role for estrogen, and identify the UPR as a pathway through which glucose and estrogen may modulate pancreatic beta cell health.

Introduction

Physiological levels of blood glucose are primarily regulated by the peptide hormones insulin and glucagon, produced by pancreatic beta and alpha cells respectively. When proper homeostasis cannot be maintained there is an increased risk of developing diabetes mellitus, a metabolic disorder characterized by chronic hyperglycemia. The incidence of diabetes has been steadily growing in recent years and it is expected to continue to soar over the next twenty years due to a significant increase of predisposing risk factors such as overweight/obesity and sedentary lifestyles, among the general population (1,2). Individuals with diabetes have an increased risk of developing cardiovascular complications such as myocardial infarction and stroke, which highlights how this disease, and its complications, pose a significant burden in terms of patient management and health care costs (1–4).

The ER is a cellular organelle that is responsible for the synthesis, proper folding, and maturation of proteins that are destined for secretion or transport to the plasma membrane. Pancreatic beta cells have a well-structured endoplasmic reticulum to support their ability to synthesize and secrete appropriate amounts of insulin into the circulation. Conditions of chronic hyperglycemia induce pancreatic beta cells to synthesize and process large amounts of insulin in a short period of time. This can lead to an overwhelmed ER where the protein-folding capability of the cell is exceeded. This unbalance is known as ER stress. Cells respond to ER stress by activation of the unfolded protein response (UPR) in order to restore homeostasis (5). The UPR can be activated through three ER-resident transmembrane proteins: protein kinase-like ER kinase (PERK), activating transcription factor 6 (ATF6), inositol-requiring enzyme 1α (IRE1α) (6,7). These activators initially function to restore cell homeostasis by increasing the protein folding capacity of the ER. This is accomplished through the upregulation of expression of protein chaperones/foldases such as GRP78, GRP94, protein disulfide isomerase (PDI), and by enhancing the ER-associated degradation (ERAD) of terminally misfolded proteins. These processes are collectively known as the adaptive UPR (6). However, when ER stress is protracted and ER homeostasis cannot be achieved, such as in conditions of chronic hyperglycemia, apoptotic signals are initiated and the cell undergoes programmed cell death. This is referred to as the apoptotic UPR (5,6,8,9). Although there are several studies that have investigated the link between ER stress and the development of hyperglycemia and diabetes, the specific mechanisms by which this occurs are still unknown.

Rodent models of chronic hyperglycemia are very useful to clarify the mechanisms underlying metabolic disorders (10,11). In this experimental study we use the TALLYHO/JngJ (TH) mouse model, which is a polygenic model of type 2 diabetes and obesity showing sexual dimorphism in terms of glucose regulation (12,13). These mice have been selected for hyperglycemia over many generations from a colony of outbred mice in which some male mice spontaneously developed hyperglycemia, glycosuria and polyuria (12). Male TH mice develop chronic hyperglycemia, whereas female TH mice remain normoglycemic throughout their lifespan (12,13). This is an exaggerated model of what is observed with the prevalence and incidence of diabetes in humans, with women before menopause being less susceptible to developing diabetes, compared to men or women after menopause (14–16). The aim of this study is to better understand the role of ER stress in the development of hyperglycemia and evaluate potential sex differences in the activation of the UPR.

Materials and Methods

Mouse model

Male and female TALLYHO/JngJ (TH) mice were purchased from The Jackson Laboratory (JAX stock #005314). Experimental mice received a standard diet (2018 Teklad Global 18% Protein Rodent Diet, Harlan Teklad, Madison, WI, USA) *ad libitum*,

and free access to water. As the hyperglycemic phenotype is not fully penetrant, male mice used for breeding were checked for hyperglycemia at 8 weeks of age. Male mice that did not meet the hyperglycemic threshold (fasting blood glucose levels > 15mM) were eliminated from the study. All animal procedures were pre-approved by the McMaster University Animal Research Ethics Board. Characterization and experimental procedures started from the F7NE4F7+2 breeding generation.

Ovariectomy

Ovariectomy was performed on subsets of female TH mice (n=5-10 per experimental group) at 4 weeks of age using previously described methods (17,18). Briefly, a 3 x 3 cm incision area surrounding the iliac crest was shaved and cleaned, and a horizontal incision through the skin in the midline area was performed. Once the ovary was identified, an incision was made through the muscle layer to reach the abdominal cavity. The fat surrounding the ovary was removed and the ovary was exposed, a double ligation was performed in the uterine horn and vessels, and the ovary was removed. The remaining tissue was put back into the abdominal cavity, and the incision was sutured. The same procedure was used to remove the contralateral ovary. The wound was closed using a wound clipper. Sham operated animals (n=5-10 per experimental group) underwent a similar procedure however, ovaries were not removed.

Treatment with 4-phenylbutyric acid (4-PBA)

Subsets of female mice were either sham operated or ovariectomized at 4 weeks of age. At 5 weeks of age, male, sham female, and ovariectomized female TH mice were switched to drinking water containing the chemical chaperone 4-PBA for 15 weeks (n=5-10 per experimental group). Control male, sham female, and ovariectomized female TH mice (n=5-10 per experimental group) received regular drinking water.

4-PBA was administered at a concentration of 3.8 g/L of drinking water, corresponding to a dosage of 1 g 4PBA/kg body weight/day taking into account water intake per mouse. (19)

Fasting blood glucose levels were measured at 5, 10, 15 and 20 weeks of age. Experimental endpoint was set at 20 weeks of age, where organs and tissues were harvested and analysed, as described in the following paragraphs.

Harvesting

Experimental mice were anesthetized with isoflurane and euthanized by cervical dislocation. Blood was collected and the vasculature was rinsed with phosphate buffered saline. Pancreata, liver, and adipose tissue were harvested and fixed in 10% neutral-buffered formalin and stored at room temperature.

Glucose measurements, glucose tolerance test (GTT), lipid analysis and serum insulin quantification

Fasting blood glucose levels were measured using a glucometer (One Touch Verio Flex, LifeScan, Burnaby, BC, Canada) after mice were fasted as follows: 1 hour fasting

for 3 and 4 week old mice; 2 hour fasting for 5-6 week old mice; 4-6 hours fasting for >6 week old mice.

Subsets of experimental mice (n=4-6 per experimental group) were fasted at experimental endpoint (5 weeks of age or 20 weeks of age) for 4-6 hours. Fasting blood glucose levels (time 0) were determined, and a solution of glucose (2g/kg body weight of a 200mg/ml solution) was administered via intraperitoneal (IP) injection. Fasting glucose levels were measured after 30, 60 and 120 minutes from the injection using a glucometer (One Touch Verio Flex, LifeScan, Burnaby, BC, Canada). Because the glucometer has a maximum detection limit of 35 mM, blood samples exceeding 35 mM were determined using a colorimetric glucose assay (Infinity Glucose Hexokinase Reagent, Thermo Fisher, Middletown, VA, USA).

Fasting plasma lipid levels were quantified using the colorimetric diagnostic kits for total cholesterol and triglyceride determination (Infinity Triglyceride, Infinity Cholesterol, Thermo Scientific, Middletown, VA).

Fasting serum insulin was determined through an ELISA assay (Ultra Sensitive Mouse Insulin ELISA Kit, 90080, Chrystal Chem, Elk Grove Village, IL 60007 USA).

Pancreata analysis

Pancreata from male, sham female, and ovariectomized female TH mice were harvested at 20 weeks of age. Pancreata were embedded in paraffin and 6µm thick sections were sectioned using a microtome. A total of six sections per mouse, at 180 µm intervals, were used to perform immunohistochemical and immunofluorescent analyses.

Immunohistochemical analysis was performed for the quantification of alpha and beta cell mass and for the assessment of the UPR marker GADD153/CHOP. Immunofluorescent staining was performed to determine insulin and glucagon content and the quantification of the UPR markers GRP78/GRP94, PDI, ATF4. The following antibodies/dilutions were used: monoclonal rat IgG insulin antibody at 1:100 dilution (MAB1417, R&D Systems, Minneapolis, MN, USA); polyclonal mouse glucagon antibody at 1:100 dilution (CL8867AP-S, Burlington, ON, Canada); anti-GADD153/CHOP antibody at 1:50 dilution (monoclonal mouse GADD153 (B3) sc-7531, Santa Cruz Biotechnology, Dallas, TX, USA); anti-GRP78/GRP94 antibody at 1:250 dilution (monoclonal mouse KDEL antibody, ADI-SPA-827-J, Enzo/Cedarlane, Burlington, ON, Canada); anti-PDI antibody at 1:200 dilution (monoclonal mouse antibody, ADI-SPA-891-F, Enzo/Cedarlane, Burlington, ON, Canada); anti-ATF4 antibody at 1:200 dilution (polyclonal rabbit antibody, 10835-1-AP, Thermo Fischer, Mississauga, ON, Canada). A 1:200 dilution was used for the secondary antibodies: goat anti-rat biotinylated IgG (BA 9400, Vector, Burlington, ON, Canada), goat anti-rabbit biotinylated IgG (BA 100, Vector, Burlington, ON, Canada), goat anti-mouse biotinylated IgM (BA 2020, Vector, Burlington, ON, Canada), Alexa Fluor 488 goat anti-rat IgG (A11006, Thermo Scientific, Middletown, VA, USA), Alexa Fluor 568 goat anti-

mouse IgG (A11004, Thermo Scientific, Middletown, VA, USA), Alexa Fluor 488 goat anti-mouse IgG (A11001, Thermo Scientific, Middletown, VA, USA), Alexa Fluor 488 goat anti-rabbit IgG (A11008, Thermo Scientific, Middletown, VA, USA).

Beta cell mass (BCM) was calculated using the following formula (20):

BCM= (average of insulin-positive stained area: total area of section) x pancreatic mass (mg)

Alpha cell mass (ACM) was calculated using the following formula (20):

ACM= (average of total glucagon-positive stained area/ total section area) x pancreatic mass (mg)

Immunohistochemical and immunofluorescent images were captured on a Leitz LABORLUX S microscope connected to a DP71 Olympus camera.

Immunohistochemical quantification of GADD153/CHOP was performed by counting the brown-stained nuclei versus the total number of nuclei.

All immunofluorescent staining experiments were counterstained with DAPI (Invitrogen, Carlsbad, CA, USA) at a dilution of 1:5000. Separate sections were stained with pre-immune IgG, instead of the primary antibody, to control for non-specific staining. Threshold levels were selected to correct for non-islet fluorescence.

Immunofluorescence intensity was quantified using ImageJ software (NIH, Bethesda, MD, USA; http://imagej.nih.gov/ij), and calculated as follows:

Intensity of fluorescence = (average intensity of fluorescence of the pancreatic islets per mouse)- (average intensity of fluorescence of the pancreatic islets of the negative control)

For both immunohistochemical and immunofluorescent analysis six pancreatic sections per mouse were scanned and a total of n=12-15 islets per mouse were selected for the intensity of fluorescence analysis. Each experimental group consisted of n=4-6 mice per experimental group.

Statistical analysis

Statistical analyses for multiple groups were performed using t-test, one-way or two-way ANOVA, as appropriate. Data are presented as mean± Standard Error of the Mean (SEM). Analyses were performed using ImageJ. A value of $p<0.05$ was considered statistically significant.

Results

The TH mouse model is characterized by sexual dimorphism in terms of blood glucose regulation (12). To investigate the potential role of sex hormones in the regulation blood glucose, a subset of female TH mice was ovariectomized at 4 weeks of age. Fasting

blood glucose levels were monitored weekly. Male TH mice developed chronic hyperglycemia by five weeks of age, whereas female mice maintained the normoglycemic phenotype (Figure 1A). Ovariectomized female TH developed chronic hyperglycemia (Figure 1A). Glucose tolerance tests were performed on five week old male and female TH mice (Figure 1B,C). Female mice showed significantly improved glucose tolerance compared to the age-matched male TH mice.

Metabolic parameters in TH mice treated with 4-PBA

There were no significant differences in body weight and pancreas weight in female TH experimental groups (Table 1). Liver weight was significantly increased in female TH sham operated mice that received treatment with 4-PBA, compared to those receiving regular water. No significant differences in liver weight were observed in the ovariectomized groups. Adipose tissue was significantly increased in the female TH sham operated receiving regular water, compared to the ovariectomized counterparts. Ovariectomized TH mice presented with increased plasma cholesterol and triglyceride levels, compared to sham operated counterparts.

Male TH mice receiving regular water had significantly higher pancreas weight and adipose tissue weight, compared to those receiving treatment with 4-PBA (Table 2). Conversely, male TH receiving treatment with 4-PBA had increased liver weights than the respective controls. No significant differences were observed for body weight and plasma cholesterol and triglyceride levels.

Fasting glucose levels and GTT in TH mice treated with 4-PBA

To determine the potential role of ER stress in the development of hyperglycemia in this mouse model, five week old male, female sham operated and female ovariectomized TH mice received either regular drinking water or water containing 4-PBA (Figure 2). 4-PBA is a chemical chaperone and it prevents misfolded protein aggregation thereby alleviating ER stress (21).

Fasting glucose levels in male mice treated with 4-PBA were transiently improved at 15 weeks of age relative to males receiving regular water (Figure 3A). This effect was not significant in 20 weeks old male mice. Supplementation with 4-PBA had no effect on normoglycemic female TH controls. Ovariectomized female TH treated with 4-PBA presented with significantly reduced fasting blood glucose levels compared to ovariectomized female controls however, they still maintained hyperglycemia. Supplementation with 4-PBA significantly improved glucose tolerance in both male and female ovariectomized TH mice compared to controls (Figure 3B-E).

Alpha and beta cell mass, insulin and glucagon content, and serum insulin levels in TH mice treated with 4-PBA

Alpha and beta cell mass was assessed in all experimental groups at 20 weeks of age. Supplementation with 4-PBA did not significant effect alpha or beta cell mass in male TH mice (Figure 4A,C). Alpha cell mass was not significantly altered by ovariectomy or 4-PBA supplementation in female TH mice (Figure 4B). Beta cell mass was significantly

reduced in ovariectomized TH mice, compared to sham operated female TH mice (Figure 4D). Supplementation with 4-PBA did not significantly influence beta cells mass in ovariectomized female TH mice and respective controls. Consistently with these findings, no significant differences in insulin content, glucagon content, and fasting plasma insulin levels were observed in all TH male experimental groups (Figure 4E-G, Table 4). All female ovariectomized TH mice treated (or not) with 4-PBA showed a significant reduction in insulin content, compared to female TH controls (Figure 4H,I). Plasma insulin levels were significantly reduced in all female ovariectomized TH groups compared to those of sham operated TH females (Table 3). In accordance with what was observed with alpha cell mass, no significant differences were observed for glucagon content among all female experimental groups (Figure 4J).

Evaluation of the UPR in the pancreatic islets of TH mice

In order to assess the effects of ovariectomy and 4-PBA on beta cell function, markers of the adaptive and apoptotic UPR were quantified in the pancreata of 20 week old TH mice. GRP78 and GRP94 are endoplasmic reticulum-resident chaperones involved in aiding proper protein folding and key components of the adaptive UPR (22). GRP78/GRP94 expression was significantly increased in female ovariectomized TH mice compared to sham operated females treated with 4-PBA (Figure 5). Male TH mice receiving regular drinking water also expressed significantly higher levels of GRP78/GRP94 compared to males treated with 4-PBA.

Protein disulfide isomerase (PDI) also resides in the endoplasmic reticulum, and mediates the formation of disulfide bonds, which is a key step for proper folding and maturation of insulin (23). There were no significant differences in PDI expression across female TH experimental groups however, male TH mice receiving regular water expressed significantly higher levels of PDI, compared to male TH mice receiving 4-PBA treatment (Figure 5).

Activating transcription factor 4 (ATF4) is an apoptotic UPR marker that induces the transcription of another apoptotic marker, GADD153/CHOP, responsible of signalling to the cell to undergo apoptosis. In this study there were no significant differences observed in the expression of ATF4 in all male or female TH experimental groups (Figure 6A-D). No significant differences in the expression of GADD153/CHOP between male TH mice supplemented (or not) with 4-PBA were observed (Figure 6E,F). Female ovariectomized TH mice significantly increased the expression of this apoptotic GADD153/CHOP, compared to female controls and controls receiving 4-PBA (Figure 6G,H).

Discussion

The TH mice represent a mouse model of polygenic type 2 diabetes showing sexual dimorphism in glucose homeostasis, with male TH mice developing chronic hyperglycemia and female TH mice remaining normoglycemic throughout their lifespan

(12). This mouse model represents an exaggeration of the biological phenotype observed in humans, with premenopausal women being protected from developing diabetes, compared to men or women after menopause (14–16). A common way to study the effects of menopause or estrogen deprivation in rodents is by performing ovariectomy, as this significantly reduces the levels of circulating estrogens (10). We previously observed that in a mouse model of hyperglycemia-induced atherosclerosis ($ApoE^{-/-}$:$Ins2^{+/Akita}$), which also shows sexual dimorphism in glucose regulation, estrogen depletion induced by ovariectomy promoted a chronic hyperglycemic phenotype (18) (see chapter 2). Consistent with these findings, in this study we observed that ovariectomized female TH mice become chronically hyperglycemic with impaired pancreatic beta cell health and function (Figure 3, Figure 4). Interestingly, estrogen depletion did not have any effect on alpha cell mass and/or glucagon content in female TH mice. Beta cell mass and insulin content are very low in the islets of male TH mice suggesting that significant beta cell death has occurred by 20 weeks of age.

Significant research has been conducted to study the specific mechanisms underlying the pathogenesis of diabetes. Accumulating evidence has implicated ER stress/UPR activation as one contributing pathway to disease progression (5,8,9,24). Elevated blood glucose levels stimulate a significant increase in insulin production by pancreatic beta cells, and this high demand can exceed the protein folding capacity causing ER stress (8). Cells respond to ER stress by activating the adaptive UPR to restore the protein folding homeostasis. This involves the upregulation of protein chaperones (GRP78/GRP94) and other factors involved in protein processing (PDI) to increase ER's protein processing capacity (25–27). However, when hyperglycemia is protracted, such as what is observed in diabetes, homeostasis might not be restored. Under these conditions, pancreatic beta cells increase the expression of proteins involved in the pro-apoptotic UPR pathway, including ATF4 and GADD153/CHOP (28). The apoptosis of dysfunctional pancreatic beta cells creates a vicious cycle where surviving beta cells must compensate for lost cells, by increasing insulin biosynthesis, thus aggravating ER stress in these surviving cells. Given the known sex differences in glucose regulation observed in humans (14–16) and other rodent models (see chapter 2), we investigated alterations in UPR activation in ovariectomized female TH mice. We found that ovariectomized female TH mice had significantly increased expression of GRP78/GRP94 and PDI (Figure 5), as well as the apoptotic UPR marker GADD153/CHOP in pancreatic islets relative to intact female mice. This suggests that an increase in blood glucose levels is associated with an increase in ER stress. When hyperglycemia is protracted, the apoptotic UPR pathway is activated.

To further analyse the role of ER stress in the development of hyperglycemia and beta cell health in this mouse model, we evaluated the expression of UPR markers in males, females and ovariectomized females treated (or not) with 4-PBA. 4-PBA is a drug currently approved for use in humans to treat urea cycle disorders, a rare group of inherited dysfunctions characterized by an accumulation of ammonia or nitrogenous components (i.e. glutamine, alanine), which can ultimately lead to neurological disorders

and/or developmental disability (21). 4-PBA can also act as a chemical chaperone, aiding in protein folding and preventing protein aggregation in the ER thereby alleviating ER stress (21).

Our results show that 4-PBA significantly reduced fasting blood glucose levels and significantly improved glucose tolerance in ovariectomized female TH mice. In male TH mice, 4-PBA reduced fasting blood glucose levels but did not significantly affect glucose tolerance. Investigation of UPR markers showed that there was a reduction in the expression of GRP78/GRP94 and PDI in male but not female or ovariectomized female TH mice.

It is interesting that ovariectomized TH mice improved fasting glucose levels with 4-PBA treatment, but no significant change in the levels of ER stress was detected. Although this might be attained by increasing the dosage of 4-PBA, it is important to consider that this treatment might have potential side effects and toxicity in other organs and tissues. In fact, the metabolic parameters in our study show a significant increase in liver weight in mice treated with 4-PBA (Table 1, Table 2). Additionally, 4-PBA might exert its beneficial effects on glucose homeostasis not only on pancreatic beta cells but also in other peripheral insulin resistant organs and tissues. These advantages can be seen in a study on a mouse model of obesity and pancreatic beta cell dysfunction, where treatment with 4-PBA significantly improved glucose homeostasis and reduced hyperinsulinemia (29). Accordingly, 4-PBA improved glucose homeostasis, insulin sensitivity, and ER stress levels in Wistar rats with streptozotocin-induced hyperglycemia and fed a high fat diet (30). Another study in C57BL/6J mice fed with a high fat diet showed that treatment with 4-PBA significantly reduced ER stress in adipocytes and improved insulin sensitivity (31). These recent studies confirm previous findings conducted in cells and mice where 4-PBA was able to improve insulin sensitivity in rat hepatoma cells challenged with the ER stress inducer tunicamycin, and leptin-deficient (ob/ob) mice treated with this chemical chaperone normalized their glucose levels to those of the wild type controls and improved insulin sensitivity (32).

Taken together, the results of our study indicate that ER stress is involved in the development of hyperglycemia. Treatment with the ER stress alleviator 4-PBA improves, but does not normalize, blood glucose levels in male and ovariectomized female TH mice. These results lead us to consider the potential role of estrogens in the modulation of UPR and glucose homeostasis in the TH mice. Previous analysis in the $ApoE^{-/-}$:$Ins2^{+/Akita}$ mice showed that chronically hyperglycemic male and female ovariectomized $ApoE^{-/-}$:$Ins2^{+/Akita}$ mice presented with significantly increased levels of the apoptotic UPR marker GADD153/CHOP, compared to normoglycemic female sham operated $ApoE^{-/-}$:$Ins2^{+/Akita}$ mice (18) (see chapter 3). Additionally, supplementation of exogenous estrogen in the female ovariectomized $ApoE^{-/-}$:$Ins2^{+/Akita}$ mice correlated with an enhancement of adaptive UPR components, and an inhibition of apoptotic UPR components, leading to an improved glucose homeostasis (see chapter 3). Therefore,

circulating estrogens might be a critical component in effectively regulating glucose homeostasis by modulating the UPR pathways.

Further studies need to be conducted to improve our understanding of the UPR in pancreatic beta cells and how this response might be influenced by sex hormones.

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Tables

Mouse genotype/ intervention	TALLYHO/JngJ sham	TALLYHO/JngJ sham + 4-PBA	TALLYHO/JngJ ovx	TALLYHO/JngJ ovx + 4-PBA
Body Weight (g)	43.80± 3.10	44.14± 1.81	39.94± 1.42	43.99± 1.25
Pancreas weight (g)	0.22± 0.01	0.24± 0.07	0.21± 0.02	0.21± 0.01
Liver weight (g)	1.51± 0.15	2.37± 0.36 $	2.03± 0.06	2.14± 0.06
Adipose tissue weight (g)	3.59± 0.82	4.98± 0.20 $$	2.36± 0.34	3.26± 0.16
Plasma Cholesterol (mM)	3.73± 0.99^^^	3.29± 1.39^^^^	5.40± 0.52	6.88± 0.60^
Plasma Triglycerides (mM)	1.02± 0.23****	1.07± 0.12****	1.97±0.26	1.21± 0.22

Table 1. Metabolic parameters of 20 week old female TALLYHO/JngJ either treated or not with 4-PBA.

$ p<0.05 TALLYHO/JngJ sham vs TALLYHO/JngJ sham+ 4-PBA, $$ p<0.01 TALLYHO/JngJ sham+ 4-PBA vs TALLYHO/JngJ ovx. ^p<0.05 TALLYHO/JngJ sham + 4-PBA vs TALLYHO/JngJ ovx+ 4-PBA, ^^^p<0.001 TALLYHO/JngJ sham vs TALLYHO/JngJ ovx+ 4-PBA, ^^^^p<0.0001 TALLYHO/JngJ sham + 4-PBA vs TALLYHO/JngJ ovx+ 4-PBA ****p<0.0001 TALLYHO/JngJ sham vs TALLYHO/JngJ ovx, **** p<0.0001 TALLYHO/JngJ sham +4-PBA vs TALLYHO/JngJ ovx+ 4-PBA, $^{\#}$p<0.05 TALLYHO/JngJ sham vs TALLYHO/JngJ ovx n=4-9 per experimental group. Each average value is presented with the Standard Error of the Mean (SEM).

Mouse genotype/ intervention	TALLYHO/JngJ	TALLYHO/JngJ + 4-PBA
Body Weight (g)	29.26± 0.44	32.11± 1.59
Pancreas weight (g)	0.17± 0.01*	0.14± 0.01
Liver weight (g)	0.97± 0.29 ****	3.58± 0.18
Adipose tissue weight (g)	0.35± 0.07*	0.17± 0.02
Plasma Cholesterol (mM)	3.47± 1.29	5.15± 1.45
Plasma Triglycerides (mM)	2.91± 1.09	2.31± 0.83

Table 2. Metabolic parameters of 20 week old male TALLYHO/JngJ either treated or not with 4-PBA.

*p<0.05, ****p<0.0001. Each average value is presented with the Standard Error of the Mean (SEM). n=5-9 per experimental group.

Mouse genotype/ intervention	Serum insulin (ng/ml)
TALLYHO/JngJ sham	0.90± 0.16*
TALLYHO/JngJ sham + 4-PBA	0.63± 0.16
TALLYHO/JngJ ovx	0.35± 0.15
TALLYHO/JngJ ovx + 4-PBA	0.47± 0.15

Table 3. Fasting serum insulin levels in 20 week old female TALLYHO/JngJ either treated or not with 4-PBA.

*p<0.05 TALLYHO/JngJ sham vs TALLYHO/JngJ ovx. Each value is presented with the Standard Error of the Mean (SEM). n=5 per experimental group.

Mouse genotype/ intervention	Serum insulin (ng/ml)
TALLYHO/JngJ sham	0.29± 0.1
TALLYHO/JngJ sham + 4-PBA	0.19± 0.1

Table 4. Fasting serum insulin levels in 20 week old male TALLYHO/JngJ either treated or not with 4-PBA.

Each value is presented with the Standard Error of the Mean (SEM). n=5 per experimental group.

Figure captions

Figure 1. Glucose levels and intraperitoneal glucose tolerance test (GTT) in male and female TALLYHO/JngJ (TH) mice. Evaluation of fasting blood glucose levels through time in (**A**) male, female sham operated, and female ovariectomized (ovx) TALLYHO/JngJ (TH). ****P<0.0001 male TH vs age-matched female TH sham. § p<0.05 female TH ovx vs age-matched female TH sham, §§ p<0.01 female TH ovx vs age-matched female TH sham, §§§§ p<0.0001 female TH ovx vs age-matched female TH sham. n=4-10 per experimental group. Bars represent Standard Error of the Mean (SEM). Evaluation of intraperitoneal GTT (**B**) and related area under the curve, AUC, (**C**) in 5 week old normoglycemic female and age-matched hyperglycemic male TH. ***p<0.001. n=4 per experimental group. Bars represent Standard Error of the Mean (SEM).

Figure 2. Experimental design. The figure represents a schematic representation of the study conducted in male, female sham operated, and female ovariectomized TH mice, aimed at evaluating the role of ER stress in the development of hyperglycemia. Male, female, and female ovariectomized TH mice were given either regular drinking water or water containing the chemical chaperone 4-PBA. The treatment started at five weeks of age. Fasting blood glucose levels were monitored every five weeks until 20 weeks of age (experimental endpoint), were plasma and serum were collected to assess metabolic parameters, and organs and tissues were harvested for analysis.

Figure 3. Glucose levels and intraperitoneal glucose tolerance test (GTT) in male, female, and female ovariectomized TH mice treated or not with the chemical chaperone 4-PBA. Fasting blood glucose levels in (**A**) female sham operated, ovariectomized (ovx) and age-matched male TALLYHO/JngJ either treated or not with the chemical chaperone 4-PBA. *p<0.05 male TH control vs age-matched male TH treated with 4-PBA. * p<0.05 female TH ovx vs age-matched female TH ovx treated with 4-PBA, ** p<0.01 female TH ovx vs age-matched female TH ovx treated with 4-PBA, n=4-10 per group. Bars represent Standard Error of the Mean (SEM). Intraperitoneal GTT (**B**) and relative AUC (**C**) in 20 week old male and age-matched female sham operated, ovariectomized (ovx) (**D**, **E**) TALLYHO/JngJ either treated or not with the chemical chaperone 4-PBA. *p<0.05, ***p<0.001, ****p<0.0001. n=4 per group. Bars represent Standard Error of the Mean (SEM).

Figure 4. Alpha and beta cell mass and insulin and glucagon content in male, female and female ovariectomized TH mice treated or not with 4-PBA. Immunohistochemical analysis of alpha and beta cell mass in 20 week old (**A**, **C**) male and age-matched (**B**, **D**) female sham operated, ovariectomized (ovx) TH either treated or not with the chemical chaperone 4-PBA. NS, not significant n=5 per group. *p<0.05, **p<0.01. Bars represent Standard Error of the Mean (SEM). Immunofluorescent analysis of insulin and glucagon content in pancreatic sections from (**E**) male and (**H**) female, and female ovariectomized TH mice. Immunostaining was performed using

antibodies against insulin (green fluorescence), and glucagon (red fluorescence). Magnification=x20. Scale bars equal 100 µm. Quantification of (**F**) insulin and (**G**) glucagon staining intensity of male TH mice treated or not with chemical chaperone 4-PBA. n=60 pooled islets from n=4-5 mice/group (corresponding to n=15 islets per experimental mouse). Error bars represent Standard Error of the Mean (SEM). NS, not significant. Quantification of (**I**) insulin and (**J**) glucagon staining intensity of female and female ovariectomized TH mice treated or not with chemical chaperone 4-PBA. n=60 pooled islets from n=4 mice/group. Error bars represent Standard Error of the Mean (SEM). *p<0.05, **p<0.01.

Figure 5. Expression of adaptive UPR markers in pancreatic islet sections of male TALLYHO/JngJ mice either treated or not with 4-PBA. Immunofluorescent analysis in panels **A-D** represents the expression of adaptive UPR markers GRP78/GRP94 in pancreatic sections of male (**A, B**), female sham operated and female ovariectomized (**C,D**) TH mice treated or not with the chemical chaperone 4-PBA. n=4-5 per group. *p<0.05, **p<0.01. Bars represent standard error of the mean (SEM). Immunofluorescence staining in panels **E-H** represents the expression of the adaptive UPR marker PDI in pancreatic sections of male (**E, F**), female sham operated and female ovariectomized (**G,H**) TH mice treated or not with the chemical chaperone 4-PBA. *p<0.05, NS, not significant. n=4-5 per experimental group. Bars represent standard error of the mean (SEM).

Figure 6. Expression of apoptotic UPR markers in pancreatic islet sections of male TALLYHO/JngJ mice either treated or not with 4-PBA. Immunofluorescent analysis in panels **A-D** represents the expression of apoptotic UPR marker ATF4 in pancreatic sections of male (**A, B**), female sham operated and female ovariectomized (**C,D**) TH mice treated or not with the chemical chaperone 4-PBA. n=4-5 per group. NS, not significant. Bars represent standard error of the mean (SEM). Immunohistochemistry staining in panels **E-H** represents the expression of the apoptotic UPR marker GADD153/CHOP in pancreatic sections of male (**E, F**), female sham operated and female ovariectomized (**G,H**) TH mice treated or not with the chemical chaperone 4-PBA. *p<0.05, NS, not significant. n=4-5 per experimental group. Bars represent standard error of the mean (SEM).

Figures

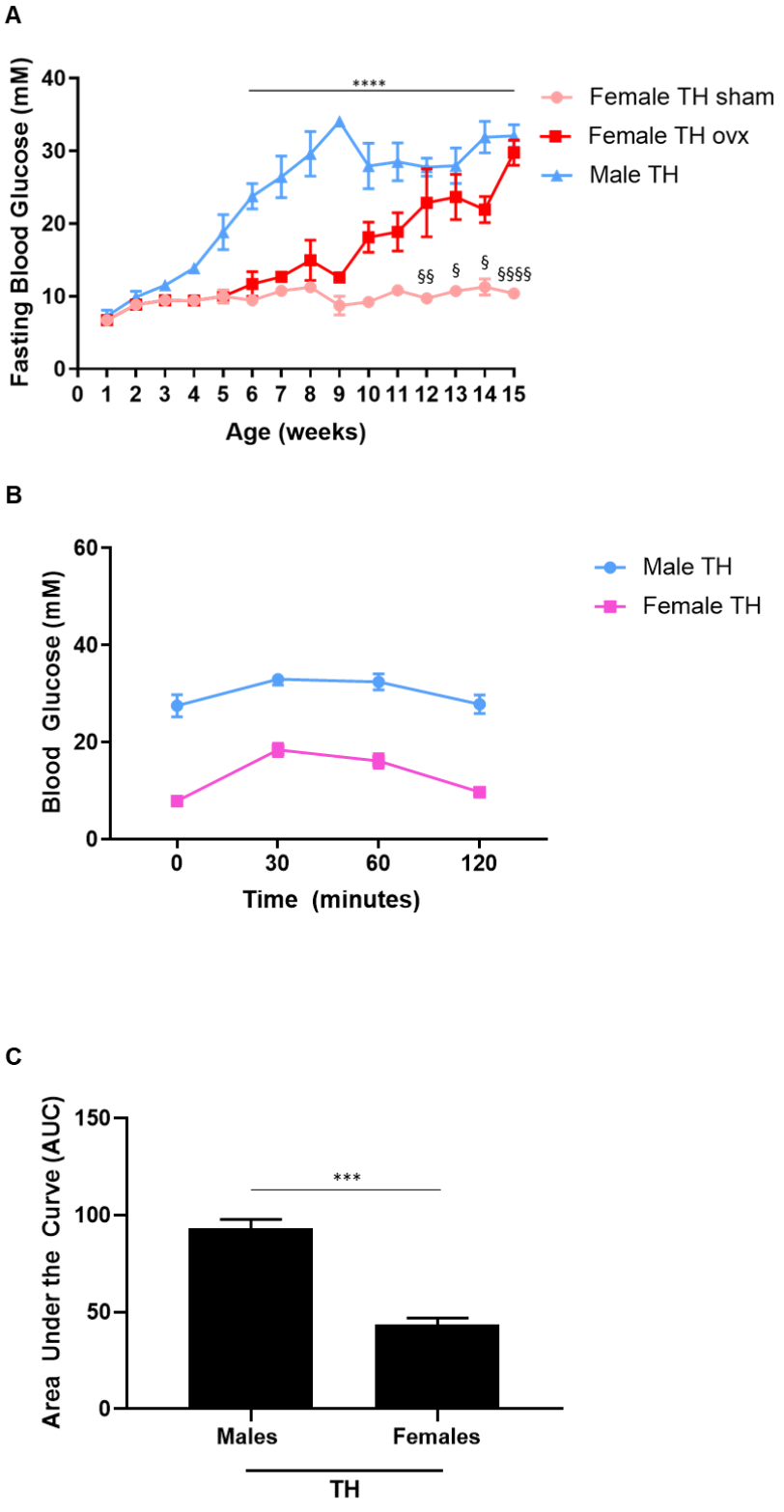

Figure 1. Glucose levels and intraperitoneal glucose tolerance test (GTT) in male and female TALLYHO/JngJ (TH) mice.

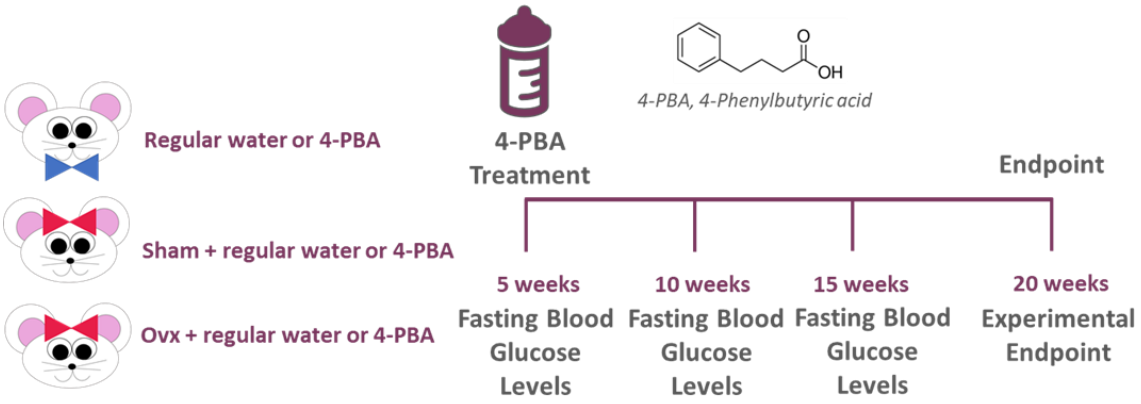

Figure 2. Experimental design.

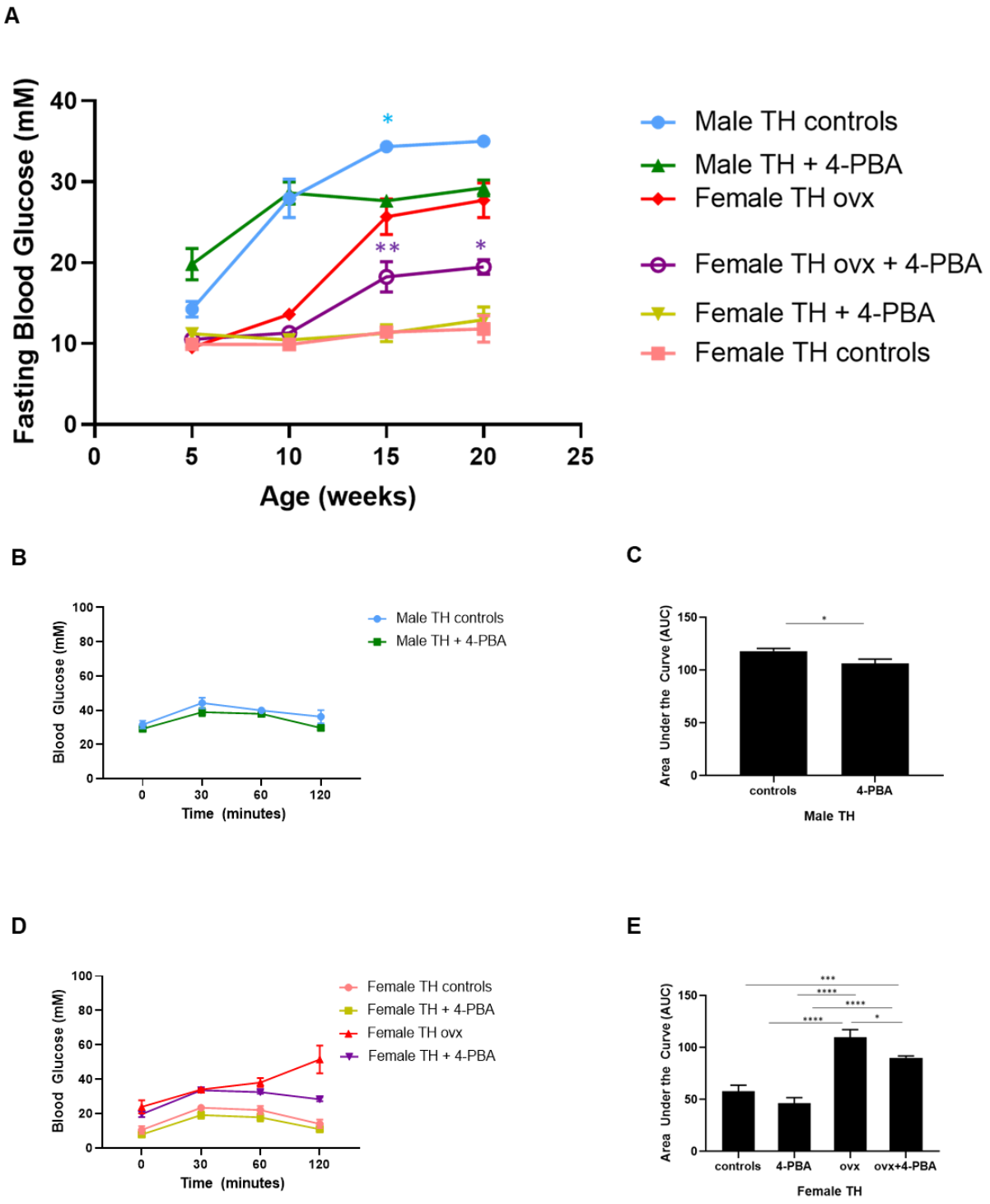

Figure 3. Glucose levels and intraperitoneal glucose tolerance test (GTT) in male, female, and female ovariectomized TH mice treated or not with the chemical chaperone 4-PBA.

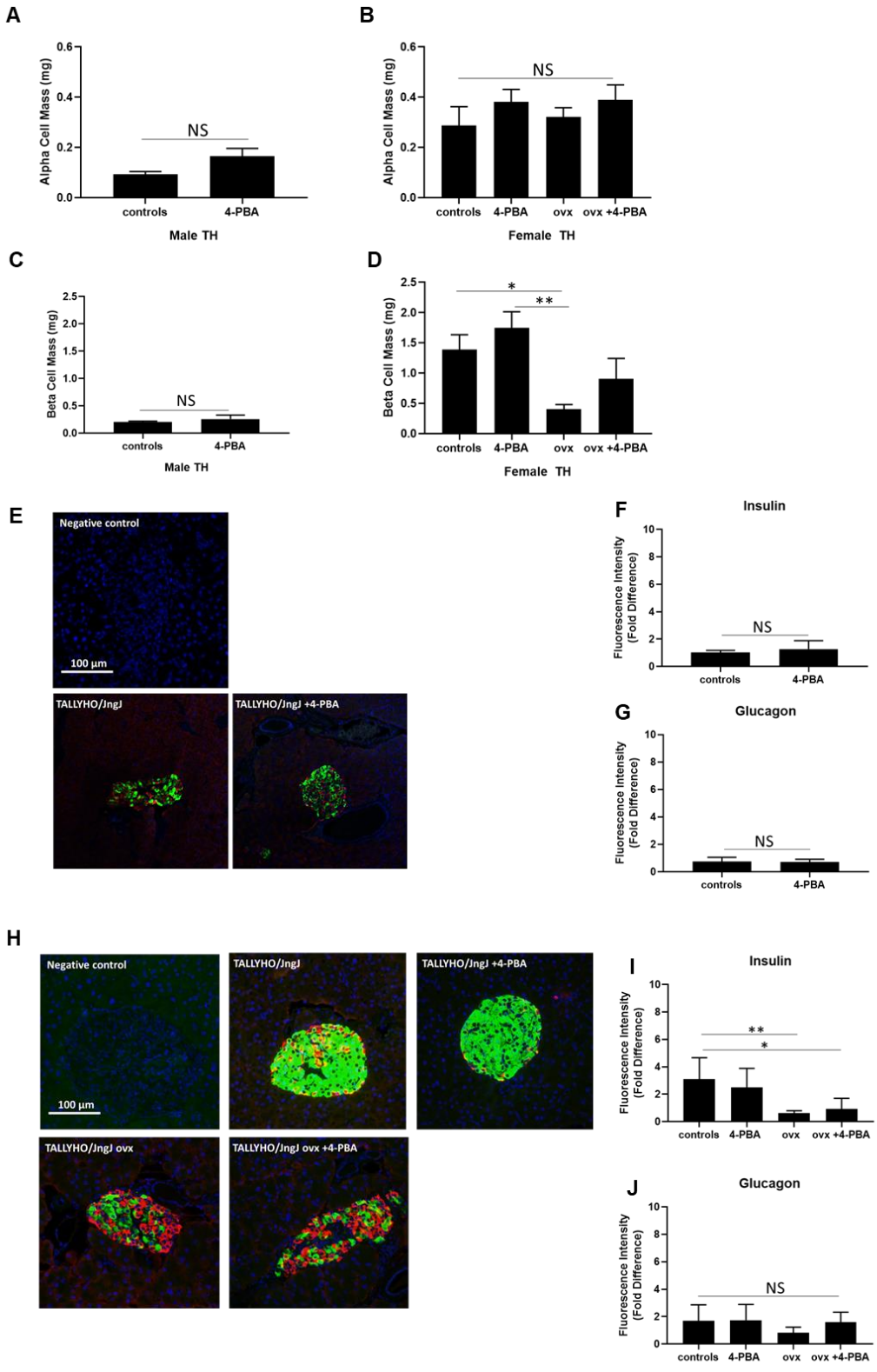

Figure 4. Alpha and beta cell mass and insulin and glucagon content in male, female and female ovariectomized TH mice treated or not with 4-PBA.

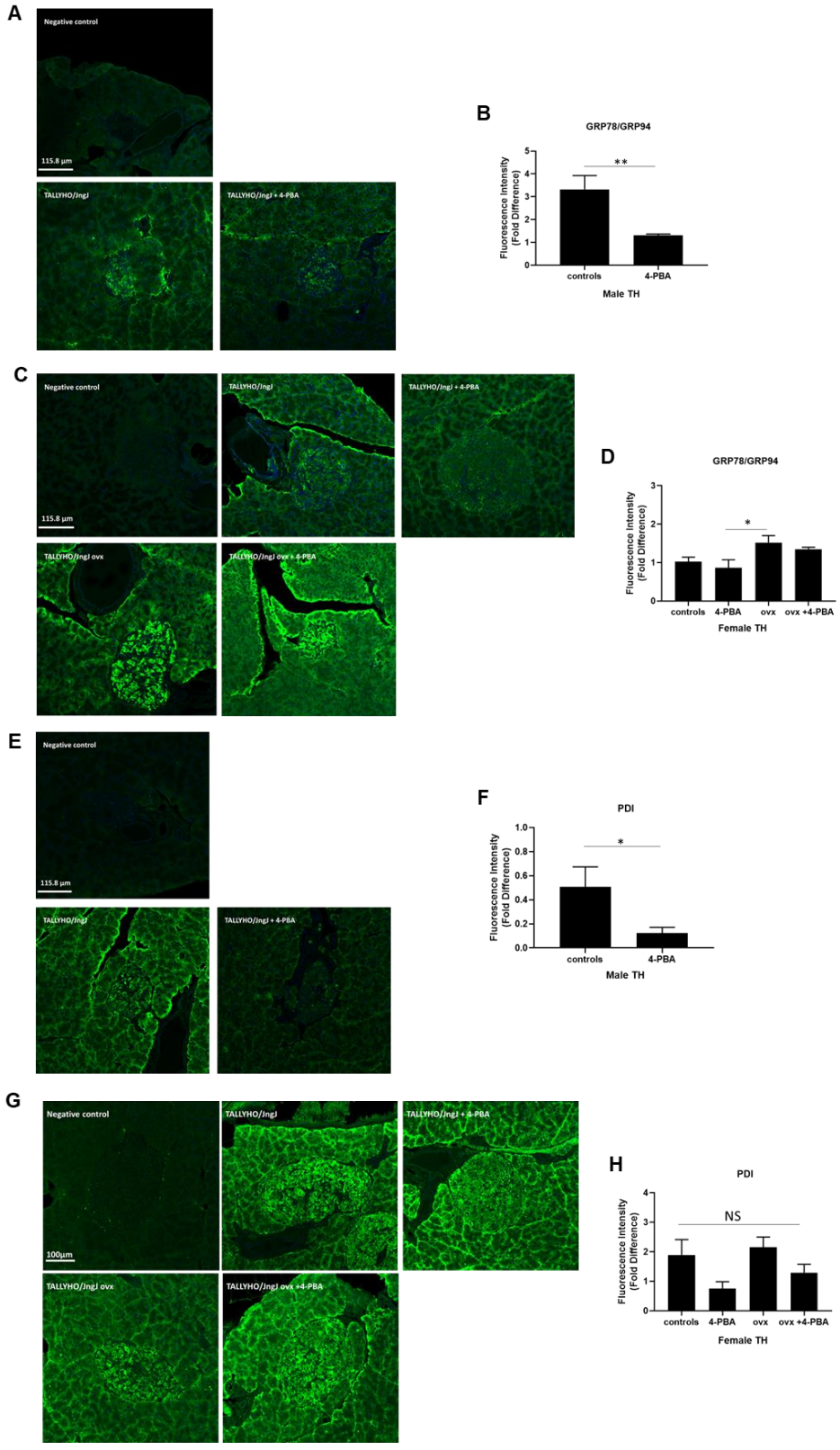

Figure 5. Expression of adaptive UPR markers in pancreatic islet sections of male TALLYHO/JngJ mice either treated or not with 4-PBA.

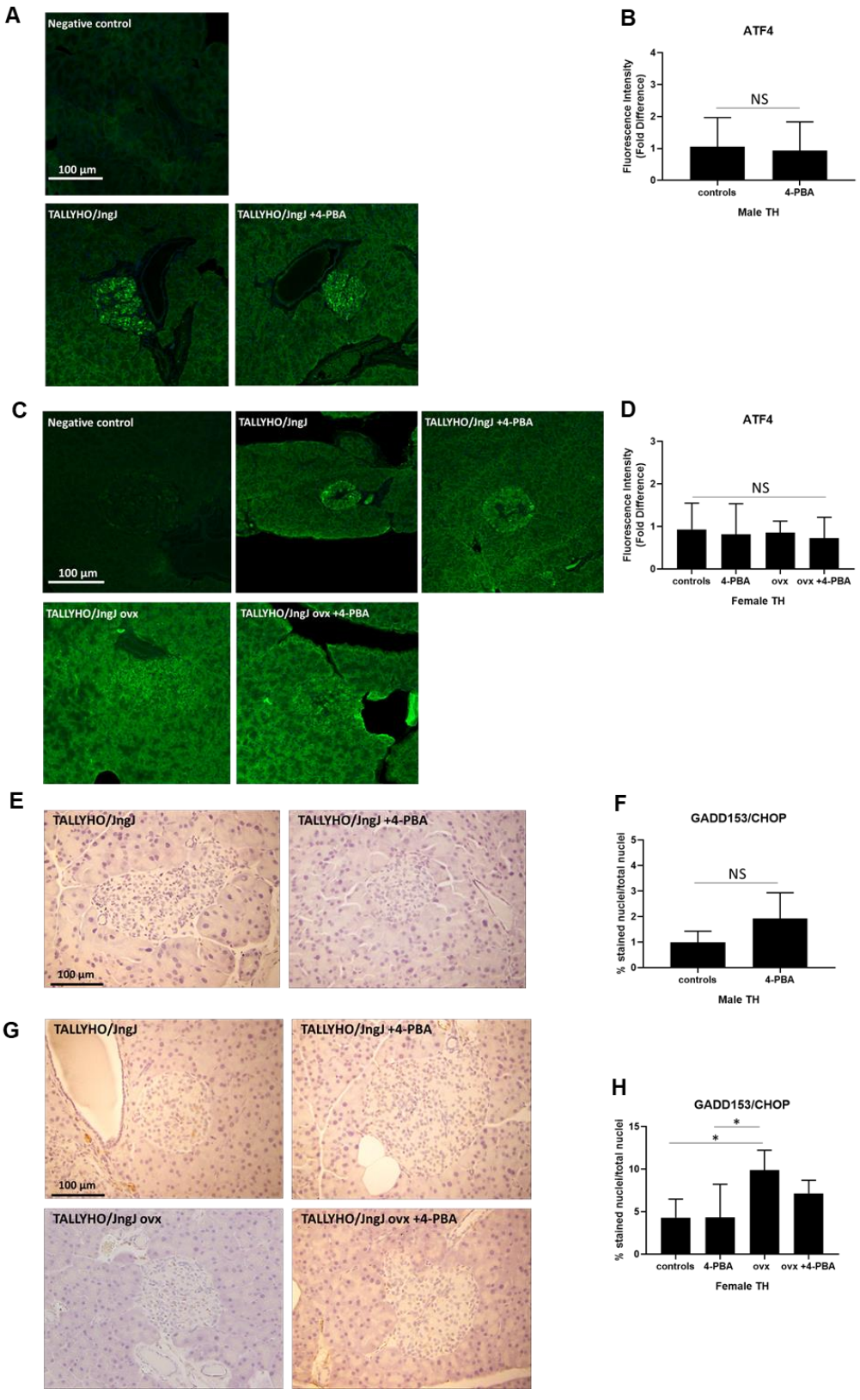

Figure 6. Expression of apoptotic UPR markers in pancreatic islet sections of male TALLYHO/JngJ mice either treated or not with 4-PBA.

CHAPTER 5 – CONCLUSION

Sex differences in DM presentation

DM encompasses a group of metabolic disorders characterized by the presence of chronic hyperglycemia. People with DM have a higher risk of developing CVDs, and a consequent higher mortality rate due to these complications, compared to people without DM (Cho et al., 2018; Ogurtsova et al., 2017). The prevalence of DM has been steadily increasing worldwide in both urban and rural areas (Kaiser et al., 2018; Mapa-Tassou et al., 2019; Ogurtsova et al., 2017). Additionally, DM mortality in low-income areas mainly occurs in people under the age of 60, causing a significant economic burden in developing countries (Ogurtsova et al., 2017). The current estimates show that the prevalence of DM will continue to grow in the years to come. It is predicted that by the year 2040 about 10% of the global population will have DM, with consequent social, financial and economic connotations (Cho et al., 2018; Ogurtsova et al., 2017).

Sex differences in the prevalence of DM do exist. Premenopausal women have a lower chance to develop DM, compared to men or postmenopausal women (Huebschmann et al., 2019; Mauvais-Jarvis, 2015; Raparelli et al., 2017; Tramunt et al., 2020). Several major studies have shown that the prevalence of DM was lower in premenopausal women than men (Huebschmann et al., 2019; J. Li et al., 2019; Zhang et al., 2021). Furthermore, a meta-analysis of 751 population-based studies from 146 countries and including over 4 million subjects, has shown that DM is more prevalent in men than premenopausal women (Kautzky-Willer et al., 2016). Another study conducted in the United States also confirmed that the prevalence of DM, corrected for age, is higher in men than premenopausal women (Peters et al., 2019). Interestingly, in all these studies it was observed that once women reach the postmenopausal stage, the prevalence of diabetes is similar to age-matched men.

Sex differences in DM-associated CVDs

DM represents a significant risk factor for the development of CVDs and people with DM are more likely to develop CVDs such as myocardial infarction and stroke (Prospective Studies Collaboration and Asia Pacific Cohort Studies Collaboration, 2018). It has been observed that sex differences in the presentation and outcome of cardiovascular diseases do exist. A study showed that men have a 3- to 5-fold increased risk of developing CHD, compared to premenopausal women (Colhoun, 2006). Furthermore, the risk of women developing CHD appears much later in life than men, at an age corresponding to the postmenopausal phase (Anand et al., 2008). However, when looking at DM-associated CVDs women tend to have a more severe prognosis. Studies indicate that DM is a stronger risk factor for CVDs in postmenopausal women as they have a two-fold increased risk of developing cardiovascular complications, such as CHD, compared to men even after accounting for other cardiovascular risk factors and/or potential undertreatment (Huxley et al., 2006; Peters et al., 2014b, 2014c; Raparelli et al., 2017). These results are supported by a meta-analysis of 102 prospective studies conducted in 25 countries, as well as a large meta-analysis of 64 cohorts, and a collaborative meta-analysis of 68 prospective studies (Peters et al.,

2014c; Prospective Studies Collaboration and Asia Pacific Cohort Studies Collaboration, 2018; The Emerging Risk Factors Collaboration, 2010). The risk of developing stroke is significantly increased in women with DM, compared with men with DM. A systematic review calculated this risk to be 27% higher in women with DM than men with DM even after accounting for other major cardiovascular risk factors (Peters et al., 2014b). Similar results showing a significantly increased risk of developing stroke in women with DM was also observed in various other studies (Ho et al., 2003; Malik, 2014; Oertelt-Prigione et al., 2015).

The increased cardiovascular risk observed in women with DM is also associated with a more severe prognosis that can translate into increased mortality, an increased risk of hospitalization, and an increased risk of developing long term disability. This has been shown by a Canadian study that calculated that mortality due to DM-associated CVDs was two times higher in women than men. Hospitalization was also significantly increased in women with DM (Roche & Wang, 2013). Population-based studies showed that survival rate after CVDs was significantly reduced in women with DM compared to men (Ballotari et al., 2015; Eriksson et al., 2012), and an analysis of patients with DM who suffered from hemorrhagic stroke showed that the intra-hospital mortality was significantly higher in women than men (Lopez-de-Andres et al., 2021).

Taken together, these findings outline two important aspects: the first is that premenopausal women appear to be protected from developing DM, compared to age-matched men. The second aspect is that postmenopausal women have an altered glucose homeostasis which increases the risk of developing DM. In addition, women also present with a significantly higher risk of developing DM-associated CVDs with a more severe prognosis, compared to men with DM. This suggests that the hormonal changes observed during menopause, and possibly the reduced levels of circulating estrogens, might be detrimental for pancreatic beta cell health and function and the regulation of glucose homeostasis, thereby promoting the development of DM. Evidence of the protective role of estrogens can be inferred by studies on menopausal women taking hormonal replacing therapy (HRT). A meta-analysis has shown that postmenopausal women taking HRT significantly improved insulin sensitivity and prevented the onset of DM (Salpeter et al., 2006). Similar results were observed in another study on postmenopausal women with or without DM, where administration of HRT was associated with improved glycemic status (J.-E. Kim et al., 2019).

There has been some controversy regarding the effects of HRT on glucose homeostasis and health outcomes (Manson et al., 2013; Santen et al., 2010, 2014; Stuenkel et al., 2015). These results can be clarified by accounting for various factors such as differences in the analysed population, the specific HRT regimen, the way HRT is administered, and eventual differences in measuring metabolic parameters (Bitoska et al., 2016). Additionally, it is important to stress the importance of the timing at which HRT is started. It has been observed that HRT is beneficial in terms of glucose homeostasis when initiated at the early stages of menopause, whereas HRT in women with established menopause appears to have no effect, or can even be even detrimental (Gupte et al., 2015). Overall, the beneficial effects of estrogen are also supported by two large, randomized, double-blind, placebo-controlled clinical trials. One of them is the Women's Health Initiative Hormonal Trial which showed that women in

the HRT treatment group had significantly improved fasting glucose levels and insulin sensitivity, compared to the placebo-treated control group (Margolis et al., 2004). The second study is the Heart and Estrogen/progestin Replacement Study, which showed that women in the placebo-treated group had significantly worse fasting glucose levels than those receiving HRT (Kanaya et al., 2003). Moreover, women in the HRT-treated group had a significantly lower incidence rate of DM than those in the placebo-treated control group (Kanaya et al., 2003).

The role of estrogens in the maintenance of pancreatic beta cell health

Accumulating evidence shows that in premenopausal women estrogens confer a protective effect in terms of glucose homeostasis, thereby reducing the risk of developing DM and its associated CVDs. However, the exact mechanisms underlying this protection are still poorly investigated and understood. With this research project we sought to better understand these mechanisms by initially analyzing the protective effects of estrogen in pancreatic beta cell health. We used two rodent models of diabetes, the ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice and the TALLYHO/JngJ mice. These mice are a monogenic and a polygenic model of diabetes respectively. Both models show sexual dimorphism in terms of blood glucose regulation, with male mice becoming chronically hyperglycemic, whereas female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice normalize blood glucose and female TALLYHO/JngJ mice maintain normoglycemia. It is interesting to note that female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice are transiently hyperglycemic and normalization of their blood glucose levels can be observed at 5 weeks of age, a timepoint corresponding to when these mice reach sexual maturity, and therefore when their estrogen levels increase. In this research project we attempted to measure circulating estrogen levels in ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice using commercially available ELISA kits, the quantification biomarker kits of Eve Technologies, and by mass spectrometry. Unfortunately, the results of these experiments were not reliable, likely because the severe dyslipidemia associated with this mouse model interfered with the results. Our inability to accurately measure estrogen is a limitation of all of these studies.

To further evaluate the role of estrogens in beta cell health, we performed ovariectomies on female mice in both mouse models. This procedure is typically used in experimental animal studies to investigate the effects induced by the lack of estrogens, as the removal of ovaries significantly decreases circulating levels. We observed that both ovariectomized female TALLYHO/JngJ mice and ovariectomized female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice maintained a chronic hyperglycemic phenotype, with a significant reduction in glucose tolerance, pancreatic beta cell mass and insulin storage within the pancreatic beta cells. Interestingly, the supplementation of exogenous estradiol in ovariectomized female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice restored normal blood glucose levels, and preserved pancreatic beta cell mass and glucose tolerance. Taken together, our findings show that estrogens do have a protective role in pancreatic beta cell health and are consistent with several other studies performed in different rodent models of diabetes. For example, the Zucker Diabetic Fatty rat (ZDF) also shows sexual dimorphism in terms of glucose regulation, with male mice developing chronic hyperglycemia, and female rats remaining normoglycemic (Shiota & Printz, 2012; Yokoi et al., 2013). However, when female rats were ovariectomized they showed a significant

impairment in glucose homeostasis and developed hyperglycemia and glucose intolerance (Weigt et al., 2015). This condition was reversed when mice received supplementation with exogenous estradiol (Weigt et al., 2015). In accordance with these findings, ovariectomy in female C57BL/6J, who are typically normoglycemic, resulted in significantly elevated fasting glucose levels, impaired insulin secretion, while supplementation with exogenous estradiol restored homeostasis (Santos et al., 2016).

Taken together, evidence from our research as well as other animal studies supports the protective role of estrogens in pancreatic beta cell health which is similar to what is observed in premenopausal women. This led us to investigate and propose a potential mechanism by which this protection is exerted.

The UPR as a proposed mechanism by which estrogens might influence pancreatic beta cell health

The central hormone responsible for the normalization of blood glucose levels is insulin. When blood glucose levels increase, there is an increased demand for insulin, which is synthesized in the ER of the pancreatic beta cell. An increased and protracted demand of insulin, such as what happens in conditions of chronic hyperglycemia, can overwhelm the ER processing machinery causing an accumulation of misfolded insulin, thereby initiating ER stress. This can lead to pancreatic beta cell death if the homeostasis cannot be restored by the UPR. Many studies consider ER stress as one of the processes involved in the pathogenesis of diabetes (Araki et al., 2003; Back & Kaufman, 2012; Cao et al., 2020; Demirtas et al., 2016; Ghosh et al., 2019). Even though a limitation of this study is that male TALLYHO/JngJ mice develop chronic hyperglycemia and do not have a correspondent normoglycemic control, we have shown that in this mouse model of polygenic type 2 diabetes, chronically hyperglycemic male and female ovariectomized TALLYHO/JngJ mice present with ER stress in their pancreatic beta cells, as shown by the significantly increased levels of adaptive and apoptotic UPR markers such as the protein chaperones GRP78/GRP94, PDI, and GADD153/CHOP. Administration of an ER stress alleviator, such as 4-PBA, which typically acts as a chemical chaperone by reducing protein aggregates and by improving protein folding, is able to significantly reduce the expression of ER stress markers, and decrease blood glucose levels, in these mice. However, even though blood glucose levels in 4-PBA treated mice are significantly reduced, they do not normalize to those of the controls. This could mean that a more potent activator of the adaptive UPR might be necessary to restore homeostasis and promote normoglycemia.

We hypothesized that estrogens are able to protect pancreatic beta cell health by modulating the UPR in response to ER stress by improving protein folding capacity through an enhanced adaptive UPR activation, and/or by repressing the apoptotic UPR. In our research project we saw that female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice have a significantly increased expression of adaptive UPR markers, compared to male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice, where apoptotic UPR markers are mainly expressed. Interestingly, when female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice are ovariectomized they show a similar phenotype to that observed in male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice, with a significant increase in apoptotic UPR markers. This is consistent with our hypothesis that estrogen improves pancreatic beta

cell health by modulating the UPR. Furthermore, exogenous supplementation of estradiol in ovariectomized female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice showed an enhancement of the expression of the adaptive UPR markers, and normalized on blood glucose levels.

To the best of our knowledge this is the first study that has investigated the role of estrogens in the modulation of UPR in response to ER stress in pancreatic beta cells using in an in vivo mouse model. Studies on this area of research have been mostly conducted in vitro. An experiment in pancreatic beta cells derived from male Ins2^{Akita} mice showed that when challenged with the ER stress inducer thapsigargin (an inhibitor of the sarcoplasmatic reticulum Ca^{2+} ATPase that reduces calcium-dependent chaperone activity), there was an increase in expression of apoptotic UPR marker GADD153/CHOP (Xu et al., 2018). However, when treated with conjugated estrogens, these cells were able to reduce the expression of various apoptotic UPR markers such as ATF4 and s-XBP1. Another study in MIN-6 cells, a pancreatic beta cell line derived from mouse insulinoma cells, demonstrated that treatment with estrogens significantly reduced the expression of GADD153/CHOP and the activation of PERK when challenged with the ER stress inducer glucosamine (Kang et al., 2014). To better elucidate the mechanisms by which estrogens protect pancreatic beta cell health and influence the UPR a study on the rat insulinoma cell line, INS-1 showed that cells exposed high levels of glucose reduced their cell viability and increased the expression of the apoptotic UPR marker GADD153/CHOP (Kooptiwut et al., 2014). However, treatment with estrogen improved cell viability and significantly reduced the expression of GADD153/CHOP. Furthermore, researchers in this study tried to further elucidate the mechanisms by which estrogen modulates the UPR. Results showed that estrogen can activate all three branches of the UPR pathway (ATF6, PERK, IRE1α), as they showed an increase in ATF6 p50, p-PERK, and s-XBP1 which are key activators of each respective branch. Additionally, they hypothesized that estrogens are able to modulate the UPR by signalling through estrogen receptors. Pancreatic beta cells do express the three estrogen receptors ERα, ERβ, and GPER. Researchers in this study showed that using an ERα and ERβ antagonist in cells treated with high glucose concentrations and estrogen significantly reduced cell viability, and similar results were also shown using the GPER antagonist. The role of estrogen receptors in pancreatic beta cell viability has also been investigated in another study that used MIN-6 cells deficient in ERα (Zhou et al., 2018). Consistent with previously discussed results, when ER stress was induced in these cells the expression of the apoptotic UPR marker GADD153/CHOP as well as pro-apoptotic marker caspase 3 were significantly induced. Another study using pancreatic beta cells from ERα-knock out female mice showed that, when challenged with the ER stress inducer thapsigargin, cell viability was significantly impaired, and GADD153/CHOP expression was significantly induced even after treatment with estrogen, compared to wild type cells (Xu et al., 2018).

The importance of estrogen receptors in pancreatic islet viability has been investigated in another study using mice deficient in either ERα, ERβ, ERα and ERβ, or GPER (Liu et al., 2009). Researchers observed that individual deletion of ERα, ERβ, or GPER predisposed these mice to chronic hyperglycemia, and viability of pancreatic islets was significantly impaired. The lack of both ERα and ERβ did not have a synergistic effect. Further analysis on cultured pancreatic islets from these mice showed that estrogen

promotes pancreatic beta cell survival predominantly via ERα. However, in the absence of both ERα and ERβ estrogen is still able to protect pancreatic beta cell survival, indicating that it might either signal through GPER or could use other distinct redundant pathways.

Research in the field of how estrogen modulates the UPR in pancreatic beta cells is currently quite limited. Further studies need to be conducted to delineate the specific pathways by which estrogen modulates the UPR and ultimately, influences pancreatic beta cell availability. These next steps will be further discussed in the last section of this chapter.

The role of estrogen in cardiometabolic disease

To investigate the role of estrogen in cardiometabolic disease we have used the ApoE$^{-/-}$:Ins2$^{+/Akita}$ mouse model. These mice represent an experimental model of hyperglycemia-induced atherosclerosis which mimics the increased risk observed in people with DM to develop cardiovascular complications. When evaluating the effects of estrogen in cardiometabolic disease progression, we came across an interesting finding in terms of atherosclerosis development. Specifically, exogenous administration of estrogen significantly reduced atherosclerosis progression in the *en face* aorta as well as the aortic sinus of male and female ovariectomized ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice. Considering the link between hyperglycemia and the development of atherosclerosis, our results show that estrogen has a direct effect in reducing glucose levels, as shown in female ovariectomized ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice. However, since male ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice remain chronically hyperglycemic even after exogenous administration of estrogen, but still show a significant reduction in atherosclerosis progression, this may suggest that estrogens also have a direct effect on the vasculature physiology.

In this specific context, estrogen is known to mediate vasodilation through the modulation of the endothelial nitric oxide synthase. Estrogen can stimulate endothelial nitric oxide synthase to increase the production of nitric oxide which is a potent vasorelaxant and has atheroprotective effects (Chakrabarti et al., 2014; Sukhovershin et al., 2015). Studies have shown that this action is mediated by estrogen receptors (ERα, ERβ, GPER) however, the exact mechanisms have yet to be elucidated (Fredette et al., 2018).

Furthermore, estrogen might be involved in promoting endothelial integrity, which is also a key factor in maintaining vasculature homeostasis. In a study conducted in Sprague-Dawley rats estrogen was able to improve endothelial healing as well as reduce the proliferation of smooth muscle cells (SMC) and therefore reduce atherosclerosis progression (C. R. White et al., 1997). Another study using human umbilical vein smooth muscle cells showed that proliferation is significantly reduced in the presence of estrogen or progesterone (Morey et al., 1997).

Estrogen might also modulate the various proinflammatory components at the basis of the atherosclerotic process. In the initial stages of this disease, there is an increased expression of adhesion molecules such as p-selectin, ICAM-1 and VCAM-1, which are responsible for promoting monocyte adhesion in the damaged endothelium. In a clinical

study in healthy male and females, the levels of p-selectin were inversely correlated with estrogen levels (Jilma et al., 1996). Specifically, high levels of estrogen observed in women correlated with lower expression of p-selectin. Consistent with this clinical trial, studies conducted in human female iliac artery endothelial cells and in cultured human endothelial cells showed that estrogen significantly reduced the expression of adhesion molecules ICAM-1 and VCAM-1 (Hou & Pei, 2015; Piercy et al., 2002). Furthermore, estrogen might be involved in modulating the expression or action of proinflammatory cytokines. An in vitro study using samples of human saphenous veins, showed that treating these segments with the proinflammatory cytokine TNFα significantly induced the proliferation and migration of smooth muscle cells and endothelial cells however, cotreatment with estrogen hindered both effects (Nintasen et al., 2012). The results of this study are in agreement with other studies conducted in C57BL/6 mice and Sprague–Dawley rats, where treatment with estrogen significantly downregulated the expression of proinflammatory cytokines such as IL-6 and TNFα, and increased the expression of anti-inflammatory cytokine IL-10 (Messingham et al., 2001; Suzuki et al., 2008).

Taken together these data show that estrogen can exert a protective vascular and antiatherogenic effect by modulating several key players in these processes however, additional studies need to be conducted to elucidate these specific mechanisms.

Possible future directions of this research

The results of this project allow us to better understand the impact of sex hormones in the molecular mechanisms that modulate UPR under conditions of ER stress, as they relate to the development and progression of diabetes and atherosclerotic disease. We observed that estrogen depletion is associated with an impairment of glucose homeostasis along with the progression of atherosclerosis. Supplementation of 17-beta estradiol restored pancreatic beta cell health as well as significantly reduced atherosclerosis progression. Furthermore, our findings show that chronic hyperglycemia induces ER stress and activates the apoptotic UPR in pancreatic beta cells. Consistent with our hypothesis, estrogen supplementation significantly induces the adaptive UPR and inhibits the apoptotic UPR in these cells.

To further increase our knowledge on the impact of estrogens in pancreatic beta cell health and cardiometabolic disease, this project paves the way for the following future directions:

- Mechanistic studies on pancreatic beta cells: this project is currently underway in our lab and has the aim of investigating the mechanistic effects of estrogen (and potentially other sex hormones) in a pancreatic beta cell line (Beta TC6) challenged with ER stress inducers (thapsigargin, tunicamycin, glucose). The expression of UPR markers will be assessed, and specific UPR pathways that might be particularly influenced by estrogen will be investigated. To evaluate whether estrogen signals through one or more estrogen receptors, additional experiments could be conducted using specific estrogen receptors' agonists and/or antagonists. These results will help elucidate the mechanisms by which

estrogens modulate UPR activation in the presence of ER stress, and has the potential to explain what was observed in the ApoE$^{-/-}$:Ins2$^{+/Akita}$ mouse model, which is a unique model of hyperglycemia-induced atherosclerosis.

- Ex vivo analysis of pancreatic beta cell function: further analysis on beta cell function and UPR activation could be conducted by culturing isolated pancreatic islets of male, female and female ovariectomized ApoE$^{-/-}$:Ins2$^{+/Akita}$ and TALLYHO/JngJ mice. Islets could be treated with estrogen (and potentially other sex hormones) and challenged with various ER stress inducers to assess glucose stimulated insulin secretion and UPR activation. Pancreatic islets could be treated with estrogen receptors' agonists and antagonists to delineate estrogen signalling pathways.

- In vivo analysis of how estrogens modulate the UPR in pancreatic beta cells: the TALLYHO/JngJ mouse model could be further characterized by implanting slow-releasing estrogen pellets in male and female ovariectomized mice to further our understanding on the role of estrogen in the regulation of glucose homeostasis. Pancreatic beta cell health and function and UPR modulation could be assessed.

- Mechanistic studies on the effects of estrogen in atherosclerosis progression: endothelial cell lines could be used to understand at a mechanistic level the cardiovascular effects of estrogen observed in our in vivo experiments on the ApoE$^{-/-}$:Ins2$^{+/Akita}$ mouse model. This could inform us on the role that estrogen plays in maintaining vascular physiology. An investigation on the effects of sex hormones in atherosclerosis progression, and specifically on whether sex hormones influence the polarization of macrophages into a pro- or anti-inflammatory phenotype, is currently ongoing in our lab. This project uses bone marrow derived macrophages from C57BL/6J mice as well as the human derived THP-1 cell line.

Significance of this research

Overall, this project and the respective future directions are clinically relevant since they will allow us to better understand the influence of sex on metabolic diseases, as well as the impact of menopause, aging and hormone replacement therapies on health outcomes. Further research in this field is needed to develop more targeted, gender-specific therapies in the management and/or prevention of chronic disease.

Identifying the mechanisms by which estrogens exert their protection in pancreatic beta cell health and cardiometabolic diseases can lead us to identify and outline the specific pathways involved with this protection. This would allow us to develop small molecules targeting only the pathways involved in exerting the desired protective effect, without

altering those that might be involved in inducing secondary sex effects or other potential side effects.

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APPENDIX 2: THE ROLE OF ESTROGEN IN DIABETES MELLITUS: A REVIEW OF CLINICAL AND PRE-CLINICAL DATA

Monica De Paoli, Geoff H Werstuck

Thrombosis and Atherosclerosis Research Institute, Department of Medicine,

McMaster University, Hamilton, Ontario, Canada

Contributions

The author, Monica De Paoli, has designed, written, edited and approved the manuscript.

Supervisor: Dr Geoff Werstuck has edited and approved the manuscript.

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Abstract

The incidence and prevalence of diabetes mellitus, and the cardiovascular complications associated with this disease, are rapidly increasing worldwide. Individuals with diabetes have a higher mortality rate due to cardiovascular diseases and a reduced life expectancy compared to those without diabetes. This poses a significant economic burden on health care systems worldwide making the diabetes epidemic a global health crisis. Sex differences in the presentation and outcome of diabetes do exist. Pre-menopausal women are protected from developing diabetes and its cardiovascular complications relative to males and post-menopausal women. However, women with diabetes tend to have a higher mortality as a result of cardiovascular complications than age-matched men. Despite this evidence, pre-clinical and clinical research looking at sex as a biological variable in metabolic disorders and their cardiovascular complications is very limited. The aim of this review is to highlight the current knowledge of the potential protective role of estrogens in humans as well as rodent models of diabetes mellitus, and the possible pathways by which this protection is conferred. We stress the importance of increasing knowledge of sex-specific differences to facilitate the development of more targeted prevention strategies.

APPENDIX 3: THE ROLE OF ESTROGEN IN INSULIN RESISTANCE: A REVIEW OF CLINICAL AND PRE-CLINICAL DATA

Monica De Paoli, Alexander Zakharia, Geoff H Werstuck

Thrombosis and Atherosclerosis Research Institute, Department of Medicine,

McMaster University, Hamilton, Ontario, Canada

237 Barton Street East

Hamilton, Ontario, Canada

Contributions

The author, Monica De Paoli, has designed, written and edited the manuscript.

The co-author Alexander Zakharia has written the paragraph “Insulin: mechanisms of action” and “Sex differences in insulin resistance in the cardiac tissue**”**. Additionally Alexander has also created Figure 1 of this manuscript.

The supervisor, Dr Geoff Werstuck, has edited the manuscript.

All authors approved the manuscript.

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Abstract

Insulin resistance results when peripheral tissues, including adipose, skeletal muscle and liver, do not respond appropriately to insulin causing the ineffective uptake of glucose. This represents a risk factor for the development of type 2 diabetes mellitus. Along with abdominal obesity, hypertension, high levels of triglycerides, and low levels of high-density lipoproteins, insulin resistance is a component of a condition known as the metabolic syndrome, which significantly increases the risk of developing cardiometabolic disorders. Accumulating evidence shows that biological sex has a major influence in the development of cardiometabolic disturbances, with females being more protected than males. This protection appears to be driven by female sex hormones (estrogens), as it tends to disappear with the onset of menopause but can be re-established with hormonal replacement therapy. Current knowledge on the protective role of estrogens in the relevant pathways associated with insulin resistance is evaluated in this review. We emphasize the importance of increasing our understanding of sex as a biological variable in cardiometabolic research to promote the development of more effective preventative strategies.